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Culture and differentiation of human mesenchymal stromal cells and human endothelial cells on polymeric scaffolds to improve bone tissue-engineered substitutes

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To OtoLab group for supporting me

To my parents for your affection and love

My amazing cousin Gian Franco Serecchia, thank you for always being there,

for your support and confidence

My friends Simone Mozzachiodi, and Alejandro Arze

My Colombians friends in Pisa, son todos una chimba!!.

My Italians friends thank you for helping me speak Italian

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Culture and differentiation of human mesenchymal stromal cells and human endothelial cells on polymeric scaffolds to improve bone tissue engineered substitutes

Abstract

The purpose of this thesis is the development of a new strategy to improve the bone substitutes obtained via tissue engineering. These substitutes are usually achieved by culturing in vitro mesenchymal stromal cells (MSCs) and differentiating into osteogenic lineage the MSCs grown on three-dimensional porous structures made of biocompatible materials, known as scaffolds. One of the problems still to be solved is that these in vitro generated substitutes must be vascularized in vivo after implantation. Post-implant vascularization is often inadequate and leads to death of the transplanted cells. In this thesis several methods were tested to develop in vitro bone substitutes already provided with an initial vascularization. Spongy scaffolds made of polyvinyl alcohol (PVA) and gelatin (G) were tested with different weight compositions of the two polymers (namely PVA/G 100/0, 90/10, 80/20, 70/30, 50/50).

The experimental work is articulated in the following phases:

1) Cross-linking of the scaffolds with glutaraldehyde to have the polymeric structures stabilized inside an aqueous environment. The morphology of the sponges was observed with a scanning electron microscope (SEM) and their stability tested by immersion in water for two weeks.

2) Selection of the type of scaffold for the culture and differentiation of human umbilical vein endothelial cells (HUVECs), as an endothelial cell model. The scaffolds were cultured for 3 days and the differentiation induced by addition of Matrigel® for 18 hours. Before completing these experiments and securing the constructs in formalin, the non-destructive viability test AlamarBlue® it was performed to evaluate the metabolic activity of the cells. The fixed samples were processed for histological analysis with hematoxylin and eosin. The results showed that the scaffolds PVA/G 80/20 were the best about viability and cell colonization.

3) Selection of the type of scaffold for culturing and osteo-differentiation of human MSCs. The MSC/scaffold constructs were cultured for 22 days and the differentiation induced by addition of ascorbic acid, dexamethasone and β-glycerophosphate for 21 days. Every

week the vitality test AlamarBlue® was performed. The samples were fixed in formalin and processed for histological analysis with hematoxylin and eosin to assess cell morphology and von Kossa to assess the formation of mineral matrix. The results showed that the scaffold types PVA/G 80/20 and 70/30 were the best suitable. Therefore, PVA/G 80/20 was chosen to perform the co-culture with HUVECs.

4) The co-culture was conducted for 3 days by adding, at the end of osteogenic differentiation (day 22), HUVECs to MSC/scaffold constructs, both in the presence and absence of Matrigel®. The constructs were fixed in formalin and processed for an extensive histological analysis. Immunohistochemistry analyses of endothelial and osteogenic markers were performed, in order to localize the two different cell types on the scaffold and understand their interaction.

5) In order to investigate the influence played by bone extracellular matrix alone on the differentiation of HUVECs, which in vitro needs Matrigel® to form the tubes, some MSC/scaffold constructs at the end of the osteogenic differentiation, were lysed with sterile distilled water so as to remove the cellular component and to observe the interaction of the HUVECs with the pre-generated bone matrix. An extensive histological analysis was carried out. The results of points 4) and 5) finally highlighted the importance of osteoblastendothelial cell interaction for endothelial differentiation.

6) We finally tested this co-culture system under a completely autologous approach. To this purpose we used mesodermal progenitor cells (MPCs), which are a subset of highly immature stem cells present in the bone marrow and capable of differentiating into both MSCs and pre-endothelial cells. The expression of some osteogenic and endothelial markers in the construct confirmed results reported in literature and the scaffold PVA/G strategy can be used to explore the use of innovative techniques in bone tissue engineering.

Keywords: **scaffold, MSC, bone substitute, HUVEC, osteogenic, endothelial, differentiation, SEM, histochemistry, pre-generated matrix, co-culture.**

Riassunto

Culture and differentiation of human mesenchymal stromal cells and human endothelial cells on polymeric scaffolds to improve bone tissue-engineered substitutes

Lo scopo di questo lavoro di tesi consiste nella messa a punto di una nuova strategia per migliorare I sostituti ossei ottenuti tramite ingegneria tissutale. Tali sostituti generalmente si ottengono coltivando in vitro le cellule stromali mesenchimali (MSCs) e differenziandole in senso osteogenico su *scaffold*, ossia strutture porose tridimensionali a base di materiali biocompatibili.

Uno dei problemi ancora da risolvere è che questi sostituti generati in vitro dovranno essere vascolarizzati in vivo dopo l'impianto. Questo processo è spesso inadeguato e porta alla morte delle cellule trapiantate. In questa tesi sono stati testati alcuni metodi per sviluppare in vitro dei sostituti ossei già dotati di una iniziale vascolarizzazione. Come *scaffold* sono state utilizzate spugne di alcool polivinilico (PVA) e gelatina, con varia composizione ponderale dei due polimeri (PVA/G 100/0, 90/10, 80/20, 70/30, 50/50).

Il lavoro di tesi si è articolato nelle seguenti fasi:

1. Reticolazione degli *scaffold* con glutaraldeide per stabilizzare le strutture polimeriche in ambiente acquoso. La morfologia delle spugne è stata osservata al microscopio elettronico a scansione (SEM) e la loro stabilità testata tramite immersione in acqua per due settimane.
2. Selezione delle tipologie di *scaffold* per la coltura ed il differenziamento delle *human umbilical vein endothelial cells* (HUVEC), come modello di cellule endoteliali. Gli *scaffold* sono stati coltivati per 3 giorni ed il differenziamento indotto tramite aggiunta di Matrigel® per 18 ore. Prima di terminare gli esperimenti e fissare i costrutti in formalina è stato eseguito il test di vitalità non distruttivo AlamarBlue® che valuta l'attività metabolica delle cellule. I campioni fissati sono stati processati per l'analisi istologica con Ematossilina ed Eosina. I risultati hanno evidenziato che gli *scaffold* PVA/G 80/20 erano i migliori.
3. Selezione delle tipologie di *scaffold* per la coltura ed il differenziamento osteogenico delle *human MSCs* (hMSCs).Gli *scaffold* sono stati coltivati per 22 giorni ed il differenziamento indotto tramite aggiunta di acido ascorbico, dexametasone e β-

glicerofosfato per 21 giorni. Ogni settimana è stato eseguito il test di vitalità AlamarBlue®. I campioni sono stati fissati in formalina e processati per l'analisi istologica con Ematossilina ed Eosina e von Kossa, per valutare la formazione di matrice minerale. I risultati hanno evidenziato che gli *scaffold* PVA/G 80/20 e 70/30 erano i migliori. Sono stati quindi selezionati gli *scaffold* PVA/G 80/20 per effettuare la co-coltura con le HUVEC.

4. La co-coltura è stata condotta per 3 giorni aggiungendo, al termine del differenziamento osteogenico (giorno 22), le HUVEC ai costrutti hMSC/*scaffold*, sia in presenza che in assenza di Matrigel®. I costrutti sono stati fissati in formalina e processati per un'analisi istologica approfondita. L'immunoistochimica è in corso per valutare i marcatori endoteliali ed osteogenici, in modo da localizzare le due diverse tipologie cellulari sugli *scaffold* e capirne l'interazione.
5. Nel prosieguo del lavoro abbiamo voluto anche indagare l'influenza che la matrice extracellulare ossea da sola può giocare sul differenziamento delle HUVEC, le quali nella coltura in vitro necessitano del Matrigel® per formare i tubi. Per questa indagine, alcuni costrutti hMSC/*scaffold* al termine del differenziamento osteogenico, sono stati lisati con acqua distillata sterile in modo da rimuovere la componente cellulare e poter osservare l'interazione delle HUVEC con la matrice osteogenica pregenerata. Le analisi istologiche: citochimici, istochimica, ed immunoistochimica sono stati fatti evidenziando così le sue caratteristiche morfologiche, strutturali e biochimici.

Keywords: *Scaffold*, MSC, Sostituto osseo, HUVEC, osteogenico, endoteliale, differenziamento, SEM, Istochimica, matrice pre-generata, co-cultura.

ACRONYMS

MSC	Mesenchymal Stromal Cells
HUVEC	Human Umbilical Vein Endothelial cells
PVA	Polyvinyl Alcohol
G	Gelatin
SEM	Scanning Electronic Microscopy
ECM	Extracellular Matrix
EPCs	Endothelial Progenitor Cells.
TE	Tissue Engineering
GTA	Glutaraldehyde
MPC	Mesodermal Progenitor Cells.
VEGFs	Vein Endothelial Growth Factor
FGFs	Fibroblast Growth Factors
TGF-β	Transforming Growth Factor-β
RUNX-2	Runt related transcription factor-2
ALP	Alkaline Phosphatase
FN	Fibronectin
BMP	Bone Morphogenetic Protein
DMEM	Dulbecco’s Modified Eagle’s Medium
EGM-2	Endothelial cells Growth Medium
BSA	Bovine Serum Albumin
DAB	Diaminobenzidine Tetrahydrochloride
EBM-2	Endothelial Cell Basal Medium-2
FBS	Fetal Bovine Serum
OPN	Osteopontin
PECAM	Platelet Endothelial Cell Adhesion Molecule
VEGF-R2	Vascular Endothelial Growth Factor Receceptor-2
VEGF	Vascular Endothelial Growth Factor
OCN	Osteocalcin

p-VEGF-R2	anti-Phosphorylated Vascular Endothelial Growth Factor Receptor 2
PBS	Phosphate Buffered Saline
MIP	Mercury Intrusion Porosometry
BMMNCs	Bone Marrow Mononuclear Cells
H&E	Hematoxylin-Eosin
IHC	Immunohistochemistry
MPC	Mesodermal Progenitor Cell

Chapter 1

1. INTRODUCTION

1.1. VASCULARIZATION IN BONE REGENERATION

Restoration of bone defects, as a consequence of trauma, tumor excision, chronic osteomyelitis, non-union, avascular necrosis and spinal fusions represent a widespread clinical problem (Amini et.al, 2012). If the defect is too large, bone regeneration cannot occur spontaneously, leading to the necessity of surgical strategies which avail themselves of bone substitutes. Materials to replace bone can be biologic or synthetic. The best choice, whenever possible, is using bone tissue of the same patient. This is performed by taking the bone from a different part of the same person and is referred as autografting. However, sometimes the lesion is too big, and auto-transplanting such a great amount of bone would generate defects and pain (known as “morbidity”) in a different body setting. Limited availability and prolonged surgical times are the drawbacks of autografting (Amini,et al, 2012). An alternative is the use of grafts taken from different individuals who are dead and decided to donate their tissues. Grafts taken from other human beings are called homografts. Finally, sometimes grafts are taken from a different species and they are named xenografts. Banked bone has become a useful source of bone substitutes in orthopedic surgery. Of course, not autologous grafts (i.e., allografts) must be preliminary treated to eliminate the donor’s or animal’s cells, so that they are just composed by bone extracellular matrix (ECM)., The use of allografts is advantageous because there is no second procedure required to remove and transfer a portion of the patient’s native bone or tissue. Surgical time may be minimized, postoperative discomfort reduced and patients may be back to normal activities more quickly. However, they place the patients at risk for infections and rejection by the immune system, due to the presence of allogeneic proteins. Moreover, to promote the vascularization it is necessary to keep the vascular connections of vessels to and from a freed segment of bone, (Derby, et.al 2013). Synthetic substitutes for bone regeneration can be obtained in the laboratory by growing the patient’s own cells on biomaterial-based scaffolds which are designed to further degrade in vivo, and transplanting the living bone construct back to the patient. However, the ex vivo procedure for obtaining these bone substitutes is very laborious and constrained by strict regulatory

issues, so that only a limited number of clinical studies have been reported so far. Substitute failures have been highlighted because of their post-implant unsuccessful vascularization (Santos et.al, 2010). The lack of a functional vascular supply after implantation put at serious risk the survival of the transplanted cells. Therefore, the development of strategies which can promote a microvasculartur have become a primary goal in bone replacenent. Some studies (Asahara et.al, 2011; Das et.al, 1999) assess that the in vivo formation of new blood vessels depends on the ordered interaction of endothelial cells with different types of cells including, macrophages, pericytes, endothelial progenitor cells (EPCs), and mesenchymal stromal cells (MSCs) (Denber et.al, 1995; Wang et.al, 1997). It is has become clear that multiple stem cell types, immunogenic cues, and cytokines participate in, and are necessary for initiating angiogenesis and supporting osteogenesis. Several strategies have been studied to foster the establishment of a functional vascular network in bone substitutes (Santos et al, 2010).They include: *(i)* scaffolds with hierarchical topology, obtained via microfabrication; *(ii)* scaffolds for angiogenic factor release, to recruit endothelial cells in vivo; *(iii)* microsurgery approach, such as flap fabrication and arteriovenous loop, *(iv)* scaffolds supporting endothelial cell attachment and *(v)* co-cultured systems, in which endothelial cells coexist with bone cells at the time of implantation. Because vascularized bone regeneration requires an interplay between multiple cell types, recent work has focused on co-culturing different cell types to facilitate healing and to most powerfully promote vascularized post-implant bone regeneration.

1.2. BONE TISSUE ENGINEERING

Bone tissue regeneration is considered an important challenge in the field of orthopedic and craniofacial surgery. Traumatic injuries and pathological diseases including osteoporosis, osteoarthritis, osteogenesis imperfecta, can impair normal bone functions and lead to bone fractures non-unions, immobility, severe pain and deformity. As such, the request for bone grafts is increasing and represents the second most common tissue transplantation procedure after blood, with over 2.2 million bone graft procedures conducted worldwide annually in orthopedics and dentistry (K.-U. Lewandrowski et.al, 2000) (Fernandez-Yague et al, 2015). Novel solutions are required to overcome the limitations of current bone grafting approaches through tissue engineering (TE) or regenerative medicine which offer promising strategies for treating bone diseases and

reconstructing bone defects TE involves the combination of cells, biomaterial scaffolds and biochemical and physical stimuli to encourage in vitro tissue formation. The development of responsive biomaterials capable of a modulated functionality in response to the dynamic physiological and mechanical environments found in vivo remains an important challenge in bone tissue engineering to achieve long-term repair and good clinical outcomes (Rose,F.R, 2002).

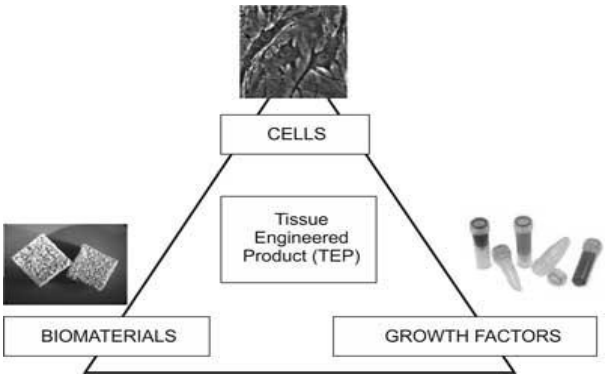

Figure 1 *The TE triad. Reproduced by Wójtowicz,2011*

Bone TE has been a field of intense research for about 20 years. However the translation to clinical practice is a challenge due to regulatory and ethical issues (Hollister et.al, 2011). Nevertheless, bone TE represents the only alternative to traditional bone autografts and allografts, and thus further research is needed to face the challenges in this field (Amini et.al, 2012). In the following paragraphs, a focus will be given to all the aspects of the TE triad which are relevant for this thesis, namely, scaffolds (and polymers), cell sources and differentiation stimuli.

1.3. SCAFFOLDS

TE scaffolds are biomaterial-based matrices that require several key features:

- Biocompatibility, to be accepted by the body without causing toxicity, carcinogenicity and inflammation
- Porosity, to host cells and ECM secretion, and to facilitate diffusion phenomena
- Pore interconnectivity, to allow cell migration, scaffold colonization and nutrient/waste trafficking
- Biodegradation, to be replaced by new tissue

A good stratagem for regenerative bone therapies is to use biomaterial scaffold that can modulate the process of healing while providing mechanical support. Successively, following initial cellular colonization, the material should degrade by cellular and enzymatic activity in parallel with the production of new bone matrix. Ideally, after total degradation of the scaffold, the tissue will recover its function without the need of clinical intervention to remove the implant. Nevertheless, this is frequently not enough to repair the complete functionality of the tissue.(Fernandez-Yague et.al,2015). The design and development of biomaterial scaffolds that will replace the form and function of native tissue while promoting regeneration without necrosis or scar formation is a challenging area of research. Different biomaterial scaffolds have been explored for bone regeneration, with the aim of identifying the most appropriate physical, chemical and biological properties to encourage *in vitro* bone production (Williams et.al,2005). Two distinct biomaterial based approaches have emerged that have demonstrated potential to support stem cell based bone regeneration; the use of polymer-based scaffolds (natural and synthetic polymers), as they are bioresorbable, and the use of ceramic scaffolds that mimic the mechanical environment of mature bone, or a combinational approach of these. Bone scaffolds for tissue regeneration require an optimal trade-off between biological and mechanical criteria. Optimal designs may be obtained using topology optimization and prototypes produced via additive manufacturing techniques(Coelho,et.al, 2015). The most common strategy employed to engineer bone is to use a scaffold combined with osteoblast cells, or cells that can mature/differentiate into osteoblasts and growth factors that promote cell attachment, differentiation, and mineralized bone formation. However, the requirements for designing and production of an ideal scaffold for bone regeneration are very complex and not yet fully understood. It is generally agreed that the scaffold must be a biocompatible, porous (more than 90% porosity and pore sizes 100-350 μm are desirable for bone regeneration), interconnected, and permeable structure in order to permit the ingress of cells and nutrients. Clearly, structures designed with fibers can meet these criteria, however also sponges can be suitable. A better understanding of the basic events during the bone healing process have provided new strategies for the development of smart scaffolds that are able to biochemically mimic the natural ECM environment of bone tissue. As well as providing tailored physicomechanical cues for induction of osteospecific function and maintenance of phenotype, these smart biomaterials can deliver key cellular components or signaling moieties through regulated release of single or multiple biofactors to promote regeneration.

1.3.1 POLYVINYL ALCOHOL (PVA).

Polyvinyl alcohol (PVA) is a water-soluble synthetic polymer, with the formula $[CH_2\text{-}CH(OH)]_n$, that is prepared by vinyl acetate polimerization. PVA has excellent film forming, emulsifying and adhesive properties. Since PVA is highly hydrophilic, its mechanical properties depend on the water content. PVA has a melting point of 230°C and 180-190°C, for the fully hydrolyzed and partially hydrolyzed grades, respectively. It is used industrially in papermaking, textiles, and in some biomedical applications. PVA is a nonhazardous material, has no negative effect on animals, and does not cause any injuries to the skin upon contact. PVA hydrogels can be obtained via freeze-thawing or chemical cross-linking (Peppas and Hansen, 1982) and have shown relevance in several TE applications, especially of soft tissues (Woerly et al., 1996; Hubbell, 1998), like arterial models (Chu and Rutt, 1997), heart valves (Jiang et al., 2004), corneal implants (Vijayasekaran et al., 1998), and cartilage (Stammen et al., 2001). Moreover, PVA nano-fibrous membranes with different compositions could have different clinical applications, especially the controlled release of drugs and bone tissue engineering (Preetti,2015). Unlike most famous aliphatic polyesters (e.g., polylactic acid, polycaprolactone) PVA is water-soluble, so it can be easily blended with most biological molecules, including growth factors and proteins.

1.3.2 GELATIN

Among proteins, gelatin (G) is widely use in TE. G derives from the hydrolysis of collagen, which is the key ECM component in the skeletal system, including skin, cartilage and bone. As such, G exerts a pivotal role in cell adhesion and is routinely used to pre-coat synthetic scaffolds upon cell seeding. Since G is soluble at 37°C, it is used blended with synthetic polymers upon fabrication. This latter approach leads to materials mixtures which are referred to bioartificial materials. In addition to the gel-forming, G shows an excellent versatility in amino acid composition, including both positively charged (arginine, lysine, and histidine) and negatively charged (glutamic acid and aspartic acid) amino acids. Biodegradability, biocompatibility, non-immunogenicity, and relatively low cost render G an appealing biomacromolecule in TE scaffolding. Chemical cross-linking is the most widely used technique to improve the thermal, mechanical and water stability. One strategy uses glutaraldehyde (GTA) for its excellent efficiency on the stabilization of collagenous

materials and reduced toxicity at low concentrations (Farris et al, 2010). The cross-linking of gelatin mediated by GTA is believed to occur via the unprotonated ε-amino groups of lysine and hydroxylysine and the amino groups of the N-terminal amino acid. G fibers produced through electrospinning found many biomedical applications, including wound or burn dressings, surgical treatments, and tissue engineering of bone, skin, and cartilage. This is mainly derived from the fact that G is a natural polymer that has well-known wound healing abilities and a biological aptitude for stimulating cell proliferation. Furthermore, G fibers can be easily extended into three-dimensional structures of woven, knitted, and nonwoven structures.

1.4. BONE MARROW STEM CELLS

1.4.1 Mesenchymal Stromal cells

Mesenchymal stromal cells (MSCs) are multipotent cells that can be isolated from several human tissues and expanded ex vivo for clinical use. Their adherent properties, immune phenotype characteristics and differentiation potential have MSC cells a population of cells able to improve in differents approach in regeneration tissue. The bone marrow contains many different types of cells. Among them are blood stem cells (also called hematopoietic stem cells; HSCs) and a variety of different types of cells belonging to a group called ‘mesenchymal’ cells. Only about 0.001-0.01% of the cells in the bone marrow are **mesenchymal stromal cells.** These cells were first isolated and characterized by Friedenstein and his colleagues in early 1974. MSCs, are a subset of non-hematopoietic adult stem cells that originate from the mesoderm. They possess self-renewal ability and multilineage differentiation into not only mesoderm lineages, such as chondrocytes, osteocytes and adipocytes, but also ectodermic cells and endodermic cells (EuroStemCells.org). MSCs exist in almost all tissues. They can be easily isolated from the bone marrow, adipose tissue, the umbilical cord, fetal liver, muscle, and lung and can be successfully expanded *in vitro.* In the lasts 10 years is rising use the MSCs in clinical trials and regenerative medicine, in countries China, Europe and United States. (Stlotz et.al,2015)

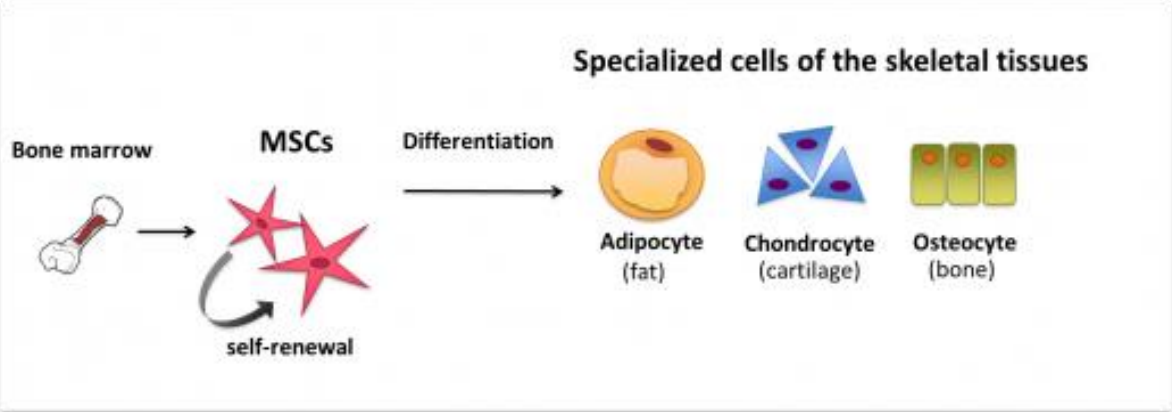

***Figure** 2 MSCs. Reproduced by EuroStemCells.org*

1.4.2 Mesodermal progenitor cells (MPC).

Mesodermal progenitor cells (MPCs) are a subset of stem cells present in the bone marrow that have recently been isolated when culturing human bone marrow derived cells in the presence of autologous serum (hAS) (Petrini et.al 2008). These adherent cells have been identified among MSCs when cultured in a medium supplemented with hAS. MPCs are more immature than MSCs and differ from MSCs in terms of their morphology (large, round cells) and of their quiescent status (Ki67-negative). MPCs are able to differentiate into MSCs when cultured in a medium supplemented with FBS or human cord serum, and have been proven to be capable of generating colon forming unit-fibroblasts (CFU-F) which can further differentiate into mesodermal lineages (adipocytes, chondrocytes, and osteoblasts), as described for MSCs (Bruder et.al,1997). MPCs do not directly differentiate into the mesodermal lineage (osteogenic, chondrogenic, adipogenic) even in the presence of appropriate stimuli. Moreover, these cells may differentiate into endothelial cells when cultured in an appropriate vascular endothelial growth factors (VEGFs)-containing medium similar to those used in the differentiation of embryonic cells.

MPCs are positive for CD105 (but negative for CD90), express high levels of aldehyde dehydrogenase (ALDH) activity, and are positive for several markers, such as SSEA4, Oct-4, and NANOG, which are also expressed in embryonic cells. They show strong plastic adherence and trypsin resistance.

1.5. ENDOTHELIAL CELLS

The Human Umbilical Vein Endothelial Cells (HUVECs) are cells derived from the endothelium of veins from the umbilical cord, these cells offer a classic model system to study the different aspects associated to endothelial function and disease, such as normal, abnormal and tumor-associated angiogenesis, oxidative stress, hypoxia and inflammation related pathways in endothelia under normal and pathological conditions, cardiovascular-related complications associated with various diseases, mode of action and cardiovascular protection effects of various compounds, and so on (Park,et al 2006) In previously works, the HUVECs have been selected and tested to demonstrate stimulation-dependent angiogenesis and key endothelial cell signaling pathways (e.g., phosphorylation of VEGFR, Akt, MAPK, and expression of Tie2, eNOS, Axl and Etk/Bmx). The obtained evidences indicated that HUVECs are an important in vitro model useful in molecular medicine including pathophysiology of atherosclerosis and plaque formation, and mechanisms for the control of angiogenesis or neovascularization in response to hypoxia and inflammation in tumors, ischemic tissue, and in embryogenesis (De Paola and Burn 2005)(Park et.al, 2006).

It is know that the culture of HUVECs on Matrigel®, an extract of endothelial basement membrane, results in the formation of honeycomb-like structures that simulate tube formation by endothelial cells in vivo. Other in vitro assays which are based on Matrigel® involve cell migration, proliferation, cell invasion, protease activity and tubule formation. The in vitro formation of capillary-like tubes by endothelial cells on a basement membrane matrix is a powerful in vitro method to screen for various factors such as VEGFs, fibroblast growth factors (FGFs), angiopoietins, transforming growth factor-β (TGF-β), platelet-derived growth factors (PDGFs), tumor necrosis factor-α (TNF-α), epidermal growth factor (EGF), interleukin-8 (IL-8), and angiogenin, which are secreted by inflammatory cells, pericytes, keratinocytes, or tumor cells that promote or inhibit angiogenesis. The use of Matrigel® was primarily described in 1988 (Kubota et.al 1988), numerous uses of the assay have been developed, such as determining angiogenic or antiangiogenic factors, genes regulating and pathway angiogenesis regulation (Arnaoutova et.al, 2010).

Cells initially attach to the matrix, then migrate toward each other, align and form tubes, but for the formation of these tubes the protein synthesis, as well as collagen synthesis and proteases are essential.

While VEGF is also responsible for increasing vascular permeability, Ang1 through its receptor Tie2 inhibits vascular leakage, but also is chemotactic to endothelial cells and induces endothelial cell sprouting and secretion of proteases plasmin and MMP-2, stimulating angiogenesis, Ang1 is constitutively expressed in the adult (Raundhaug et.al,2005)

1.6. OSTEOGENIC DIFFERENTIATION

MSCs have been shown to possess the capability to differentiate into a variety of skeletal cell types, including adipocytes, osteoblasts, chondrocytes, and myoblasts upon a proper stimulation. Although it is currently believed that the therapeutic benefits of MSCs are due to more complicated mechanisms, they have been indicated to be able to differentiate into osteoblasts, cardiomyocytes and other tissue-specific cells also after their *in vivo* systemic infusion in the treatment of osteogenesis imperfecta and myocardial infarction in both animals and humans (Wei et.al,2013). Possibly, these cells can target the injured tissue and receive proper stimulation from the native microenvironment. MSCs can be combined together with various natural and synthetic biomaterial scaffolds. Either undifferentiated or differentiated MSCs can be loaded onto scaffolds before their implantation into damaged tissue sites (Wei et,al,2013). In vitro, chemical factors as well as mechanical stimuli and architectural cues of the scaffold have been discovered to be able to address the fate of MSCs, thus demonstrating that MSCs are highly responsive to the surrounding environment. Such technologies have been successfully applied in cartilage repair and long bone repair, with the generation of well-integrated and functional hard tissues (Amado,2005). The advantage of a tissue-engineered MSC delivery system relies on the ease of controlling and manipulating the implanted cells and tissues, with reduced side effects impacting other organs and tissues. Current improvements in delivery vehicles and compatibility between the scaffolds and MSCs will help to develop a mature technology for clinical applications. The osteogenic potential of MSCs is well known and subsequently these cells have a great application value for promoting bone regeneration and relate process. Molecularly, the osteogenic differentiation is transcriptionally regulated by runt related transcription factor-2 (RUNX2) (Rocca, 2013;Krause et al, 2001) and nuclear localization of β-catenin through canonical WNT signaling (Bruschini et al, 1991)(Bruschini et al, 1992). Together this leads to downstream activation of the canonical bone genes –

osterix, osteopontin, and osteocalcin [19] and [20] – and suppression of chondrogenesis (Janeke,1975). The expression of Runx2 is noticed first in osteocondroprogenitors at early bone development. The levels of Runx2 increased in later stages of osteoblastic differentiation, with a maximum of observed in osteoblast mature expression. Runx2 acts in stages of induction, proliferation, and maturation of osteoblasts also controls the bone differentiation by binding to regulatory elements of the considered marker gene promoters for different stages of differentiation osteoblastic (Rocca,2011). Furthermore, is know that Collagen type I molecule present during the osteogenic differentiation is the primary product of osteoblasts in the course of the formation of the bone matrix, which constitutes approximately 90% of the total bone mature organic ECM. The protein is also evidently expressed at the preosteoblast stage, and moreover, it has been documented that the increase of collagen type I expression occurs before the increase of any other matrix molecule and before the alkaline phosphatase (ALP) (Stein,et al 1990) and (Clarke,2008) Osteopontin is a protein involved in cell aggregation process and is specifically localized in lamellar bone extracellular matrix during endochondral ossification and intramembranous. Among osteoblastic markers, osteocalcin is currently considered the most specific and the last to be expressed. Fibronectin (FN) is an adhesion molecule localized predominantly in the pericellular matrix and characterized by a portion capable of binding to collagen. FN is a multifunctional, extracellular matrix glycoprotein composed of two nearly identical disulfide bound polypeptides of molecular weight 220 kDa is involved in the processes of migration, adherence to the matrix and organization of bone cells.

In vitro osteogenesis of MSCs is typically initiated in the plastic adherent cell population by dispensation of ascorbic acid, β-glycerol phosphate, and dexamethasone or bone morphogenetic protein (BMP).

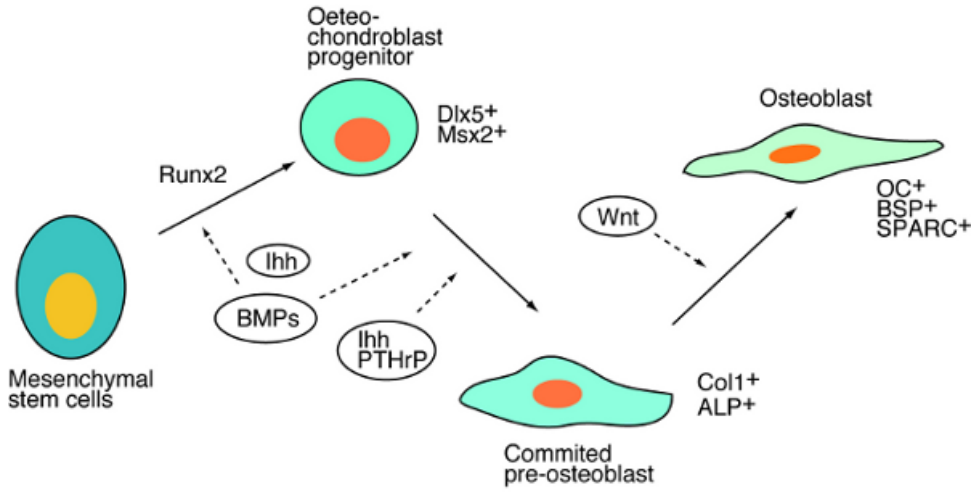

Figure 3 Osteoblast differentiation

Tissue engineering approaches comprise the combination of cells, biomaterial scaffolds and specialized culture conditions incorporating biochemical and physical stimuli to encourage in vitro bone formation for the adequate functionality. The advance of responsive biomaterials capable of modulated functionality in response to the dynamic physiological and mechanical environments found in vivo remains an important challenge in bone tissue engineering to achieve long-term repair and good clinical outcomes (Fernandez-Yague,2015).

1.7. CO-CULTURE AND PRE-VASCULARIZED BONE SUBSTITUTES

Bone is a complex tissue which comprises many cell populations. Therefore, it is expected that the co-culture of heterogeneous cell types will mimic more closely the in vivo environment [Fuchs et al, 2009]]. One of the fundamental heterotypic cross-talks in bone cells is that between osteoblasts and endothelial cells. A number of studies assessed this strong relationship and designed strategies based on the contemporaneous culture of these two cell types to regenerate prevascularized bone sobstitutes (kaigler et al. 2003; Fuchs et al. 2009). Co-cultures of human osteoblasts and HUVECs were performed on scaffolds made of different polymers, like polyurethane, starch/polycaprolactone and collagen gel (Santos et al. 2008). Human osteoblasts were also co-cultured with endothelial progenitor cells on polycaprolactone/hydroxyapatite scaffolds leading to the formation of capillary networks and osteoids (Vandervord et al. 2005). Finally, MSCs and endothelial cells were cultured together using a scaffold-free approach and on poly(lactic-

co-glycolic acid) scaffolds. All these studies reported that the co-culture strategy promotes micro-vasculature formation, which was stable in vitro up to 40 days, thus highlighting the importance of osteoblast-endothelium cross-talk in recreating the bone microenvironment. Among the ECM molecules, collagen type I was highly expressed in these co-cultures. Collagen type IV, the main component of the basement membrane was detected in the perivascular regions. In addition, endothelial cells can enhance the expression of ALP activity, a marker of osteogenesis. The process leading to capillary formation seemed to be hindered by adding the osteogenic culture media to the endothelial cells, and by a separation of the two cell population using a membrane, while it was amplified by direct cell-cell contacts (Finkenzeller et al. 2006). This interesting finding was explained by the formation of gap-junctions between osteoblasts and endothelial cells, that were studied through the expression of connexin 43 (Cx43) (Villars et al. 2002). Although the co-culture method seems a promising strategy, further studies should determine whether these microcapillary-like structures pre-formed in vitro within the biomaterial scaffolds will be able to integrate with the host microvasculature.

Chapter 2

2. MATERIALS AND METHODS

2.1. MATERIALS AND WORK PLAN

List of reagents and materials

Alamar Blue, Gibco by Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA, product code: DAL1025.

Alcian blue kit, Bio-Optica, Milan, Italy, product code: 04-161802.

Aluminium sulphate hydrate, Sigma-Aldrich, St. Louis, MO, USA, product code: 227617.

Ascorbic acid, Millipore, Temecula, CA, USA, product code: 2004011.

Biotinylated goat anti-mouse secondary antibody, Vektor Lab, Burlingame, CA, USA, product code: BA-9200.

Biotinylated goat anti-rabbit secondary antibody, Vektor Lab, Burlingame, CA, USA, product code: BA-1000.

Bovine Serum Albumin (BSA), Sigma-Aldrich, St. Louis, MO, USA, product code: A2153.

Dexamethasone, Millipore,Temecula,CA,USA, product code: 90357.

Diaminobenzidine tetrahydrochloride (DAB), Amresco, Solon, OH, USA, product code: E733.

Differentiation Basal Medium-Osteogenic, Lonza, Basel, Switzerland, product code: PT-3924.

Di-sodium hydrogen phosphate anhydrous, Carlo Erba, Milano, Italy, product code: 480141.

DPX mountant, Sigma-Aldrich, St. Louis, MO, USA, product code: 06522.

Dulbecco's modified Eagle's medium (DMEM), Gibco by Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA, product code: 11880-028.

Endocult StemCell Techologies, Vancouver, Canada, product code: 05900

Endothelial Cell Basal Medium-2 (EBM-2) Clonetics by Lonza, Basel, Switzerland, product code: CC-3156.

Endothelial cells Growth Medium (EGM-2) medium, Clonetics by Lonza, Basel, Switzerland, product code: CC-3156.

Eosin B, Sigma-Aldrich, St. Louis, MO, USA, product code: 45260.

Ethanol 99.8%, Sigma-Aldrich, St. Louis, MO, USA, product code: 02860.

Fetal Bovine Serum (FBS), Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA, product code: 10500064.

Ficoll Paque Plus, GE healthcare, Hatfield, UK, product code: 17-14440-02.

Fluconazole, Fresenius Kabi, Verona, Italy, product code: ATC J02AC01.

Fluromount, Sigma-Aldrich, St. Louis, MO, USA, product code: F4680.

Gelatin Bovine Skin B, Sigma-Aldrich, St. Louis, MO, USA, product code: G9391.

Glutaraldehyde (25%), Sigma-Aldrich, St. Louis, MO, USA, product code: 49630.

Glycine, Sigma-Aldrich, St. Louis, MO, USA, product code: 410225.

Goat Serum, Vektor Lab, Burlingame, CA, USA, product code: S-1000.

Histoplast LP, Thermo Fisher Scientific, Waltham, MA, USA, product code: 8332.

Human Fibronectin, BD Bioscience, Erembodegem, Belgium, product code: 356008

Human umbilical vein endothelial cells (HUVEC), Promocell, Heidelberg, Germany, product code: C-12200.

L-glutamine, Sigma-Aldrich, St. Louis, MO, USA, product code: G7513.

Matrigel, BD Bioscience, Erembodegem, Belgium, product code: 354234

Mayer's Hematoxylin solution, Sigma-Aldrich, St. Louis, MO, USA, product code: MHS32.

Methanol, Sigma-Aldrich, St. Louis, MO, USA, product code: 32213.

Mouse monoclonal antibody Alkaline Phosphatase (ALP), Santa Cruz Biotechnology, Santa Cruz, CA, USA, product code: sc-166261.

Mouse monoclonal primary antibody anti-Osteopontin (OPN), Santa Cruz Biotechnology, Santa Cruz, CA, USA, product code: sc-21742.

Mouse monoclonal primary antibody anti-Platelet/endothelial cell adhesion molecule-1 (PECAM), Santa Cruz Biotechnology, Santa Cruz, CA, USA, product code: sc-81158.

Mouse monoclonal primary antibody anti-Vascular endothelial growth factor receptor 2 (VEGF-R2), Santa Cruz Biotechnology, Santa Cruz, CA, USA, product code: sc-6251.

Mouse monoclonal primary antibody anti-Vascular endothelial growth factor (VEGF), Santa Cruz Biotechnology, Santa Cruz, CA, USA, product code: sc-7269.

NaCl solution (0,9%), Fresenius Kabi, Verona, Italy, product code: B100407.

NH OsteoDiff Medium,Miltenyi Biotec, Bergisch.Gladbach, Germany,product code:130-091-677

NH CondroDiff Medium Miltenyi Biotec, Bergisch.Gladbach, Germany,product code130-091-679

NH AdipoDiff Miltenyi Biotec, Medium Bergisch.Gladbach, Germany,product code: 130-091-677

Neutral Buffered Formalin (10% aqueous solution containing formaldehyde 4% w/v), Bio-Optica, Milan, Italy, product code: 05-k01009.

Nuclear Fast Red, Sigma-Aldrich, St. Louis, MO, USA, product code: 60700.

Osmium tetroxide (4% w/v solution), Electron Microscope Sciences, Hatfield, PA, USA, product code: 19170

Osteogenic differentiation medium, Lonza, Basel, Switzerland product code: PT-4120.

Penicillin/streptomycin, Sigma-Aldrich, St. Louis, MO, USA, product code: P0781.

Periodic acid, Sigma-Aldrich, St. Louis, MO, USA, product code: 77310.

Phosphate-buffered saline (PBS) 1X, Gibco by Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA, product code: 14190169.

Poly(vinyl alcohol) (PVA) 99+% hydrolyzed, Mw 89000-98000, Sigma-Aldrich, St. Louis, MO, USA, product code: 341584.

Pyrogallol , Sigma-Aldrich, St. Louis, MO, USA, product code: 16040.

Rabbit polyclonal primary antibody anti-Collagen I, abCam, Cambridge, MA, USA, product code: ab34710.

Rabbit polyclonal primary antibody anti-Osteocalcin (OCN), Santa Cruz Biotechnology, Santa Cruz, CA, USA, product code: sc-30044.

Rabbit polyclonal primary antibody anti-Phosphorylated vascular endothelial growth factor receptor 2 (p-VEGF-R2), Santa Cruz Biotechnology, Santa Cruz, CA, USA, product code: sc-101820.

Rabbit polyclonal primary antibody anti-Transforming Growth Factor beta-1 (TGFβ-1) Santa Cruz Biotechnology, Santa Cruz, CA, USA, product code: sc-146.

Schiff's reagent, Titolchimica, Rovigo, Italy, product code: TC71800PP.

Silver nitrate, Sigma-Aldrich, St. Louis, MO, USA, product code: 209139.

Sodium chloride, Carlo Erba, Milano, Italy, product code: 479687.

Sodium dihydrogen phosphate, Carlo Erba, Milano, Italy, product code: 480087.

Sodium dodecil sulphate, Sigma-Aldrich, St. Louis, MO, USA, product code: 71725

Sodium thiosulphate pentahydrate, Sigma-Aldrich, St. Louis, MO, USA, product code:S8503.

Triton x100, Sigma-Aldrich, St. Louis, MO, USA, product code: X100.

Vascular endothelial growth factor (VEGF), Miltenyi Biotec, Cologne, Germany, product code: 130-094-029

Vectastain Elite ABC Kit Standard, Vektor Lab, Burlingame, CA, USA, product code: PK6100.

Xylenes, Sigma-Aldrich, St. Louis, MO, USA, product code: 534056.

β-glycerol 2-Phosphate.Millipore, Darmstadt, Germany, product code: 2004010.

Work plan

The experimental campaign is organized according to the following steps, as shown in Figure 2.1:

1. PVA/G scaffold preparation with different compositions: 100/0, 90/10, 80/20, 70/30 and 50/50, and characterization
2. Selection of the best suitable scaffold for HUVECs
3. Selection of the best suitable scaffold for osteogenic differentiation of MSCs
4. Co-culture of osteoinduced MSCs and HUVECs on the selected scaffold, and identification of controls to analyze the outcomes. In particular:
    i) verification of the necessity of Matrigel® in presence of bone ECM (+ Matrigel and – Matrigel)
    ii) verification of necessity of osteoblast-endothelial cell interaction for microcapillary formation (+ live differentiated MSCs, - live differentiated MSCs: this was done by lysis of the osteoinduced constructs before HUVEC seeding and will be named “pre-generated ECM”)
5. Optimization of the outcomes

Generation of a fully autologous pre-vascularized bone substitute using MPC-derived pre-endothelial cells and MPC-derived osteoinduced MSCs.

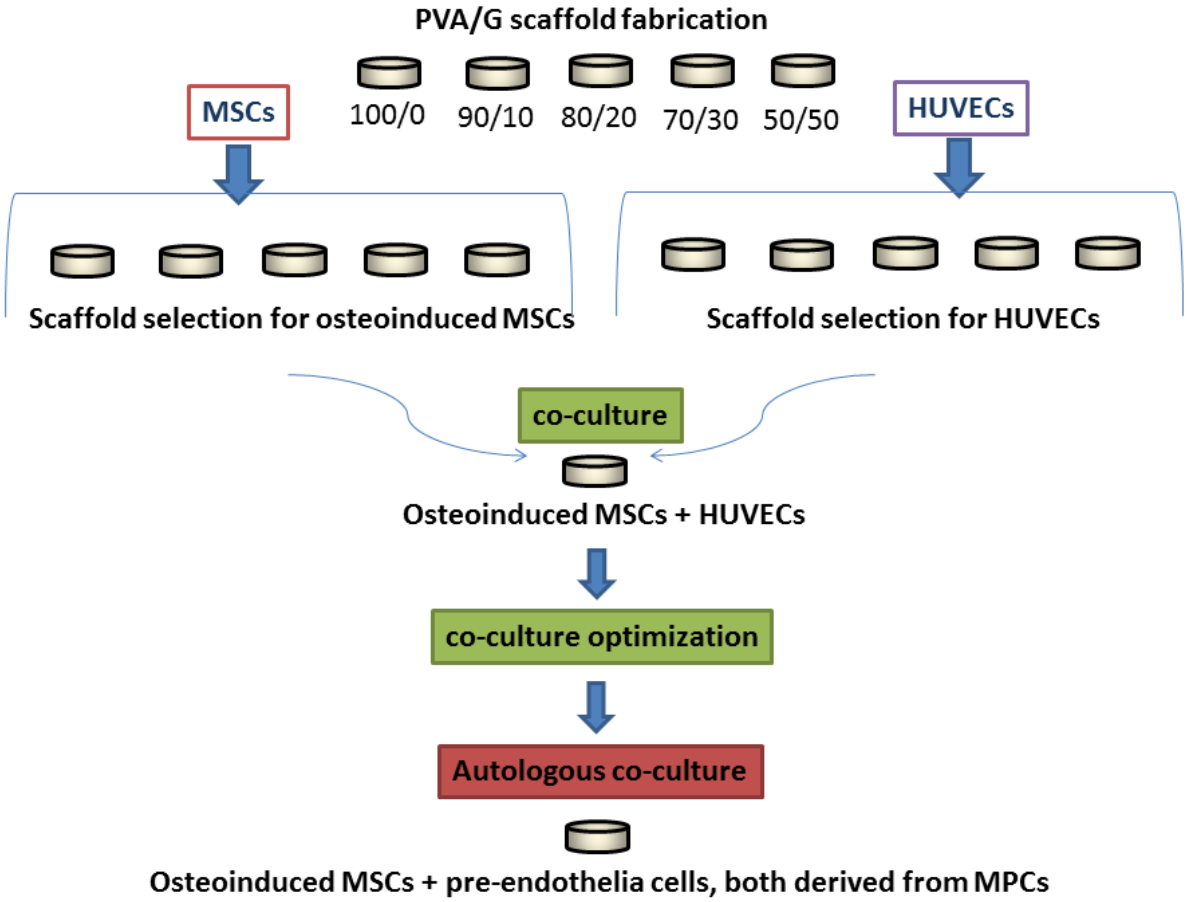

Figure 2.4*.Experimental work plan of the thesis*

2.1.1 OBJECTIVES

1) Development of a new strategy to improve the bone substitutes obtained via tissue engineering.

2) MSC/HUVEC culture and differentiation on three-dimensional porous structures made of biocompatible materials, known as scaffolds.

3) Perform co-culture system under a completely autologous approach using MPC differentiating into both MSC and pre-endothelial cells.

2.2 METHODS

2.2.1. Scaffold fabrication

Bioartificial PVA/G sponges were fabricated via emulsion and freeze-drying followed by chemical crosslinking. Specifically, an 11.72% aqueous solution of PVA prepared via autoclaving 1 h at 121°C was cooled down to 50°C under stirring at 1000 rpm inside a thermostatic bath. G was added to the PVA solution to reach the following weight ratios between the synthetic polymer and the biomolecule: PVA/G 100/0, 90/10, 80/20, 70/30 and 50/50 w/w. For each composition, a soft paste was thus obtained heating up the mixture to 71°C for 10 min. Thereafter, the bath temperature was cooled down again to 50°C, and 0.36% (w/v) of SDS was added to the mixture whereas stirring at 1000 rpm was continued for 10 min, leading to the obtainment of a voluminous dense foam that was spread on petri dishes with a large spatula, frozen at -80°C and lyophilized. The dried foams were stabilized by crosslinking via GTA vapors for 72 h at 37°C in a sealed cabinet and then flushed under the chemical hood for 48 h, thus obtaining biostable sponge-like materials. For in vitro experiments, the scaffolds were cut into cylinders of 5 mm diameter and 2 mm thickness using a puncher. and the samples were sterilized in absolute ethanol overnight, soaked in 2% Glycine for one hour to block the un-bound sites of GTA, rinsed three times with sterile 1× phosphate-buffered saline (PBS) of 15 minutes each, and finally dried under laminar flow cabinet inside Well-plates. All scaffolds will seeded with MSC and HUVECs cells to select the best performing scaffold.

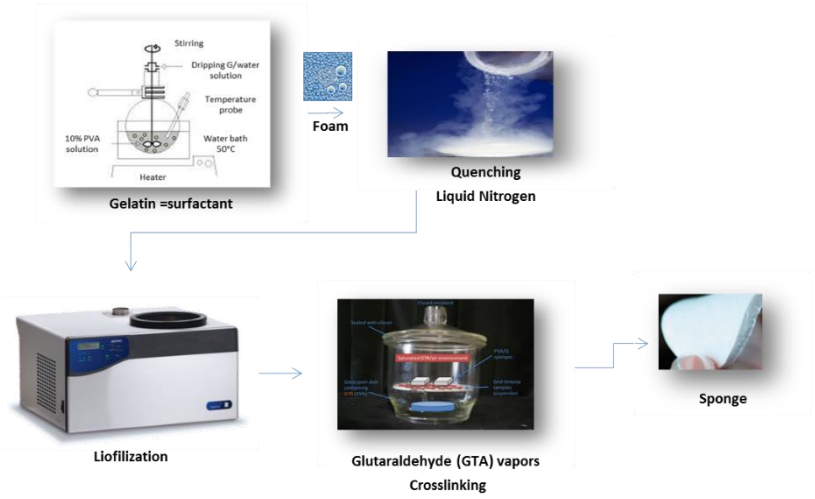

***Figure 5.2**. Scaffold fabrication*

2.2.2. Morphological analysis of the scaffolds.

Scanning electron microscopy (SEM) was used to analyze scaffold morphology, as well as cell attachment, morphology and spreading on to the scaffolds. The biological samples were dehydrated with two washings in 80% ethanol for 10 minutes, followed by one washing in 90% ethanol for 15 minutes, one in 95% ethanol l and finally three washing, each one of 20 minutes, in absolute ethanol. The biological samples were dried in oven at 37°C for two hours prior to sputter coating, Differently, plain scaffolds were obtained after freeze-drying, so they could be sputter coated directly. All the dried samples were thus mounted on aluminum stubs, sputter-coated with gold (Sputter coater Emitech K550, Quorum Technologies Ltd, United Kingdom.), and observed via SEM. (JSM-5200, JEOL Ltd, Tokyo, Japan; Zeiss EVO MA 10, Carl Zeiss Industrielle Messtechnik GmbH, Oberkochen, Germany).

2.2.3. Pore size analysis of the scaffolds

Pore size and distribution of the lyophilized sponges were investigated via mercury intrusion porosometry (MIP). An Hg intrusion porosimeter (Pascal 140; Carlo Erba, Pomezia, Italy) equipped with an automatic recording of intruded Hg volume was used to assess pore size ranging in 0.007–300 μm diameters (n = 2). The distribution of pore volumes was obtained from the derivative curve of the cumulative intruded volume, as a function of pore diameter, using the following equation (Washburn, 1921):

$$d = 10 \cdot 4\gamma \cos\frac{\theta}{P}$$

which states that the diameter d (μm) of the Hg-filled pores (i.e., open pores), considered with a cylindrical shape, is inversely proportional to the intrusion pressure P (kg/cm^2), if the Hg surface tension γ and the contact angle θ between Hg and the material are constant. In our experimental conditions, γ = 0.48 N/m and θ = 141°.

2.2.4. Cell isolation and expansion of MSC

The human MSCs cells were obtained for bone narrow aspirates from patients undergoing hip surgery at the Orthopedic Division of “Azienda Ospedaliera Universitaria Pisana”. Bone marrow samples were collected after informed consent, treated anonymously and in conformity to the principles expressed by the Declaration of Helsinki. Briefly, the bone marrow aspirate was diluted 1:4 in sterile saline and layered on Ficoll as a density gradient inside sterile tubes. The mononuclear cell layer), selected via centrifugation at 400×*g* for 15 min, was suspended in culture medium, containing low-glucose D-MEM, 2 mM L-glutamine, 100 IU/mL penicillin, 100 mg/mL streptomycin, 5 µg/ml levofloxacin and 10% (v%) heat-inactivated FBS. The mononuclear cells were counted using a Burker chamber and plated at a density of 200,000 cells/cm^2 in tissue-culture polystyrene flasks. After 24 hours, the MSCs were purified from other non-adherent mononuclear cells via washing with sterile saline. When the cultures reached about 80% confluence, the MSCs were detached by using 0.25% trypsin and replated at a density of 1000 cells/cm^2 to allow their expansion. Cell cultures were carried out in incubator under standard conditions, namely 37°C, 95% relative humidity, and 5% CO_2/95% air environment. The MSCs were seeded at passage 2 on the PVA/G scaffolds at a density of 200.000 cells/scaffold (1st experiment) and 250.000 cells/scaffold (2nd experiment) for selecting the best scaffold composition.

2.2.5. MSCs characterization

Once isolated, the MSCs were committed towards multi lineage differentiation in order to assess their multipotency, and thus confirming their constitutive stemness. The MSCs were committed towards three well-known mesodermal lineages: osteogenic, adipogenic and chondrogenic.For inducing adipodifferetiation 50.000 cells/ml were risuspended in NH Adipodiff medium and 1,5 ml of cellular suspension was seeded which corresponds to 75.000 cells. the medium was changed every three days and the culture was performed 2-3 weeks, until the formation of fatty droplets inside the cytoplasm was visible under the inverted light microscope. After, the cells were fixed with 1 %Formalin and stored for further analysis. For chondrogenic differentiation, 250.000 MSCs were resuspended in 1 ml of NH Chondrodiff medium and centrifuged at 150×*g* for 5 minutes (600 rpm) to obtain a cell pellet which was cultured for 3 weeks upon medium change every three days. At the endpoint, the pellet was fixed using 1%Formalin. To assess osteodifferentiation, the MSCs were cultured using Osteodiff Medium. The MSCs were plated at a density of of 60.000

cells/2 ml, and the medium was replaced evrey three days for three weeks. The cells were fixed using 1%Formalin.As functionality assays for these three protocols, Osmium tetroxide staining, which stains fatty droplets in black, Alcian Blue staining at pH 1, which stains sulphated glycosaminoglycans in cyan, and von Kossa staining, which stains calcium salts in black , and were performed for adipogenesis, chondrogenesis and osteogenesis (Figure 3.5)

Adipogenic differentiation was detected by incubating the cells with 1% w/v OsO4 PBS 0.1 M pH 7.2 (for 1 h. This staining shows the presence of lipid droplets in the cytoplasm, typical of the adipocytes. The other cytochemical staining were performed as described o later in paragraph.

2.2.6. Isolation and culture of MPC

Bone marrow aspirates (5-10 ml) were obtained under local anesthesia from orthopedic patients, undergoing routine total hip replacement surgery, after written consent. Bone marrow mononuclear cells (BMMNCs) were collected by density gradient centrifugation on Ficoll-Paque Plus, washed twice in sterile saline and plated in DMEM-LG supplemented with 10% of pooled human AB sera (AB human sera, male only, from Lonza (Euroclone) at 0.8x106/cm^2 on hydrophobic untreated 25 cm^2 flasks (Sarsted). After 8-10 days the cells were detached with TrypLE Select (Life Technology) for 15-20 min at 37°C, 5% CO2 and counted.

2.2.7. Endothelial differentiation of MPC

To perform endothelial differentiation, MPC were plated at 10,000 cells/cm^2 in EndoCult on 25 cm^2 tissue culture flasks coated with human fibronectin at 5 µg/cm^2 for 30 minutes at room temperature, then rinsed, and cultured for 2-3 weeks. Pre-endothelial cells were then detached with trypsin digestion and counted. At the same time, MPCs were also cultured for 2-3 weeks in EGM-2 medium on human fibronectin coated 25 cm^2 flasks (10.000 cells/cm^2). Such cells, supposed to be pre-endothelial cells, were detached with trypsin digestion and counted. Pre-endothelial cells obtained from both EGM-2 and Endocult cultures, were seeded on scaffolds (500.000 cells/scaffold) which were subsequently coated with Matrigel (100 µl/scaffold) (Becton Dickinson), incubated at 37 °C, 5% CO_2 for

1 hour, then covered with EGM-2 medium containing 50 ng/ml of VEGF and incubated again for 20 hours at 37°C, 5%CO_2.

Parallel cultures were performed on 8-well chamber slides (1,5 cm^2/well) previously coated both with human fibronectin (100 µl/well) and with Matrigel (100 µl/well), the latter to obtain terminal differentiation. Slides with MPC and MSC were performed as controls.

Slides from pre- and terminal differentiation were fixed for 10 min in Buffer formalin 1 % at 4°C and constructs (scaffolds MPC/Endothelial cells) were fixed in Neutral Buffer formalin 4 % overnight for immunocytochemistry analysis.

2.2.8. Culture differentiation of HUVEC

HUVEC were purchased from Promocell© Laboratories. Briefly, cryopreserved cells were thawed and seeded in a 25 cm^2 flask previously filled with EGM-2 medium and incubated for 30 minutes at 37°C, 5%CO_2. After 24 hours the medium was changed medium and when confluence (70-90%) was reached, cells were trypsinized and replated for further expansion.

2.2.9. Culture and differentiation of HUVEC on PVA/G scaffolds

HUVEC were loaded on different PVA/G (100/0, 90/10,80/20, 70/30 and 50/50) scaffolds (200.000 cells/scaffold). First, scaffolds were sterilized in absolute alcohol for minimum 24 hours, rinsed with 2% glycine solution for 1 hour, following three washes of 15 minutes with PBS. Then, HUVEC were detached with 1 ml of Trypsin 0.25 % (v/v) for 5 minutes in CO_2 incubator and centrifuged for 5 minutes. The cell pellet was resuspended at 200.000 cells/20 µl in gelatin solution 2% and 20 µl of cell suspension were loaded on each scaffold, which were let previously dry in a 6 well plate under the biohood. The plate was then incubated for 1 hour at 37°C, 5% CO_2 and, subsequently, 1 ml of EGM-2 medium was added.

2.2.10. Culture and differentiation of MSC on PVA/G scaffold

MSC (250.000 cells) were loaded on different types of PVA/G scaffolds (100/0, 90/10,80/20, 70/30 and 50/50). First, scaffolds were sterilized in absolute alcohol for minimum 24 hours, rinsed with glycine 2% for 1 hour, following three washes of 15

minutes with PBS. Then, MSC were detached with 1 ml of Trypsin 0.25 % (v/v) for 5 minutes in CO_2 incubator and centrifuged for 5 minutes. The cell pellet was resuspended at 250.000 cells/20 µl in 2% gelatin solution and 20 µl of the cell suspension was loaded on each scaffold, previously dried in 6 well plate. The plate was then incubated for 1 hour at 37°C, 5% CO_2 and, subsequently, 1 ml of DMEM-LG supplemented with 2 mM Glutamine, 10 % FBS, 100x penicillin/streptomycin, 5 mg/ml Levofloxacin and 100x fluconazole. The next day The medium was changed and differentiation factors such as dexamethasone (0.1 µM), β-glycerophosphate (10 mM), and ascorbic acid (50 µg/ml) were added twice a week to the medium for 3 weeks.

2.2.11. Co-culture of osteodifferentiated MSCs and HUVECs on the selected PVA/G scaffold

The co-culture was performed on the selected scaffold, which resulted best suitable for both HUVEC and MSC, On this scaffold, the osteodifferentiation process was performed for 21 days before HUVEC seeding, in order to have a mature bone ECM. The MSC/scaffold constructs were let rest 1 day inside a culture medium constituted by 1/1 osteogenic medium/EGM-2 prior to HUVEC seeding. HUVEC were seeded at a density of 200.000 cells per scaffold, and differentiated for 24 hours, according to the following conditions:

1) On live osteoblast/scaffold constructs, HUVEC were seeded (i) with Matrigel® (100 µl of Matrigel® previously warmed at 37°C for 1 hour); and (ii) without Matrigel®.
2) On lysed osteoblast/scaffold constructs, HUVEC were seeded i) with Matrigel® (100 µl of Matrigel® previously warmed at 37°C for 1 hour); and (ii) without Matrigel®.

The first condition served to assess the necessity for Matrigel® for HUVEC differentiation in presence of bone ECM, while the second condition served to assess the influence of osteoblast-endothelial cell cross-talk for HUVEC differentiation. In this case, the HUVEC were seeded on a scaffold containing only a bone pre-generated ECM, without living osteoblasts. The cell lysis was performed adding to the constructs 2 ml of sterile distilled water inside sterile tubes, then freezing (at -81°C for 1 day) and thawing (at 37°C for 30 min) under sonication. The pre-generated ECM scaffolds were rinsed with saline and dried under the biohood before seeding.

After seeding HUVEC, 100 µl of EGM-2 medium were added to the constructs and pre-generated ECM scaffolds, and placed in incubator (37°C, 5% CO_2) for 30 minutes. In the samples treated without Matrigel® , 500 µl of EGM-2 medium was added. After 18 hours of differentiation, the samples were fixed with 4% neutral buffered formalin overnight.

2.2.12. Co-Culture of MSC and pre-endothelial cells derived from MPC

MSC and pre endothelial cells were obtained from bone marrow samples, the latter via MPC, as described earlier in paragraphs 2.2.6 and 2.2.7. On the selected PVA/G scaffold, MSC were osteodifferentiated for 14 days using OsteoDiff Medium (which accelerates the differentiation with respect to regular osteogenic medium) and live osteoblast constructs were used for endothelial cell seeding and differentiation. Specifically, the constructs were improved adding more MSCs (500.000 cells) and endothelial cells (400.000 cells) with respect to 2.2.11 paragraph, namely Co-culture of osteodifferentiated MSC and HUVEC on the selected PVA/G scaffold.The experiment as described in paragraph 2.2.11 was repeated using:

1) HUVEC with (i) undiluted Matrigel® and, (ii) 1:1 diluted Matrigel®, to optimize the previous results;
2) MPC-derived pre-endothelial cells culture with EGM-2 and Endocult media, to assess the possibility to assemble a completely autologous pre-vascularized bone substitute.

Treatments	Cellular density
EGM-2/Endothelial	25.000
Endo/MPC	250.000
HUVEC	25.000
MPC/EGM-2	25.000

Table 1. *Cellular density use for each treatment*

The chambers slide after culture were fixed for 10 minutes in 4% phosphate-buffered formalin for immunohistochemically analysis.

2.2.13. ALAMAR BLUE™ assay.

The metabolic activity of the cells grown on the scaffolds was monitored along the culture time using the alamarBlue® assay. This bioassay incorporates a REDOX indicator resulting in color change of the culture medium according to cell metabolism. Moreover, it can be performed multiple times on the same samples on account of its negligible toxicity. The data were acquired according to the manufacturer's instructions and expressed as percentage of reduced alamarBlue® ($\%AB_{red}$). Briefly, samples (n = 2) and negative controls (n = 2), namely, media plus dye without cells, by using equation #1 from the manufacturer, were incubated for 3 h at 37°C with the alamarBlue® dye diluted in 3 ml of culture medium for each scaffold, according to the manufacturer's recommendations. This method for calculation against negative controls is useful to assess $\%AB_{red}$ increase of the same samples along a timeline. The total quantity of dye solution per well was chosen in theoretical excess, in order to accomplish any metabolic activity increases all the samples in long term cultures. Viability tests were performed every week after seeding for MSC and prior and post differentiation for HUVEC. At each time-point, 100 µL of supernatant from sample or control was loaded into 96-well plates; then, excess supernatant was removed from the cultures and replaced with fresh CM. The absorbance (λ) of supernatants was measured with a spectrophotometer (Victor 3; PerkinElmer, Waltham, MA, USA) under a double wavelength reading (570 nm and 600 nm). Finally, $\%AB_{red}$ was calculated by correlating the absorbance values and the molar extinction coefficients of the dye at the selected wavelengths, following the protocol provided by the manufacturer. The equation applied (Eq. 1) is shown below, in which: λ = absorbance, s = sample, and c = negative control:

$$\%AB_{red} = 100 \cdot \frac{\left(117{,}216 \cdot \lambda_{s(570\,nm)} - 80{,}586 \cdot \lambda_{s(600\,nm)}\right)}{\left(155{,}677 \cdot \lambda_{c(600\,nm)} - 14{,}652 \cdot \lambda_{c(570\,nm)}\right)}$$

The data were processed using InfoStat© Sotfware for statistical analysis

2.2.14. Fixation, Paraffin Embedding and sectioning

Chambers slides seeded with HUVEC and MPC cells and 6-well culture plates seeded with MSC differentiated towards osteogenic and adipogenic lineages were fixed in 1% v/v/

neutral buffered formalin diluted in PBS 1X for 10 min at 4°C and, after formalin removal, they were air dried and stored at 4°C.

Seeded PVA scaffolds and chondrogenic pellet were fixed in neutral buffered formalin 4% w/v overnight at 4°C, washed with PBS 1X 4 times for 15 minutes and stored in 70% Ethanol. Then, they were dehydrated with a graded series of ethanol: 80% ethanol for 30 min, 95% ethanol for 45 minutes, absolute ethanol 3 h (three changes of 1 h each one), then they were clarified in Xylene twice or 45 min. All the steps were performed in a bath at 40°C. Subsequently, specimens were rinsed in liquid paraffin in a vacuum oven at 60°C for 2h, placed in wax embedding box and air dried. Paraffin blocks were sectioned with a standard microtome to obtain 6-μm-tick sections that were mounted on glass slides and stored in a bath at 37°C for 1 day, in order to permit a correct adhesion of the paraffin sections on the slides.

2.2.15. Cytochemistry

von Kossa

This staining was performed on MSC differentiated towards osteogenic lineage in order to reveal, in black, deposits of calcium phosphate, typical of bone mineralized matrix.

After two washing in distilled water, the cells were incubated with 1% w/v silver nitrate exposed to light for 15 min, 0,5% w/v Pyrogallol for 2 minutes and 5% w/v sodium thiosulphate for 2 min. All the solutions were maked in distilled water. After each passage washings in distilled water were performed. The counterstaining was performed incubating cells with 0,1% w/v nuclear fast red diluted in a distilled water solution containing 5% w/v aluminium sulphate, for 5 min and washing in tap water for 5 min in order to reveal the reaction.

Osmium tetroxide

This staining was performed on MSC differentiated towards adipogenic lineage in order to reveal, in black, the presence of lipidic drop in the cytoplasm, typical of the adipocytes.

The cells were incubated with 1% v/v osmium tetroxide diluted in PBS 1X for 1 h, then washed twice in PBS1X for 10 min.

2.2.16. Histochemistry

Before each staining, sections of both seeded PVA and chondrogenic pellet were deparaffinized rinsing them in xylene twice for 7 min, then they were rehydrated by absolute ethanol three times for 7 min and washing them in distilled water for 5 min; after each staining, the sections were dehydrated by three washes in absolute ethanol and clarified by three washes in xylene (5 min for each step), then they were mounted with a coverslip using a mountant medium diluted in xylene (DPX mountant).

All stainings were performed on seeded PVA; on sections of chondrogenic pellet, only Alcian Blue pH1 was performed.

Hematoxylin-Eosin (H&E)

H&E staining was performed in order to reveal cell general morphology and scaffold colonization.

Sections were incubated with Mayer's hematoxylin for 5 min and washed with tap water to reveal the staining; then, they were incubated with 0.003% w/v eosin in distilled water for 1 min and washed in distilled water.

von Kossa

von Kossa staining was performed as previously described.

Alcian Blue pH1 and pH2.5

This staining showed, in cyan, the presence of sulphated acid (pH1) and generic acid (pH2.5) glycosaminoglycans in the samples.

Sections were incubated by alcian blue solutions for 30 min, then with revealing solutions for 10 min, according to manufacturer's instructions. After washing in distilled water, specimens were counterstained with Nuclear fast red as previously described for von Kossa staining.

Periodic acid Schiff (PAS) reaction

PAS staining revealed, in magenta, the presence of glycoproteins in the samples.

Sections were incubated with 1% w/v periodic acid solution in distilled water for 10 min and air dried prior to incubate them with Schiff reagent 15 min. The reaction was revealed by washing in tap water for 10 min. Counterstaining was performed incubating the sections

with Mayer's hematoxylin for 5 min and revealing the reaction washing them in tap water 5 min.

2.2.17. Immunocytochemistry

After two washing in distilled water, chamber slide seeded with MPC and HUVEC were permeabilized by 0.2% v/v Triton X-100 in PBS 1X for 10 min and washed twice in PBS 1X for 5 min. Quencing of endogenous peroxidases was performed by incubation with 0.6% H_2O_2 in methanol for 15 min in the dark. After washing in PBS 1X for 10 min, samples were incubated with 20% v/v Goat serum diluted in PBS 1X for 20 min at 37°C in order to block aspecific binding sites of the secondary antibodies. After washing in PBS 1X for 5 min, sections were incubated with primary antibodies diluted in 0.1% w/v BSA/PBS 1X solution in moisted chamber at 4°C overnight: anti-PECAM 1:50, anti-VEGF-R2 1:50, anti p-VEGF-R2 1:50, anti VEGF 1:200.Negative controls were performed incubating sections with 0.1% w/v BSA/PBS 1X solution only.
Next day, specimens were incubated with goat anti-rabbit or goat anti-mouse biotinylated secondary antibodies diluted 1:200 in 1.5% v/v goat serum-PBS 1X solution for 60 min, then with streptavidin solution for 30 min, prepared according manufacturer's instructions. In order to reveal the reaction, the sections were incubated in the substrate-chromogen solution (0.5 mg/mL 3,3 min-diaminobenzidine tetrahydrochloride containing 0.02% H_2O_2) for 5 min in the dark, counterstained with Mayer's hematoxylin for 1 min and washed in tap water for 1 min. Finally, the sections were mounted with an aqueous mountant medium. In the second part of reaction, after each passage, washings in 0.01% v/v Triton/ PBS 1X and PBS 1X solutions were performed.

2.2.18. Immunohistochemistry (IHC)

Sections of seeded PVA were deparaffinized rinsing them in xylene twice for 7 min, then they were rehydrated by absolute ethanol three times for 7 min and washing them in distilled water for 5 min. The IHC protocol steps are the same reported in the previous part. The employed primary antibodies were: anti-ALP 1:100, anti-OPN 1:2000, anti-Collagen I 1:1000, anti-Osteocalcin 1:400, anti-TGFβ-1 1: 500, anti-PECAM 1:100, anti-VEGF-R2 1:50, anti p-VEGF-R2 1:50, anti VEGF 1:200. After the counterstaining the sections were dehydrated by three washes in absolute ethanol and clarified by three

washes in xylene (5 min for each step), then they were mounted with a coverslip using a mountant medium diluted in xylene.

All the histological analysis were observed with a Nikon Eclipse Ci microscope (Nikon Instruments, Amsterdam, The Netherlands) and the images were acquired by a digital camera equipped on the microscope.

Chapter 3

3.RESULTS

3.1. SCAFFOLD CHARACTERIZATION

The five composition types of PVA/G scaffolds were characterized using SEM to assess their morphology and with MIP to assess their pore size distribution. SEM images of all scaffolds showed highly porous structures, with round interconnected pores (Figure 3.1). In addition, these diverse PVA/G compositions resulted to display decreasing surface roughness, having 100/0 the roughest and the 50/50 the smoothest surfaces.

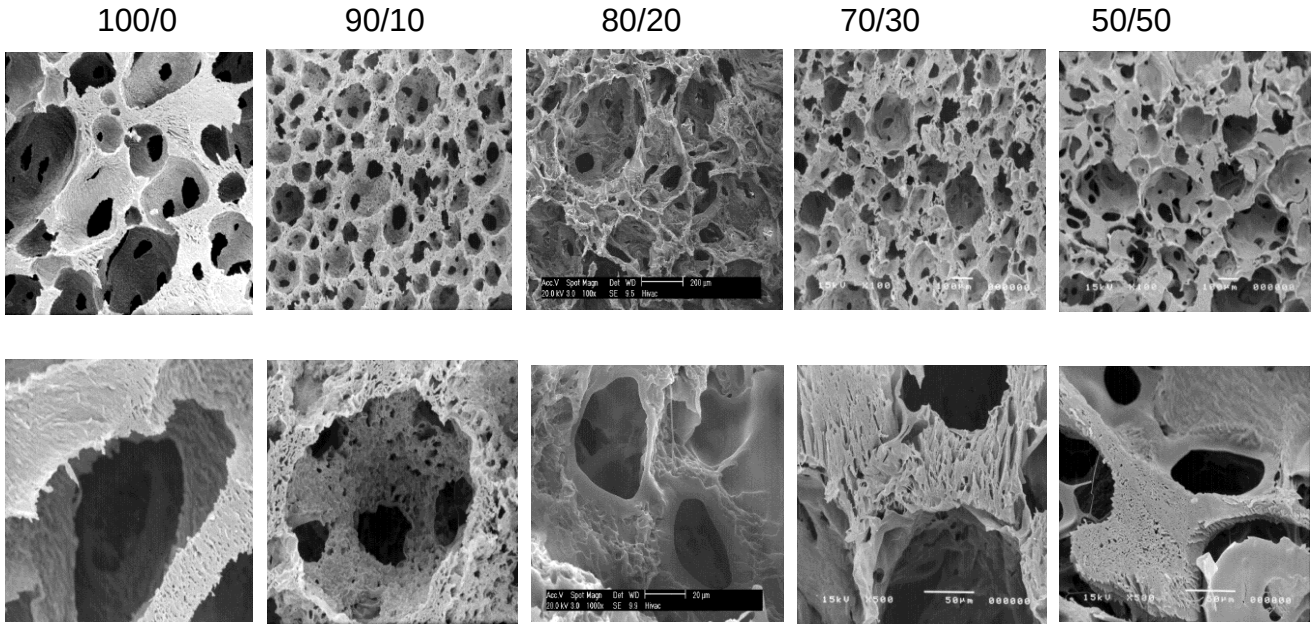

***Figure 3.6**. SEM morphology of PVA/G scaffolds at various compositions. The micrographs showed scaffold pores (upper line), and pore interconnectivity and surface roughness (lower line). The PVA/G 100/0 scaffold showedhe highest while 50/50 the smothest surface roughness.*

These PVA/G scaffolds also showed a diverse pore size distribution (Figures 3.2 and 3.3). Specifically, the higher G content scaffolds retained the higher relative volume % of pores belonging to higher diameter classes, such as 300-100 μm and 100-30 μm, while the lower G content scaffolds had higher relative volume % of pores belonging to lower diameter classes, such as 30-10 μm and 10-3 μm (and also 1-0.3 μm).

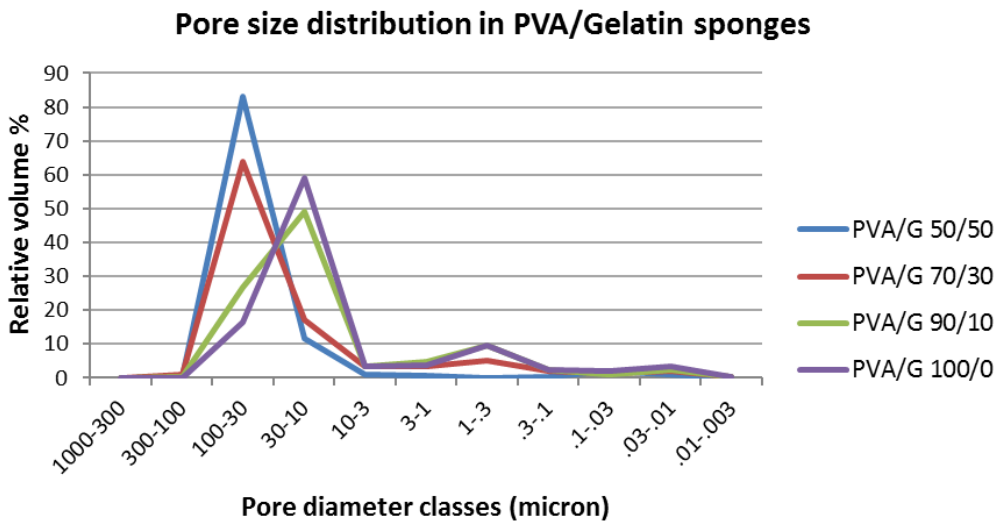

***Figure 3.2**. MIP analysis of PVA/G scaffolds with compositions 100/0, 90/10, 70/30 and 50/50 showing pore size distribution according to pre-defined pore size classes*

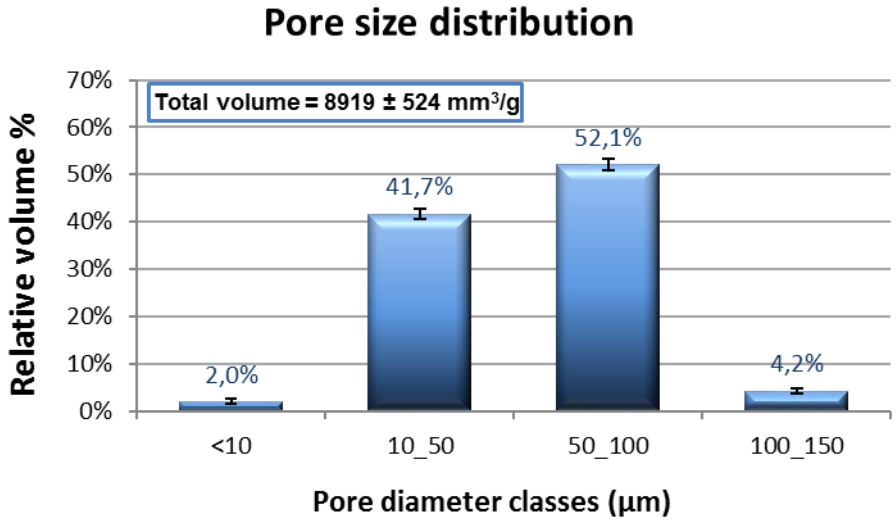

***Figure 3.3**. MIP analysis of PVA/G scaffolds with compositions 80/20 showing pore size distribution according to pre-defined pore size classes*

3.2. MSC CHARACTERIZATION

Morphology of MSCs in culture highlighted big spindle-shaped adherent cells arranged into colonies

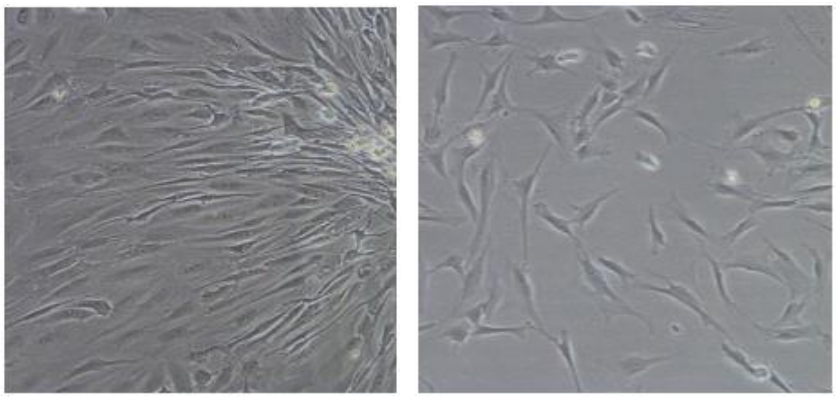

***Figure 3.4**. Inverted microscope images showing MSCs with spindle-shaped morphology, assembled into colonies, adherent cells on flasks.*

MSC were characterized through their capability of differentiating towards the three well-known mesodermal lineages (osteogenic, adipogenic and chondrogenic). The results of the differentiation assays are shown in Figure 3.5.

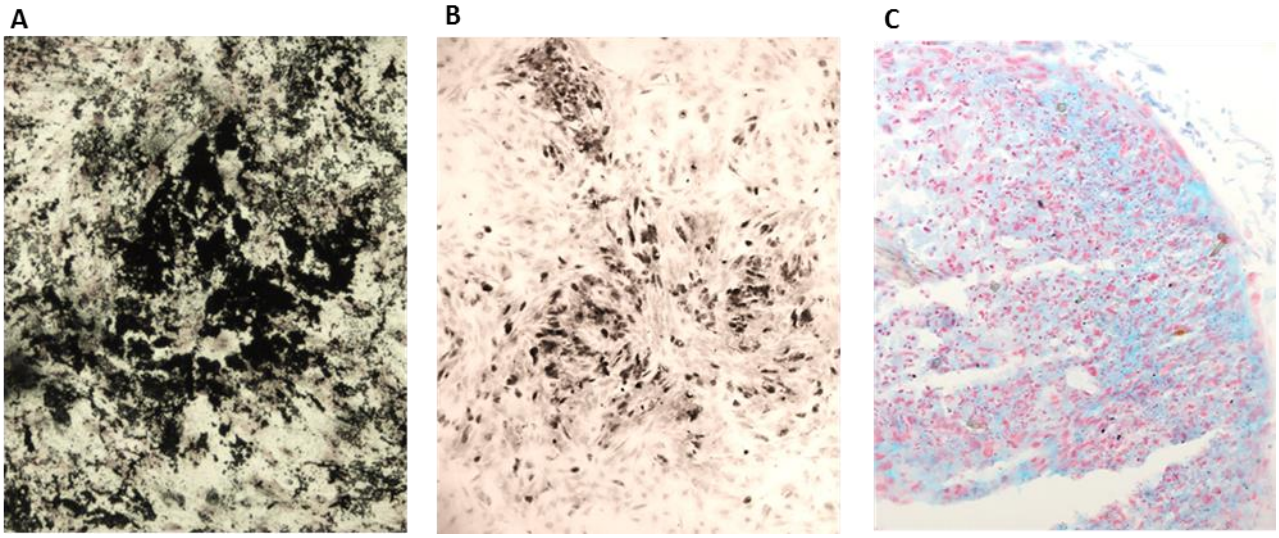

***Figure 3.5.** Citochemistry on MSC multilineage differentiation assays. Black mineralization granules, in black, in osteodifferentiated MSC (A); fatty vacuoles, in black, in MSC differentiated towards adipogenic lineage (B); sulphated glycosaminoglycans, in cyan, in MSC differentiated towards chondrogenic lineage (C).*

Osteogenic differentiation was assessed using von Kossa staining that showed mineral matrix deposition in black; adipogenic differentiation was highlighted by presence of fatty vacuoles, stained in black by osmium tetroxide; chondrogenic differentiation was displayed by the presence of sulphated glycosaminoglycans, which are stained in cyan by Alcian Blue staining at pH 1

3.3. ENDOTHELIAL CELL CHARACTERIZATION.

HUVECs showed round morphology during expansion. Upon culture in EGM-2 medium HUVECs displayed the formation of tubes or pre-vascularized structures on the chambers slides (Figure 3.6).

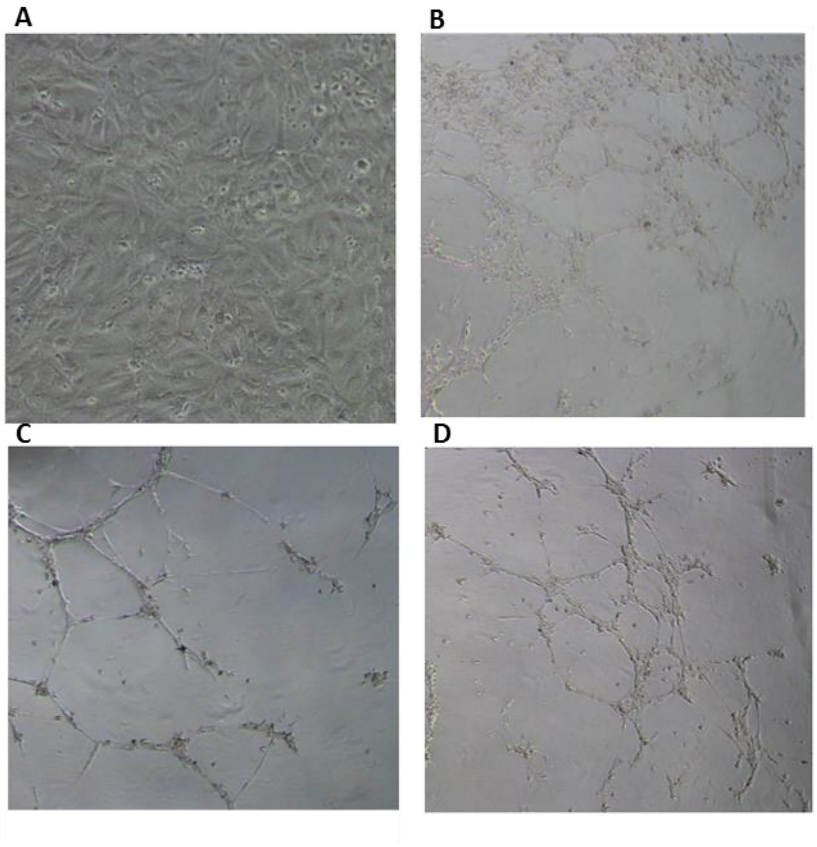

Figure 3.6 *Tube formation using EGM-2 medium. A) HUVEC in total confluency on flask ;B-D) Formation of endothelial tubes. Magnification x100.*

3.4. ENDOTHELIAL DIFFERENTIATION OF MPC AND HUVEC

Immunocytochemical analysis for endothelial markers was performed on MPC and HUVEC differentiated towards endothelial lineage in different conditions. (Figure 3.7 and 3.8)

Undifferentiated MPC: the cells showed strong positivity (++/++++; +++) for VEGF, VEGF-R2 and PECAM. No tube formation was observed.

MPC cultured in EGM2: good positivity (++) was observed for VEGF and VEGF-R2; many cells showed weak positivity (-/+; +) for PECAM, but some spindle shaped cells showed strong positivity. Negative cells were also observed. P-VEGF-R2 was not expressed. No tube formation was present.

MPC cultured in EGM2 on fibronectin: all endothelial markers were weakly expressed. Negative cells were also observed. No tube formation was observed.

MPC cultured in EGM2 on matrigel: all endothelial marker expression was negative. Tube formation was observed.

MPC cultured in Endocult on fibronectin: all endothelial markers were not expressed. No tube formation was observed.

MPC cultured in Endocult on matrigel: only few cells showed good expression of p-VEGF-R2. PECAM expression was negative. Tube formation was observed.

HUVEC cultured on fibronectin: weak expression of PECAM and p-VEGF-R2 was observed. No tube formation was revealed.

HUVEC cultured on matrigel: PECAM and p-VEGF-R2 expression was negative. Tube formation was observed.

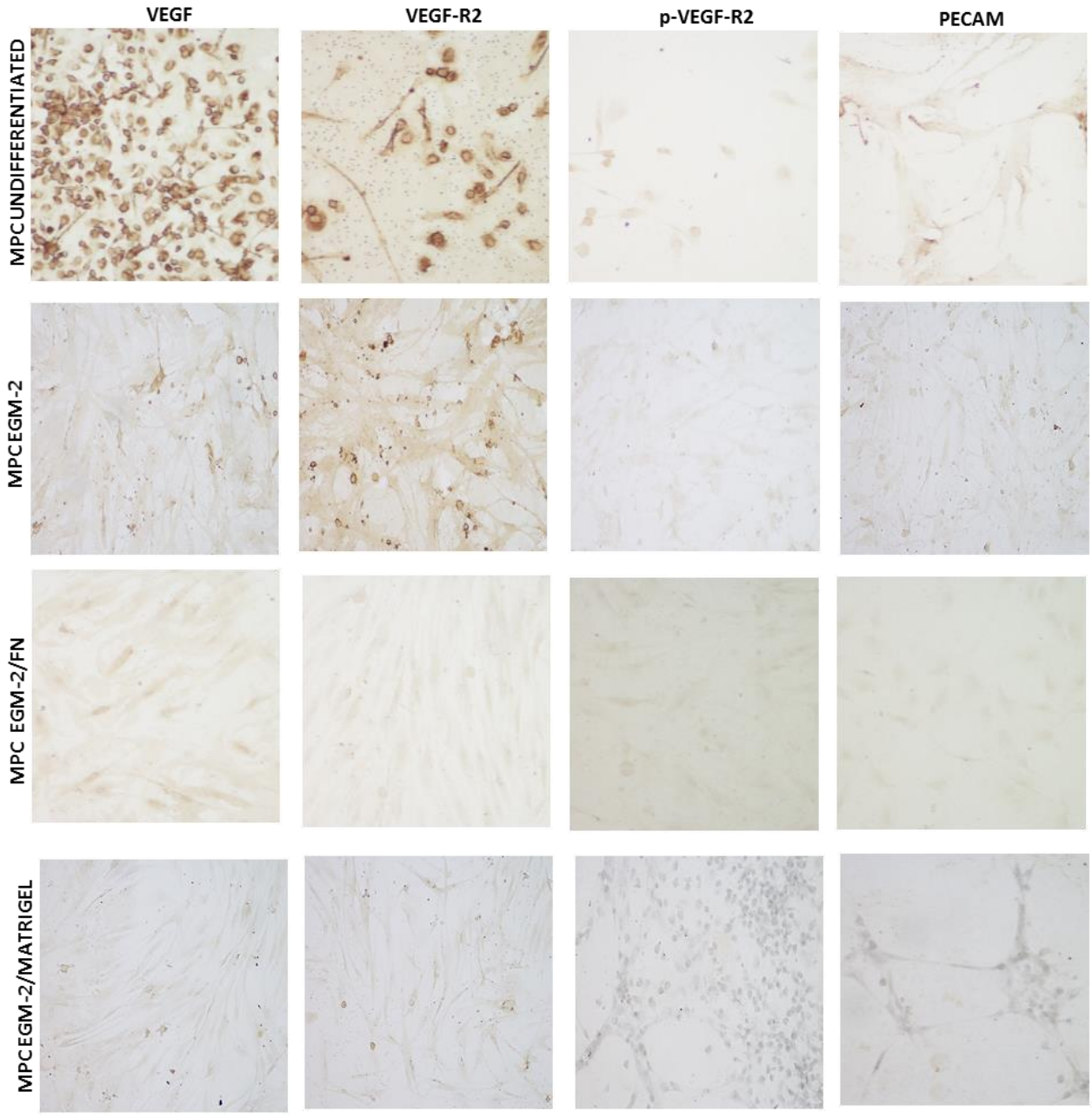

Figure 3.7. *Immunohistochemistry on MPC differentiated on endothelial cells First column: VEGF; second column: VEFG-R2; third column: p-VEGF-R2; fourth column: PECAM. First line: MPC undifferentiated; second line: MPC cultured in EGM-2; third line: MPC cultured in EGM-2 with fibronectin; fourth line: MPC cultured in EGM-2 with Matrigel. Magnification 200x*

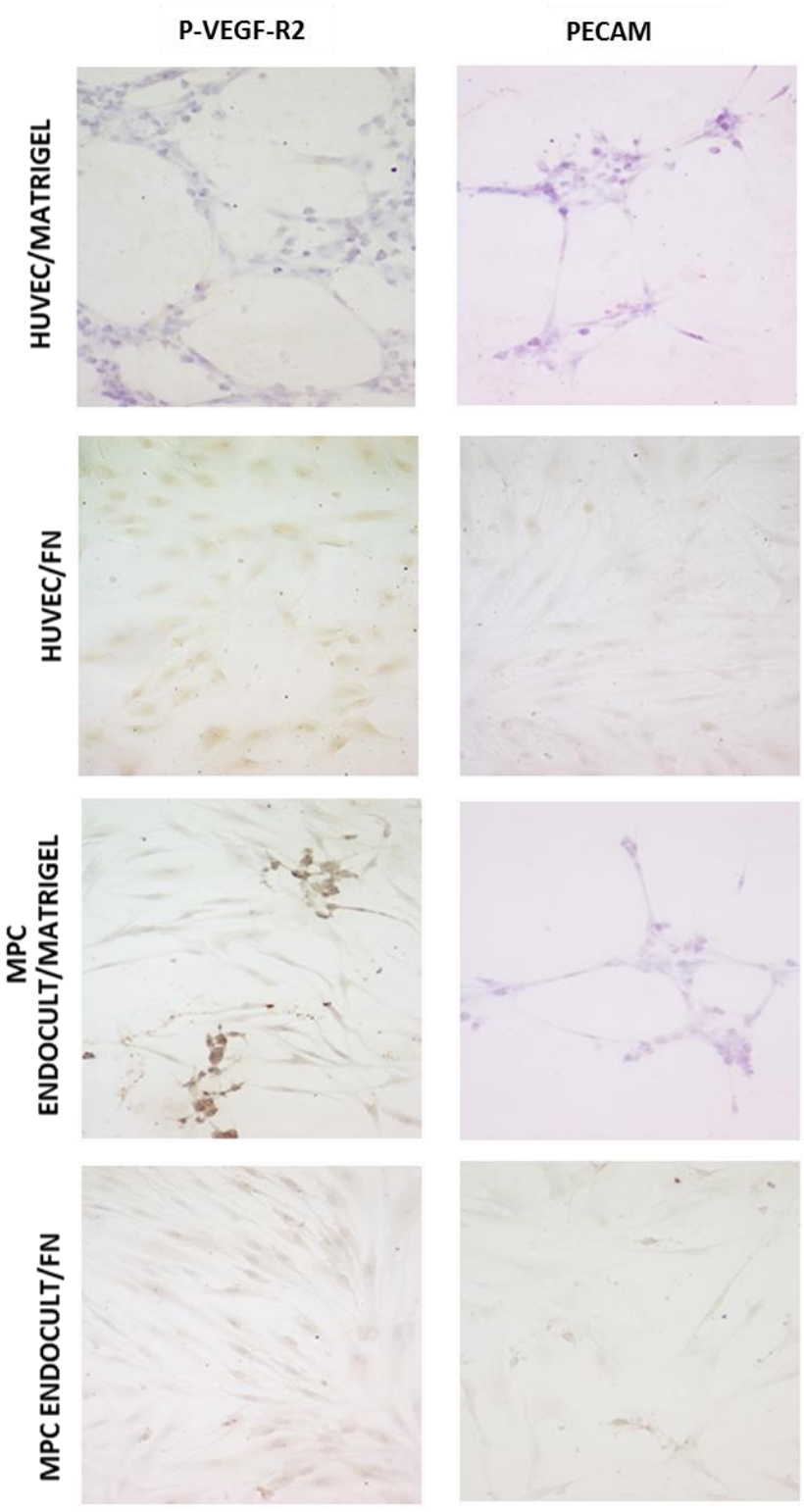

Figure 3.8. *Immunohistochemistry on endothelial differentiation of MPC and HUVEC. First column: p-VEGF-R2; second column: PECAM; First line: MPC cultured in endocult on fibronectin; second line: MPC cultured in endocult on matrigel; third line: HUVEC cultured on fibronectin; fourth line: HUVEC cultured on matrigel. Magnification 200x*

3.5 CHARACTERIZATION OF HUVECS CULTURED ON PVA/G SCAFFOLDS.

HUVECs were seeded and differentiated on the different PVA/G scaffold types to evaluate the best suitable PVA/G composition for this cell type. In the first experiment 150.000 cells/scaffold were seeded. This number was probably low, however some cells were observed on each type of scaffolds and also tube formation could be sometimes imaged (Figure 3.9). In PVA/G 50/50, the HUVECs showed a round shape, while in 100/0 they were rarely imaged. The highest presence of cells with elongated morphology was detected in the PVA/G 80/20 scaffold.

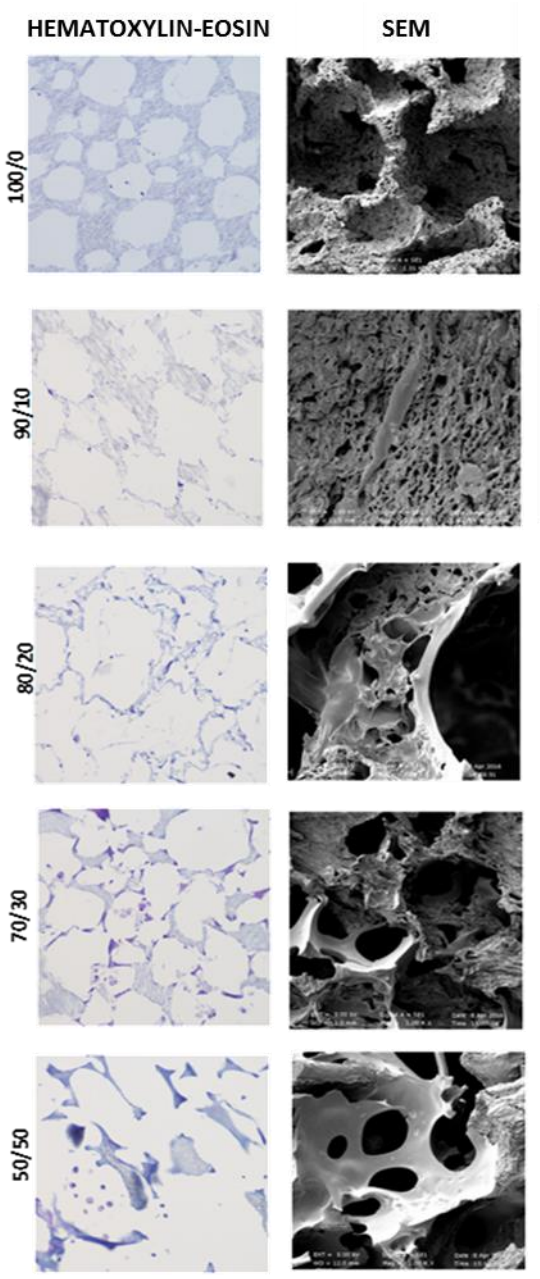

Figure 3.9. *HUVECs cultured on PVA/G scalffolds. First column: Hematoxylin-Eosin; second column: SEM images. First column magnification x200; second column: magnification x1000-3000*

3.6. CHARACTERIZATION OF MSCS OSTEODIFFERENTIATED ON PVA/G SCAFFOLDS

MSC were differentiated towards the osteogenic lineage on the PVA/G scaffolds with different compositions to assess the best suitable scaffold for bone. At the endpoint, cell morphology and mineral matrix formation were investigated using histology and SEM. The osteoinduced MSCs were able to colonize all the scaffolds and produce mineral ECM, as shown by SEM and von Kossa staining (Figure 3.10). On PVA/G 100/0 scaffold a reduced number of osteodifferentiated MSCs were observed. Von Kossa staining showed mineralization in the scaffold wall, SEM analysis confirmed scarcity of cells. On PVA/G 90/10 scaffold suffering cells were observed, granules of ECM were present in the scaffold and SEM analysis confirmed the formation of mineral matrix deposition. On PVA/G 90/10 80/20 scaffold, a good presence of cells in the pores was observed, having mainly spindle-shaped morphology while round shaped cells were scarcely detected, many pores were colonized by cells and the formation of mineralization granules was corroborated with SEM analysis and von Kossa staining. The PVA/G 70/30 scaffold showed cells in good number. The MSCs did not colonize all the scaffold and where grouped into clusters; in some cases they appeared scattered with roundish morphology. The von Kossa staining highlighted the formation of mineralization granules. In the PVA/G 50/50 scaffold the cells had round morphology and represented in limited number. However, the presence of mineral ECM was detected.

After analyzing the diverse characteristics, the distribution and localization of the cells on all the scaffolds, the PVA/G80/20 and 70/30 scaffolds appeared the most promising for reproducing a bone substitute in vitro.

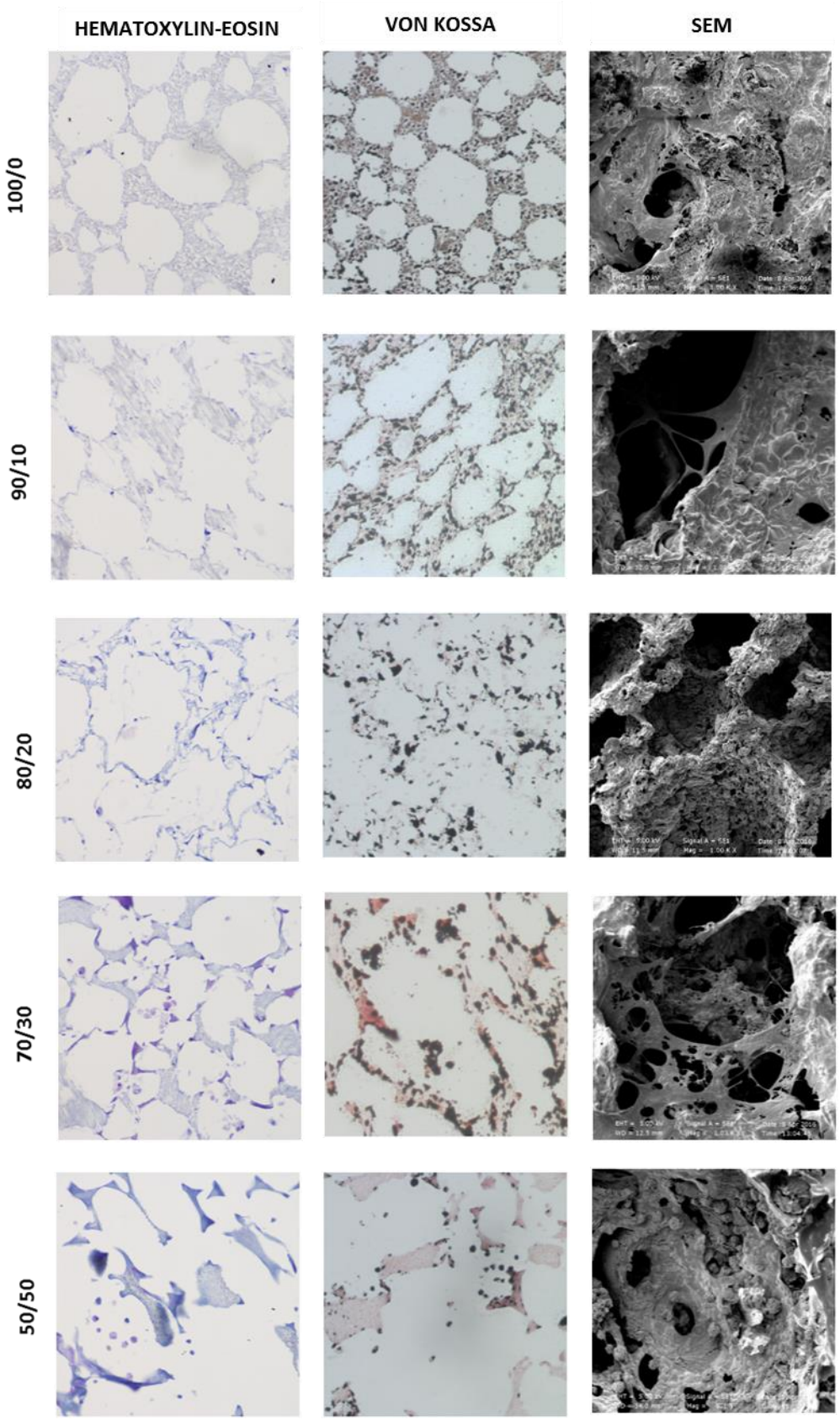

Figure 3.10. *MSCs osteodifferentiated on PVA/G scaffolds: first column: Hematoxylin-Eosin; second column: SEM images; third column: von Kossa. First line: PVA/G 100/0; second line: PVA/G 90/10; third line: PVA/G 80/20; fourth line: PVA/G 70/30; fifth line: PVA/G 50/50. First and second columns magnification x200; third column: magnification x500-1000.*

3.6.1 Alamar Blue™ Assay Results.

Results are evaluated by plotting the fluorescent (or absorbance) signal versus compound concentration and calculous percent reduction of metabolic activity. The amount of fluorescence produced is proportional to the number of living cells.(Fig 3.9 and 3.10)

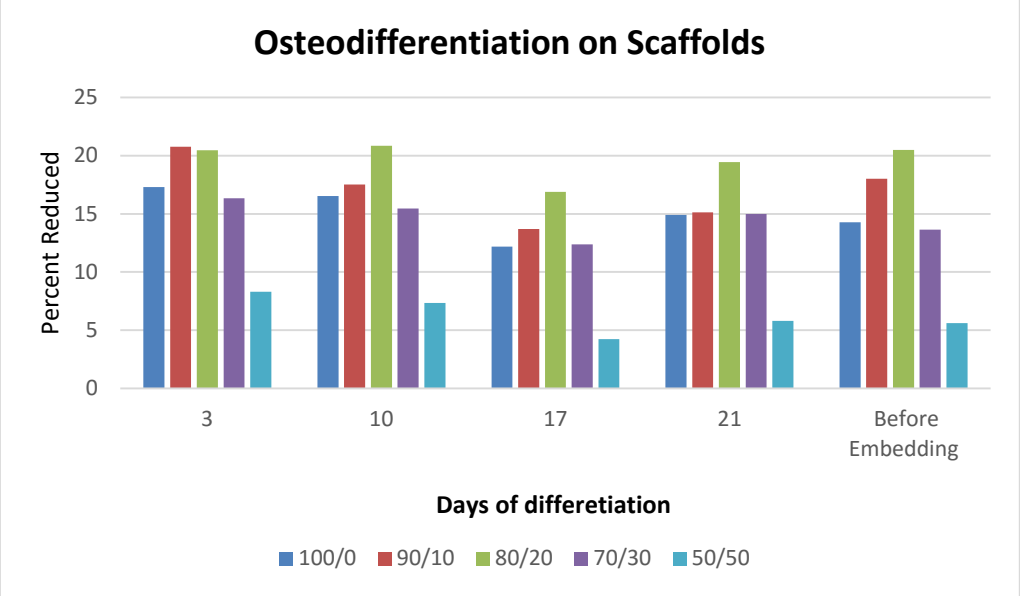

Figure 3.11 *Test of vitality Percent of metabolic reduction on PVA/G scaffold 100/0, 90/10, 80/20, 70/30 and 50/50. Average Value of six readings in one scaffold.*

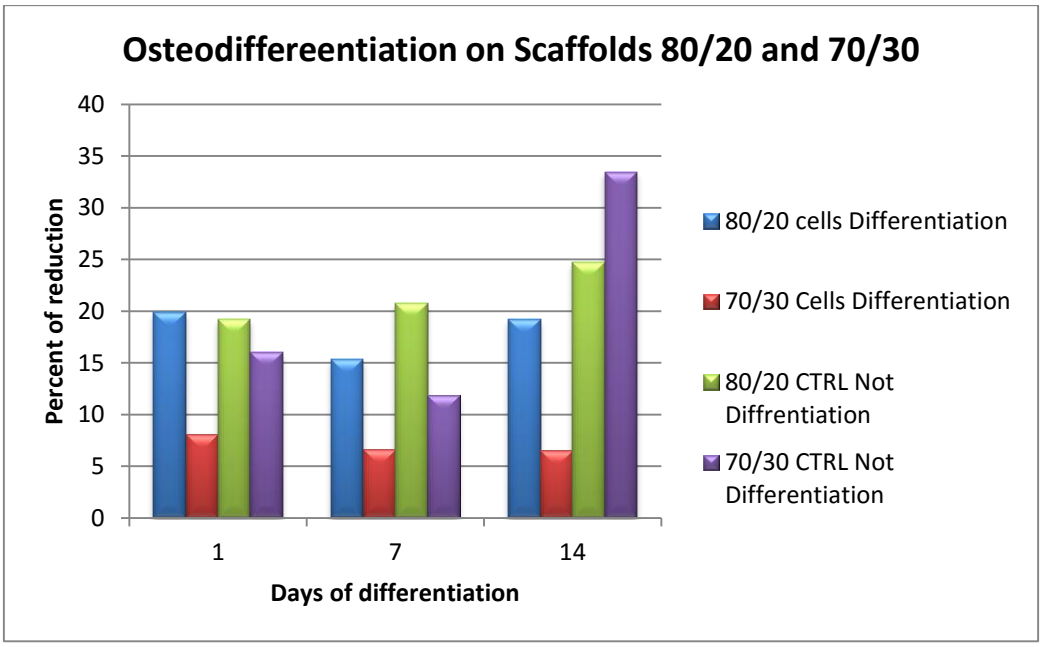

Figure 3.12*. Test of vitality Percent of metabolic reduction on PVA/G scaffold 80/20 and 70/30. Average Value of six readings in one scaffold.*

3.7. CHARACTERIZATION OF CELLULARIZED AND DECELLULARIZED PVA/HUVEC CONSTRUCTS.

3.7.1. Histochemistry

Co-culture on scaffolds of MSCs osteodifferentiated in osteoblast with HUVECs was performed and HUVEC cells was seeded 200.000 cells each scaffold in co-culture media of DMEM: ECGM/ supplement mix. Decellurarization and cellularization scaffolds were performed presenting the follow histological characteristics (Figure 3.13 and 3.14).

General morphology: H&E staining showed that, in PVA/HUVEC decellularized constructs without and with matrigel, very scarce cell colonization was evident. In PVA/HUVEC cellularized construct without matrigel some cell in the scaffold pores were observed and in PVA/HUVEC cellularized construct with matrigel small spindle-shaped cells adherent to the scaffold pore surfaces were highlighted.

Glycoprotein expression: PAS reaction performed on PVA/HUVEC decellularized constructs without and with matrigel showed that only few cells were positive (+) for this staining, highlighting scarce presence of glycorpoteins. In PVA/HUVEC cellularized construct without matrigel some cells showed good positivity (++), many others appeared negative. In PVA/HUVEC cellularized construct with matrigel the cells adherent to the scaffold pores were negative; the positivity (+) was limited to some cells present in the pore scaffold.

GAG expression: Alcian Blue staining at pH1 revealed that sulphated acid GAGs were not expressed in all the constructs. Alcian Blue staining at pH2.5 showed that in PVA/HUVEC decellularized constructs without and with matrigel, generic acid GAGs were not expressed. In PVA/HUVEC cellularized construct without matrigel, weak positivity was evidentiated. In PVA/HUVEC cellularized construct with matrigel the cells adherent to the scaffold pores and some cells present in the pores showed good positivity; some negative cells were observed.

Mineralized matrix: von Kossa staining revealed that mineralized matrix granules were deposited on the scaffold surfaces in all the constructs, mainly in the PVA/HUVEC cellularized constructs.

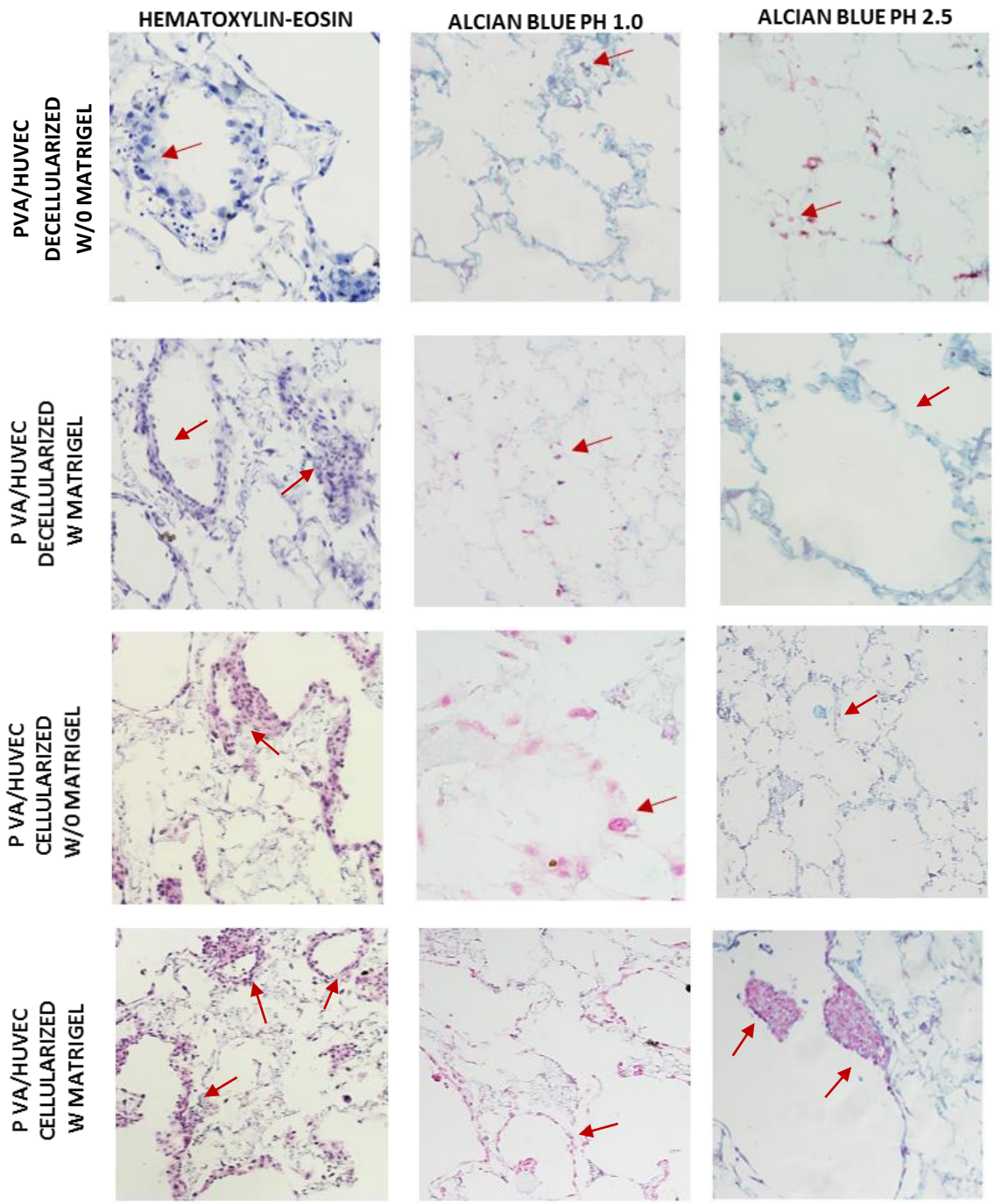

Figure 3.13. *Histochemistry on PVA/G scaffolds 80/20 with osteodifferentiated MSC and with HUVEC. First column: Hematoxylin-Eosin; second column: Alcian Blue pH 1.0; third column: Alcian Blue pH 2.5. First line: PVA/HUVEC decellularized construct without Matrigel; second line: PVA/HUVEC decellularized construct with Matrigel; third line: PVA/HUVEC cellularized construct without Matrigel; fourth line: PVA/HUVEC cellularized construct with Matrigel. Red arrows = cells. Magnification x200.*

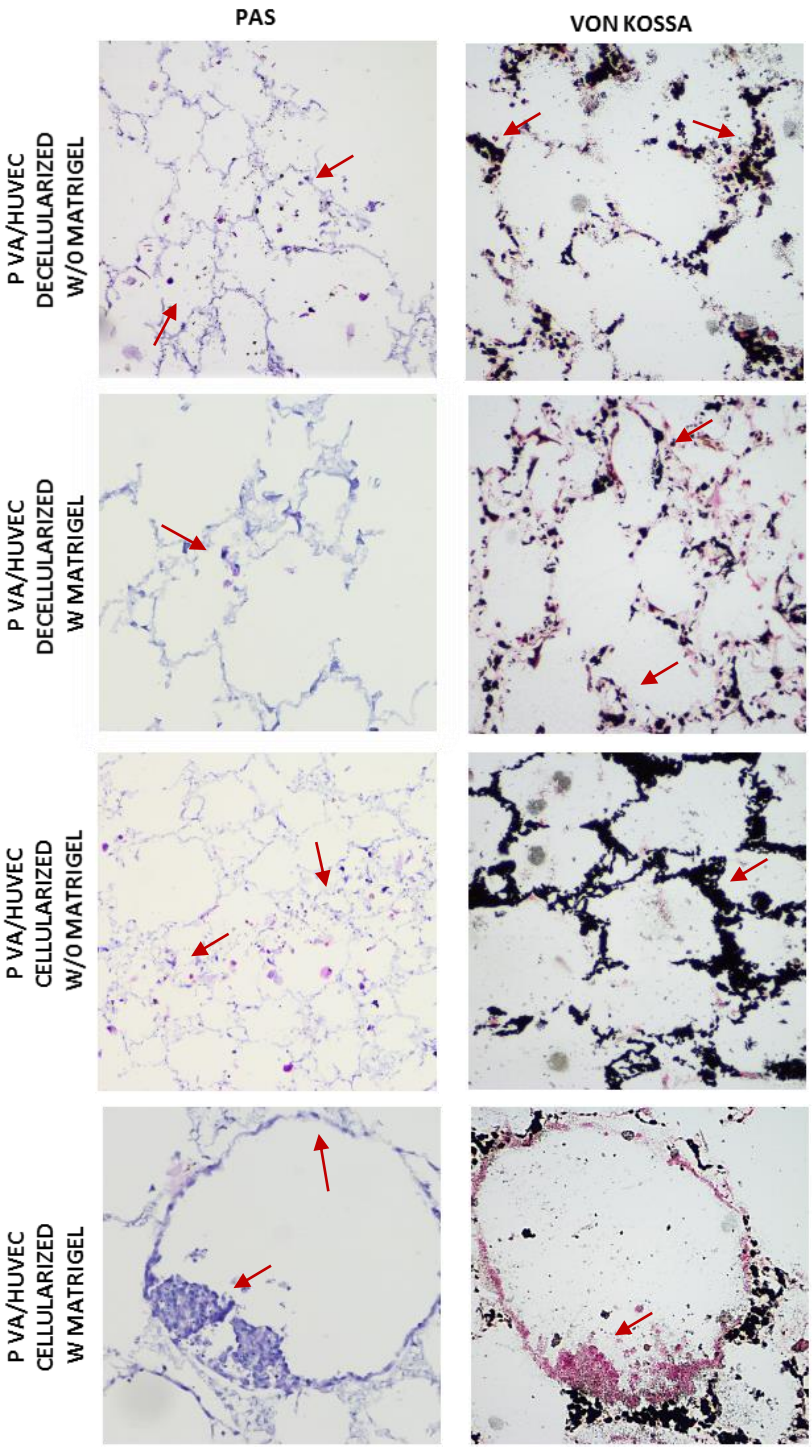

Figure 3.14. *Histochemistry on PVA/G scaffolds 80/20 with osteodifferentiated MSC and with HUVEC. First column: PAS reaction; second column: von Kossa. First line: PVA/HUVEC decellularized construct without Matrigel; second line: PVA/HUVEC decellularized construct with Matrigel; third line: PVA/HUVEC cellularized construct without Matrigel; fourth line: PVA/HUVEC cellularized construct with Matrigel. Red arrows = cells. Magnification x200*

3.7.2. Immunohistochemistry

Many osteogenic and endothelial markers were tested on PVA constructs, as reported on the table 1, in order to evaluate the most suitable for construct characterization. Due to very scarce cell presence in some constructs, not all the markers were detectable.

Between osteogenic markers detected, it was observed weak ALP expression in PVA/HUVEC cellularized constructs and weak positivity for TGFβ-1 in PVA/HUVEC decellularized construct without matrigel.

Between endothelial markers detected, strong positivity for VEGF in PVA/HUVEC cellularized constructs and PVA/HUVEC decellularized construct without matrigel was observed. Moreover, VEGF-R2 was weakly expressed in PVA/HUVEC cellularized construct without matrigel.

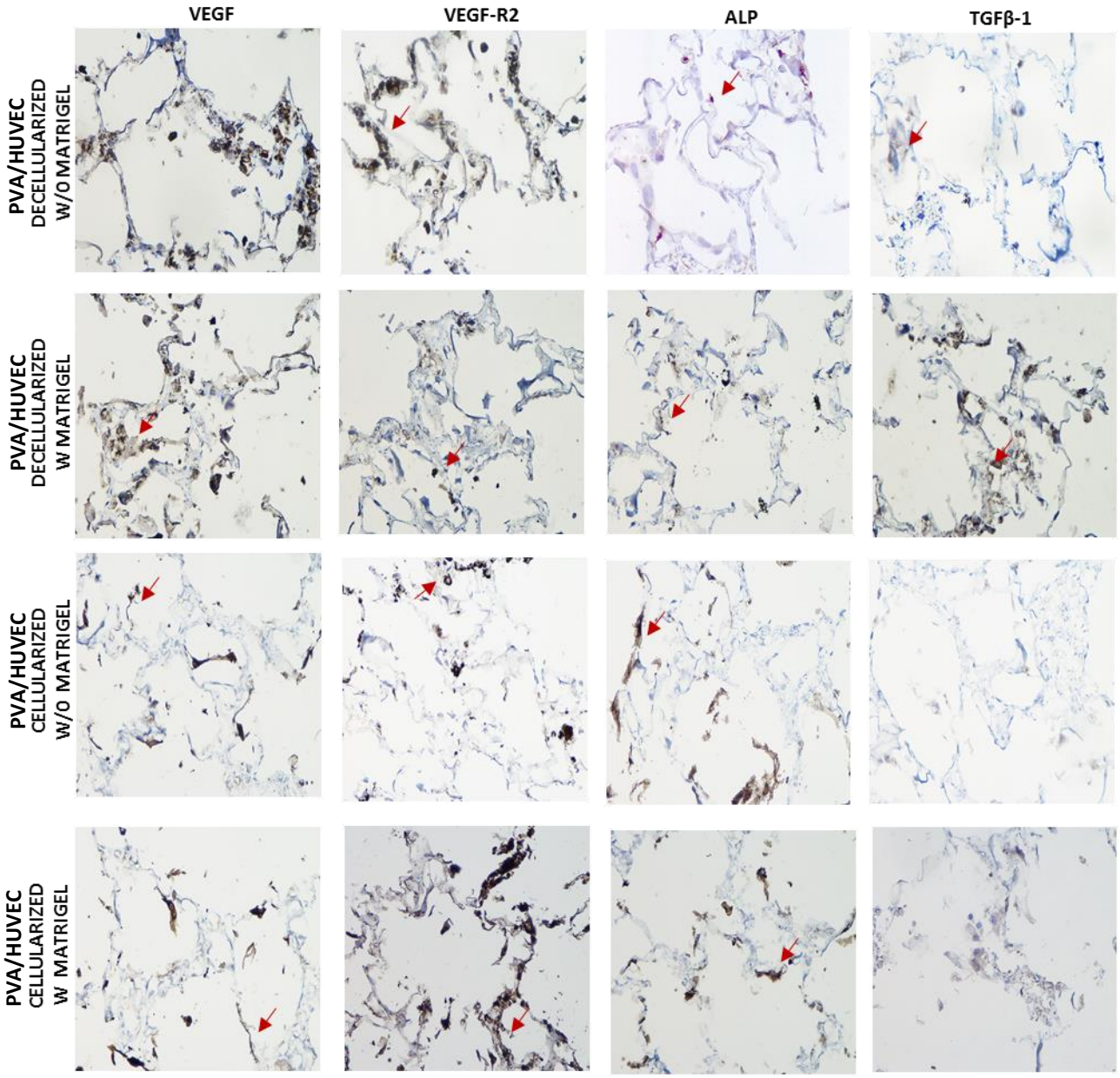

***Figure 3.15**. Immunohistochemistry for endothelial and osteogenic markers on the constructs. First column: VEGF; second column: VEGF-R2; third column: ALP; fourth column: TGFβ-1. First line: PVA/HUVEC decellularized construct without Matrigel; second line: PVA/HUVEC decellularized construct with Matrigel; third line: PVA/HUVEC cellularized construct without Matrigel; fourth line: PVA/HUVEC cellularized construct with Matrigel. Red arrows = cells. Magnification x200*

Constructs / ***Osteogenic markers***	**PVA/HUVEC Decellularized w/o Matrigel**	**PVA/HUVEC Decellularized w Matrigel**	**PVA/HUVEC Cellularized w/o Matrigel**	**PVA/HUVEC Cellularized w Matrigel**
ALP	-	ND	+	+
OPN	ND	ND	+++	+++
COLL I	ND	ND	ND	ND
TGFβ-1	-/+	ND	-	-
OCN	-/+	ND	ND	ND
Endothelial markers				
VEGF	+++	ND	+++	+++
VEGF-R2	ND	ND	-/+	-
p-VEGF-R2	-	ND	-	-
PECAM	+;-	ND	-	-

Table 1. *Osteogenic and endothelial markers tested on the constructs by IHC. ND = not detectable; - = negativity. -/+; + = weak positivity. ++ = good positivity. +++ = strong positivity.*

3.8. CHARACTERIZATION OF PVA/HUVEC AND PVA/MPC CELLULARIZED CONSTRUCTS.

3.8.1. Histochemistry

General morphology: H&E staining showed a strong scaffold colonization in the PVA/ HUVEC constructs with diluted and not diluted matrigel. The cells having a spindle-shape morphology were located in the scaffold pores as well as were adherent to the pore walls. The cells having round morphology were located in the scaffold pores only. In PVA/MPC in EGM2, both isolated and clustered cells were observed having spindle-shape and round morphology. In PVA/MPC in Endocult strong scaffold colonization was observed: the cells, having different shape, colonized scaffold pores and cell strips were detected to the scaffold periphery.

Glycoprotein expression: PAS reaction showed that in PVA/HUVEC construct with diluted matrigel glycoproteins were scarcely expressed. In PVA/HUVEC construct with not diluted matrigel, strongly PAS-positivity was observed in rounded cells, while spindle-shaped cells were weakly positive or negative. In PVA/MPC in EGM2 and in Endocult both strongly positive and negative cells were observed, but in the latter construct PAS-positive cells were more expressed than PAS negative cells.

GAG expression: Alcian Blue staining at pH1 revealed no presence of sulphated acid GAGs in all constructs. Alcian Blue staining at pH2.5 showed that in PVA/HUVEC constructs with matrigel some cells expressed weak positivity for acid GAGs, others good positivity. In PVA/MPC constructs some cells showed good positivity, others were negative.

Mineralized matrix: von Kossa staining showed that in all constructs some cells produced mineralization matrix granules that covered the cells and the scaffold also.

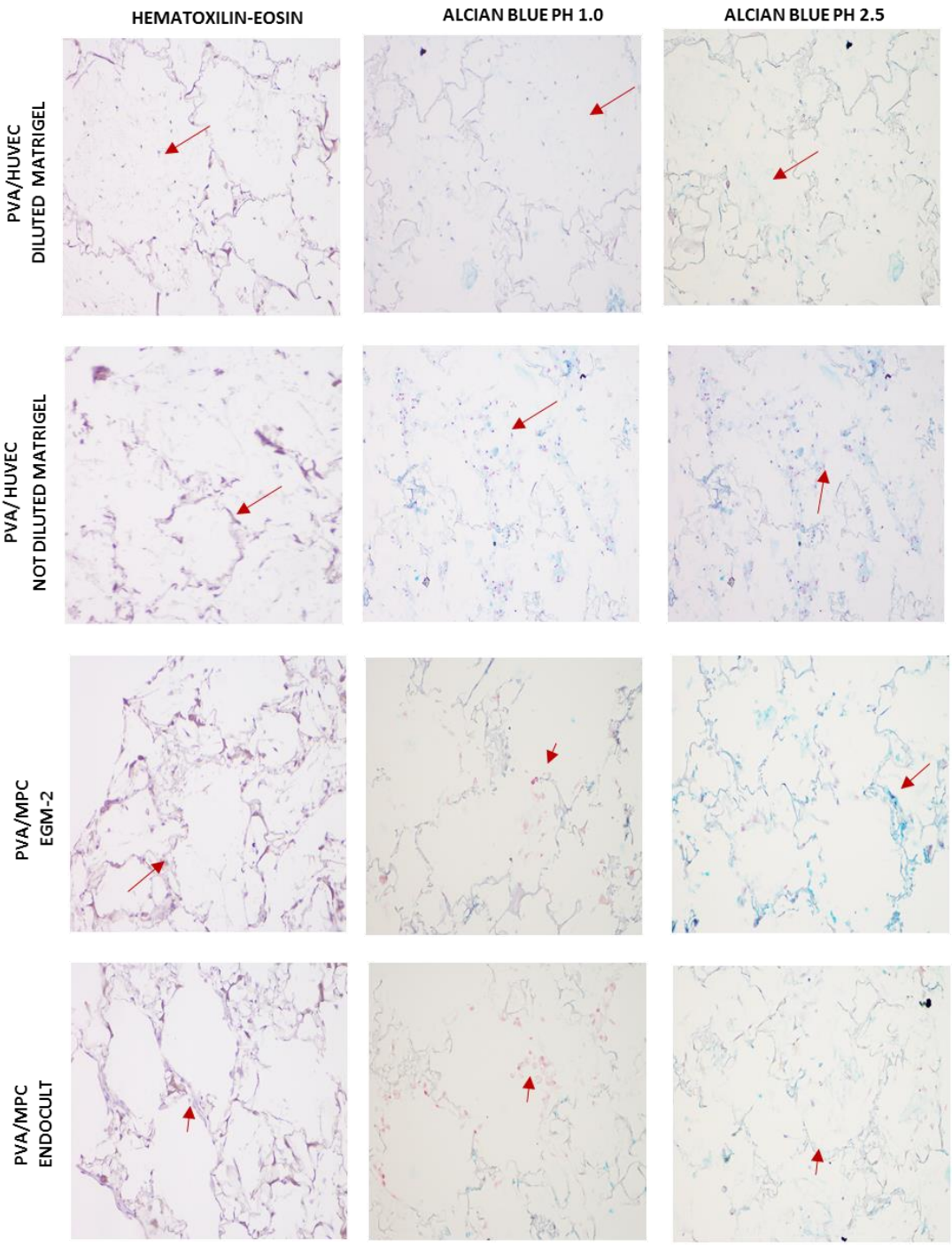

Figure 3.16 *Histochemistry on PVA/G constructs with HUVEC and MPC. First column: Hematoxylin.Eosin; second column: Alcian Blue pH 1.0; third column: Alcian Blue pH 2.5. First line: PVA/HUVEC construct with diluted matrigel:; second line: PVA/HUVEC construct with not diluted matrigel; third line: PVA/MPC construct in EGM-2: fourth line: PVA/MPC construct in Endocult. Magnification x200*

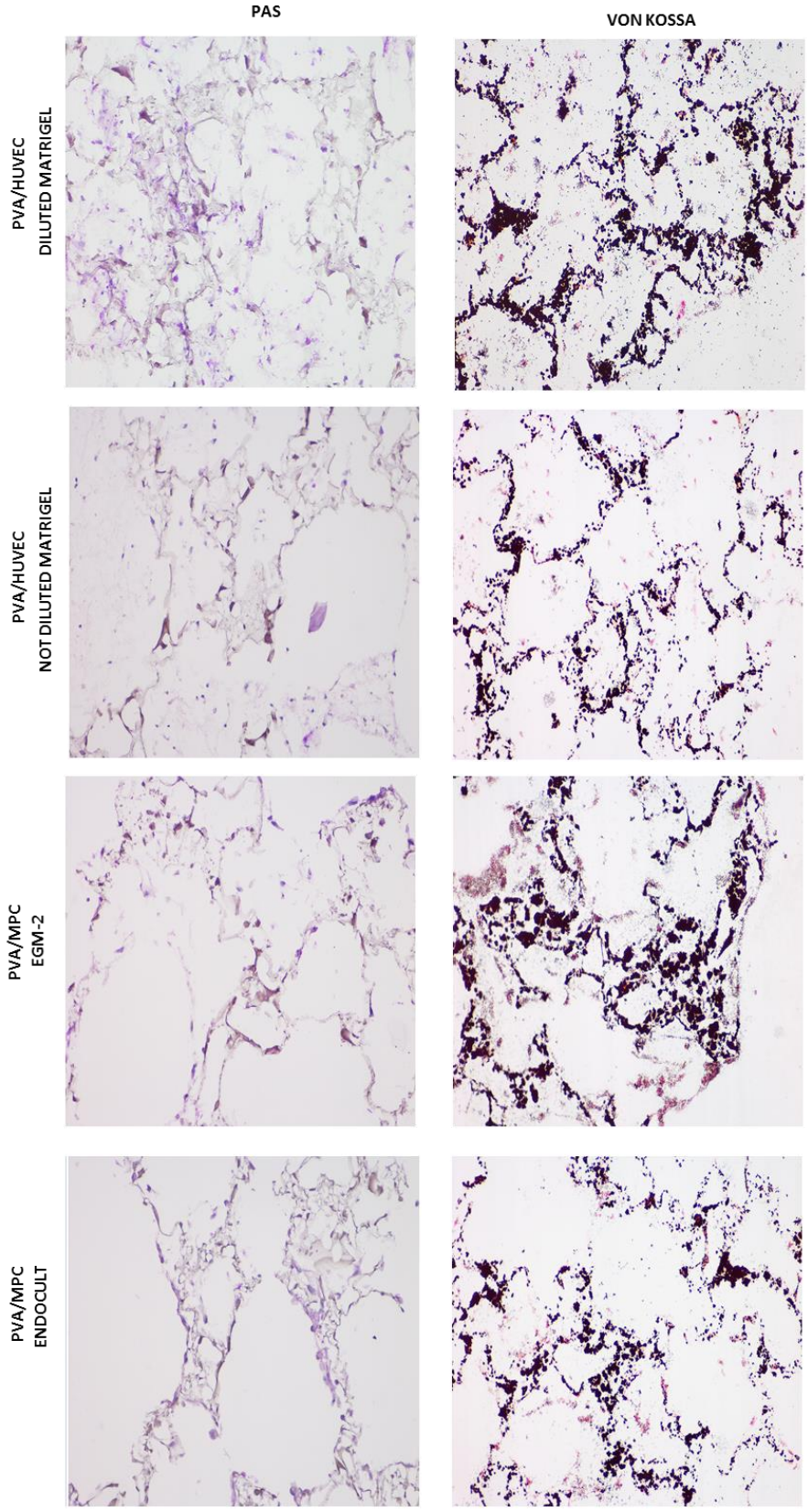

Figure 3.17. *Histochemistry on PVA/G constructs with HUVEC and MPC. First column: PAS reaction; second column: von Kossa. First line: PVA/HUVEC construct with diluted matrigel:; second line: PVA/HUVEC construct with not diluted matrigel; third line: PVA/MPC construct in EGM-2: fourth line: PVA/MPC construct in Endocult. Magnification x200*

3.8.2. Immunohistochemistry

Osteogenic markers

In all the constructs, cells expressed good amount of ALP osteogenic marker were observed, but some cells negative were also present. Good immunopositivity for Collagen I was highlighted in PVA/HUVEC construct with diluted matrigel. In the other constructs, the expression of this marker was weak. Negative cells were also present. In PVA/HUVEC with diluted matrigel, no positive cells for TGFβ-1 were showed. In PVA/HUVEC with not diluted matrigel, different positivity was observed: some cells showed good expression for this marker, others weak expression and others were negative. PVA/MPC in EGM2 and PVA/MPC in Endocult showed good an strong expression, respectively, for TGFβ-1.(Figure 3.18)

Endothelial markers

In PVA/HUVEC with diluted matrigel, only few cells expressed VEGF, but with good expression levels; in PVA/HUVEC with not diluted matrigel, both cells with scarce and strong positivity were observed; in PVA/MPC in EGM2, VEGF expression was weak, while was good in the cells of the PVA/MPC in Endocult construct. No presence of VEGF-R2 was observed in PVA/HUVEC with not diluted matrigel and in PVA/MPC in EGM 2 constructs, while this marker showed good expression in PVA/HUVEC with diluted matrigel and in PVA/MPC in Endocult constructs. Finally, PECAM was expressed only in few cells present in PVAHUVEC constructs, while in the others constructs was not expressed.(Figure 3.19).

Constructs / ***Osteogenic markers***	**PVA/HUVEC w diluted Matrigel**	**PVA/HUVEC w Matrigel**	**PVA/MPC in EGM2**	**PVA/MPC in Endocult**
ALP	++;-	++	++;-	++;-
COLL I	++;-	+;-	+;-	-/+
TGFβ-1	-	++;-/+;-	++	+++;-
Endothelial markers				
VEGF	++;-	+++;+	-/+	++
VEGF-R2	++;-/+	-	-	++
PECAM	+- ;	+;-	-	-

Table 2. *Osteogenic and endothelial markers tested on PVA/HUVEC and PVA/MPC constructs by IHC analysis. - = negativity. -/+; + = weak positivity. ++ = good positivity. +++ = strong positivity.*

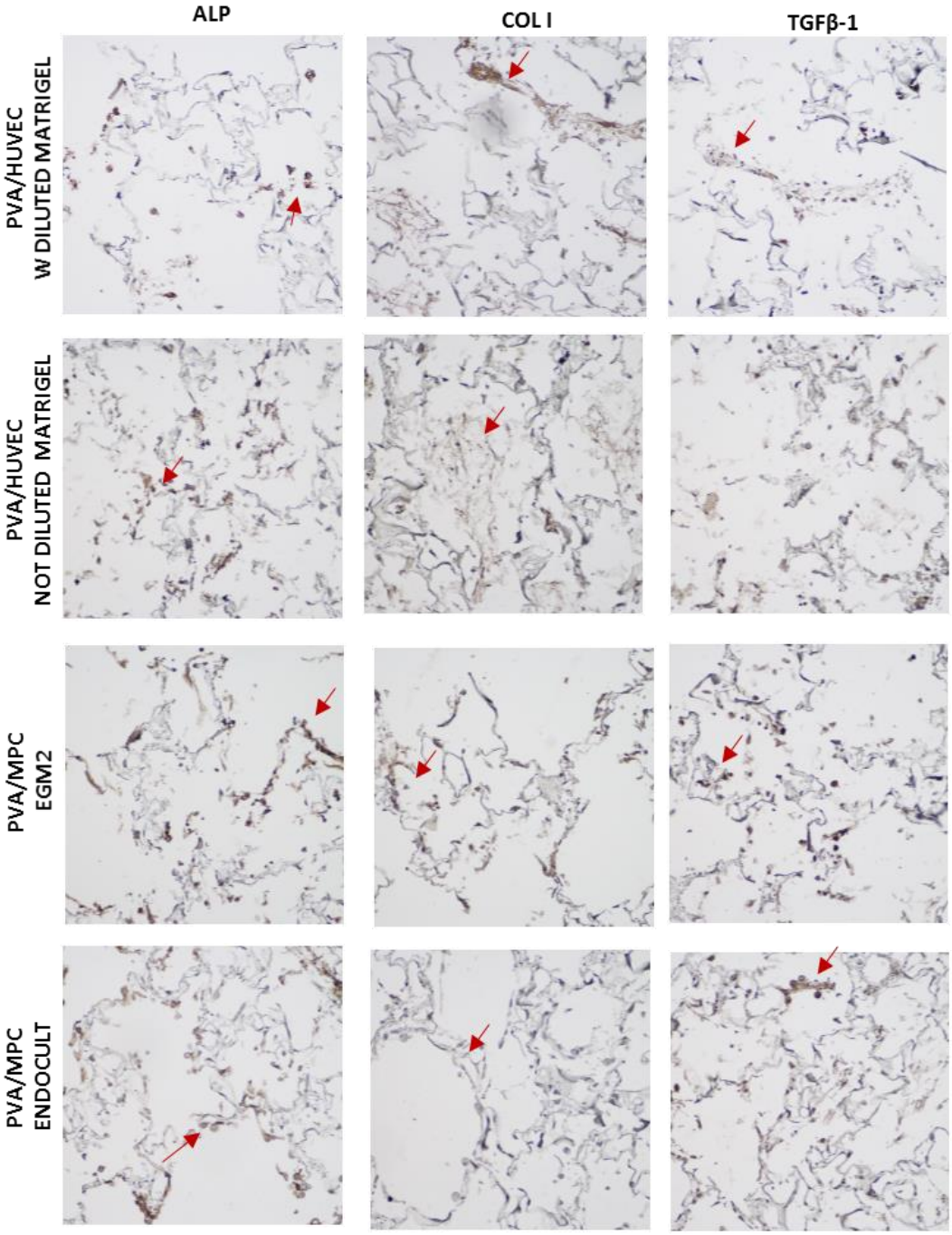

Figure 3.18 *Immunohistochemistry for Osteogenic markers on PVA/G constructs with HUVEC and MPC. First column: ALP; second column: Col I; thid column: TGF-β1. First line: PVA/HUVEC construct with diluted matrigel:; second line: PVA/HUVEC construct with not diluted matrigel; third line: PVA/MPC construct in EGM-2: fourth line: PVA/MPC construct in Endocult. Magnification x200*

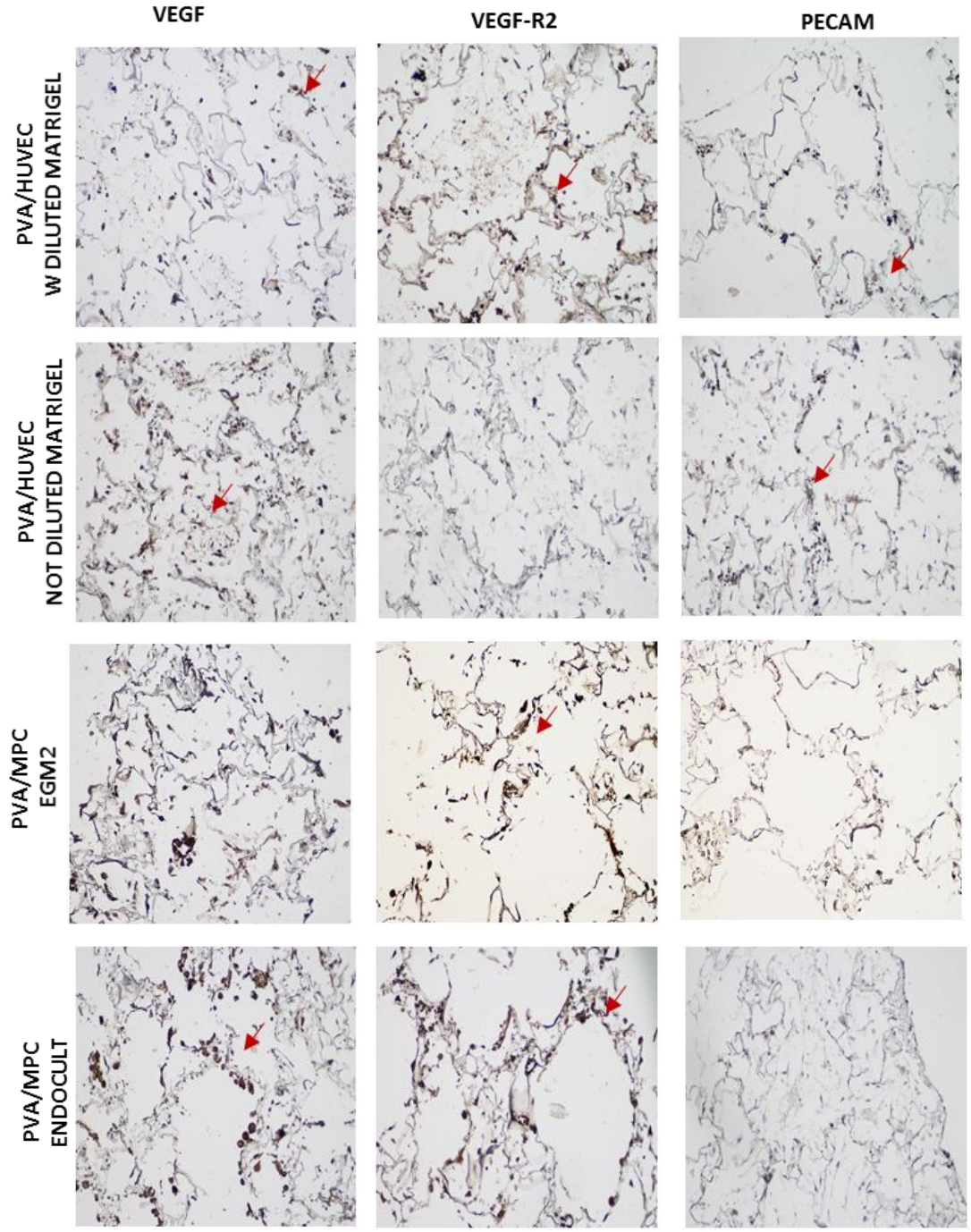

Figure 3.19. *Immunohistochemistry for endothelial markers on PVA/HUVEC and PVA/MPC constructs. First column: VEGF; second column: VEGF-R2; thid column: PECAM. First line: PVA/HUVEC construct with diluted matrigel:; second line: PVA/HUVEC construct with not diluted matrigel; third line: PVA/MPC construct in EGM-2: fourth line: PVA/MPC construct in Endocult. Magnification x200*

3.9 Statistical Analysis

Statistical analysis was performed using variance analysis and Tukey test by InfoStat software (p,0.05) was considered to be significant. Results showed that exist difference significate between treatments. The different treatment is 5 (50/50), all others have similar characteristics. (See supplements)

Chapter 4

4.1 DISCUSSION

The aim of this work was to develop an improved scaffold/cell construct for bone TE applications, constituted by PVA sponges seeded with osteodifferentiated MSCs and endothelial cells. The scaffolds behave as engineered matrices that provide structural support for 3D bone tissue formation (Rodriguez et al., 2011). The scaffolds can be designed with properties able to induce and direct cellular attachment, proliferation, migration, and differentiation, making them prominent tools in the bone TE field (Wojtowicz et al., 2010; Nyugen et al., 2012). In bone TE, the scaffolds serve as temporary structural supports for cell interactions and formation of bone ECM fundamental process for the bone formation. Furthermore, bone TE scaffolds have been used to deliver growth factors encapsulated within their structures and in some cases they resulted to facilitate the vascularization of the neo-tissue (Nyugen et.al, 2012). PVA is a synthetic polymer, water soluble and nonhazardous thus easy to handle, which can be used in several clinical applications (Peppas and Hansen, 1982; Preetti, 2015). PVA can be processed in several structures, and stabilized via physical and/or chemical crosslinking, thus becoming water insoluble. Porosity and pore interconnectivity are among the most important characteristics of a TE scaffold, because they greatly affect cell migration, ingrowth and nutrient diffusion for cell survival, can facilitate bone formation and the creation of a vascularization process. In general, scaffolds with large pore sizes can accommodate cells, cell aggregates and the secretion of ECM, while small pore sizes play a role in facilitating small molecule trafficking, such as nutrients, oxygen and metabolic waste. In this view, the production of scaffolds containing both macropores and micropores is highly recommended to promote an optimal microenvironment for cell growth and differentiation (Karageorgiou et al., 2005). It was demonstrated that optimum pore sizes should be larger than 100 μm for regeneration of vascularized bone tissue (Hulbert et al., 1970). In our study, we fabricated via emulsion and freeze-drying, and tested with MSCs and endothelial cells, PVA scaffolds containing different weight percentages of G (PVA/G 100/0, 90/10, 80/20, 70/30, 50/50), which ultimately resulted in different pore size distributions. Interestingly, the void volume was distributed into larger diameter pores in the scaffolds with higher G content. The best composition that we experimentally selected through independent cultures with MSCs and

HUVECs was the PVA/G 80/20. In this scaffold composition, 4.2% of the relative void volume was distributed into pores with 150-100 μm diameters, suitable for forming vascularized bone, 52.1% into 100-50 μm diameters, suitable for osteoblast housing, 41.7 % into 50-10 μm diameters, suitable for endothelial cell colonization and 2% into pore diameters smaller than 10 μm, useful for molecule trafficking. It can be hypothesized that the combination of the polymeric blend composition (i.e., PVA/G 80/20), this specific pore size distribution, and the related surface roughness, altogether rendered this scaffold the most suitable for osteoblast and endothelial cell culture.

We studied the capability of the PVA/G scaffolds with different compositions, to support MSC osteogenesis and new bone formation *in vitro*, to develop a model of bone TE scaffold on which endothelial cells could be cultured, so as to improve bone TE substitutes. The first investigations were performed culturing and differentiating HUVECs on the different PVA/G scaffolds. Two different cell densities were tested and the histological and SEM observations finally highlighted that PVA/G 80/20 and 70/30 were the best suitable scaffolds for HUVEC colonization. The second goal was to define the most suitable scaffold types for MSC osteodifferentiation. H&E staining showed several osteoblast-like cells colonizing all the scaffolds, but the best cellularized samples were the 80/20 and 70/30 PVA/G compositions. Using von Kossa staining, mineralization evidence was also detected on all the scaffold types, possibly for the high interconnected porous scaffold characteristics that permitted an ideal environment for cell proliferation, vascularization and new bone formation to be generated (Nyugen et al, 2012). The morphological observations conducted with SEM showed that the cells attached and proliferated well on 80/20 and 70/30 PVA/G compositions, and these findings were also corroborated by the AlamarBlue® assay for the metabolic activity, which finally allowed the selection of PVA/G 80/20 as the best scaffold among all, to be used in further co-culture studies. These osteodifferentiated MSC/scaffold constructs were thus seeded with HUVECs. The first objective was to establish if bone ECM produced by the osteodifferentiated MSCs was sufficient to HUVEC differentiation or still needed Matrigel® as an endothelial differentiation supplement. Moreover, we tested if the endothelial differentiation occurred in presence of viable osteodifferentiated MSCs or without them, decellularizing the constructs prior to seeding them with HUVECs. The histological results showed that the best condition occurred when both viable osteodifferentiated MSC and Matrigel® were present on the constructs, giving rise to the formation of microcapillary-like

structures. These results are in line with other findings reported in literature (Santos et al., 2010). These authors highlighted the complex interplay existing between angiogenesis and osteogenesis both *in vivo* and *in vitro*, that is fundamental for the vessel formation and new bone generation, and was confirmed by to gap junction formation between osteoblasts and endothelial cells (Santos et al, 2010). Also Wang et al. demonstrated the important relationship between osteoblasts and endothelial cells in co-culturing human osteoblast-like cells (HOBs) with HUVECs with an increase of ALP occurring only in co-culture (Wang et al., 1997). In our HUVEC+MSC/scaffold constructs, an enhanced ALP positivity and improved endothelial cell morphology were detected in presence of live osteodifferentiated MSCs, thus confirming the fundamental role of osteoblast-endothelial cell cross-talk for an effective bone vascularization. Basing on these outcomes, the second objective was to optimize the co-cultured constructs and it was performed increasing the number of cells seeded on the scaffolds and the Matrigel® amount. The results showed that amorphous ECM molecules, osteogenic and endothelial markers were expressed more intensely than in previous constructs. In a similar fashion, enhanced pore colonization and endothelial cell differentiation were observed with respect to the previous experiments, in accordance with some authors who demonstrated that the ECM produced by bone-forming cells may promote the creation of microcapillary-like structures (Unger, et al., 2005; and Stanhl et al., 2003).

Among the endothelial markers, VEGF is an important factor for the vessel formation, as well as a key player in physiological and pathological angiogenesis (Dvorak, 2000; Carmeliet, 2000; Ferrara et al.,1997,and 2003). VEGF can be functional to rise stem cell recruitment to damaged or diseased bone tissue and fracture healing (Beamer et al. 2010; Keramaris et al., 2008). In our constructs, VEGF was detected with good positivity in the cellularized and decellularized constructs without Matrigel®. PECAM-1 is another endothelial marker expressed by HUVECs, known to be crucial for vessel formation and maintenance. A weak PECAM positivity was observed on all our constructs. Networks of tubes or microcapillary in 2D HUVEC culture were observed in our experiments. The capacity of HUVECs to form these tubes is a relevant characteristic that may depend on the properties of the underlying ECM (Soucy, 2009). However, co-culture of endothelial cells with osteoblasts derived from osteoinduced MSCs can facilitate the tube formation *in vitro* on 3D scaffolds due to the production of cytokines and angiogenic growth factors (Deckers et al., 2008; Furumatsu et al., 2003). It is interesting to note that, among the

tested markers, ALP, VEGF and VEGF-R2 showed in many cases good expression, which is in line with the findings of Wang and coworkers, who reported an increase of these markers in co-cultures of osteoblasts and HUVECs (Wang et al.,1997).

The ultimate objective of this study was to show the possibility to create a fully autologous prevascularized bone substitute using TE. This is theoretically possible, for example using the same stem cells to obtain osteoblasts and endothelial cells. Our strategy relied on the use of MPCs, a subset of immature stem cells present in the bone marrow, as they were proved to differentiate into MSCs and pre-endothelial cells (Petrini et al., 2009 ; Trombi et al.,2009) and to apply the most successful culture approach as obtained from the previous objective. The histological results showed very good expression of amorphous ECM molecules, osteogenic and endothelial markers, demonstrating that it could be possible, in perspective, to obtain a fully-autologous construct.

4.2 CONCLUSIONS

1) PVA/G 80/20 resulted the most suitable scaffold formulation for MSC and HUVEC culture, as revealed by viability assay and histo-morphological analyses.

2) Co-culture method can represent a suitable strategy to improve TE constructs for bone regeneration studies.

3) The presence of live osteodifferentiated MSCs and ECM molecules improved the endothelial differentiation in the 3D constructs.

4) Co-culture of MSCs and MPCs could be useful to obtain a fully autologous construct, useful, in perspective, for clinical applications.

5) The expression of some osteogenic and endothelial markers in the constructs confirmed the results reported in literature, but at this stage of investigation, they were not as selective as expected to indisputably discriminate between the two cell types seeded on the scaffold.

4.3 ACKNOWLEGMENT

OtoLab, AOUP (Prof. Berrettini, Dr. D'Alessandro, Dott.ssa Luisa Trombi, Dr. Claudio Ricci) for MSC facilities. Hematology Division, AOUP (Prof. Petrini) for MPC characterization. Histology Division, University of Pisa (Dr. Moscato), for paraffin-sample sectioning. The BioRobotics Institute, Scuola Superiore Sant'Anna, for SEM analysis. ISE-CNR Pisa (Dr. Pini) for Mercury Intrusion Porosimetry

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4.5 SUPLEMENTARY

Variance Analysis by InfoStat

At Day 3

Nueva tabla : 27/05/2016 - 12:11:46 a.m. - [Versión : 23/02/2016]

Análisis de la varianza

Variable	N	R^2	R^2 Aj	CV
Respuesta	30	0,95	0,94	50,39

Cuadro de Análisis de la Varianza (SC tipo III)

F.V.	SC	gl	CM	F	p-valor
Modelo.	2061307413671520,00	4	515326853417881,00	115,25	<0,0001
Tratamientos	2061307413671520,00	4	515326853417881,00	115,25	<0,0001
Error	111786874699365,00	25	4471474987974,61		
Total	2173094288370890,00	29			

Abbreviation.

T1: Scaffold 100/0

T2: Scaffold 90/10

T3: Scaffold 80/20

T4: Scaffold 70/30

T5: Scaffold 50/50

Nueva tabla : 27/05/2016 - 12:20:46 a.m. - [Versión : 23/02/2016]

Análisis de la varianza

Variable	N	R²	R² Aj	CV
Respuesta	30	0,95	0,94	50,39

Cuadro de Análisis de la Varianza (SC tipo III)

F.V.	SC	gl	CM	F	p-valor
Modelo.	2061307413671520,00	4	515326853417881,00	115,25	<0,0001
Tratamientos	2061307413671520,00	4	515326853417881,00	115,25	<0,0001
Error	111786874699365,00	25	4471474987974,61		
Total	2173094288370890,00	29			

Test:Tukey Alfa=0,05 DMS=3585499,59185

Error: 4471474987974,6133 gl: 25

Tratamientos	Medias	n	E.E.	
Trat 1	17,30	6	863276,22	A
Trat 2	20,77	6	863276,22	A
Trat 3	2077,44	6	863276,22	A
Trat 4	207744,06	6	863276,22	A
Trat 5	20774406,28	6	863276,22	B

Medias con una letra común no son significativamente diferentes (p > 0,05)

Exist difference significate between treatments. The different treatment is 5 (50/50), all others have similar characteristics. InfoStat Analysis say that averages with a common letter is not significantly different.

At day 10

Nueva tabla : 27/05/2016 - 12:27:40 a.m. - [Versión : 23/02/2016]

Análisis de la varianza

Variable	N	R²	R² Aj	CV
Respuesta	30	0,83	0,80	14,47

Cuadro de Análisis de la Varianza (SC tipo III)

F.V.	SC	gl	CM	F	p-valor
Modelo.	601,79	4	150,45	29,74	<0,0001
Tratamientos	601,79	4	150,45	29,74	<0,0001
Error	126,46	25	5,06		
Total	728,25	29			

Test:Tukey Alfa=0,05 DMS=3,81354

Error: 5,0584 gl: 25

Tratamientos	Medias	n	E.E.		
Trat 5	7,34	6	0,92	A	
Trat 4	15,46	6	0,92		B
Trat 1	16,52	6	0,92		B
Trat 2	17,51	6	0,92		B C
Trat 3	20,85	6	0,92		C

Medias con una letra común no son significativamente diferentes (p > 0,05)

There are significant differences between treatments, treatment 5 has the highest average and is different from all other treatment 2 is the one with better results.

At day 17

Nueva tabla : 28/05/2016 - 01:07:38 a.m. - [Versión : 23/02/2016]

Análisis de la varianza

Variable	N	R²	R² Aj	CV
Resultado	30	0,83	0,81	17,31

Cuadro de Análisis de la Varianza (SC tipo III)

F.V.	SC	gl	CM	F	p-valor
Modelo.	524,56	4	131,14	31,04	<0,0001
Tratamiento	524,56	4	131,14	31,04	<0,0001
Error	105,61	25	4,22		
Total	630,17	29			

Test:Tukey Alfa=0,05 DMS=3,48506

Error: 4,2245 gl: 25

Tratamiento	Medias	n	E.E.		
Trat 5	4,22	6	0,84	A	
Trat 1	12,17	6	0,84		B
Trat 4	12,39	6	0,84		B
Trat 2	13,70	6	0,84		B C
Trat 3	16,90	6	0,84		C

Medias con una letra común no son significativamente diferentes (p > 0,05)

At 21 Days

Nueva tabla : 28/05/2016 - 01:09:48 a.m. - [Versión : 23/02/2016]

Análisis de la varianza

Variable	N	R²	R² Aj	CV
Resultado	30	0,85	0,82	14,72

Cuadro de Análisis de la Varianza (SC tipo III)

F.V.	SC	gl	CM	F	p-valor
Modelo.	599,65	4	149,91	35,04	<0,0001
Tratamiento	599,65	4	149,91	35,04	<0,0001
Error	106,97	25	4,28		
Total	706,62	29			

Test:Tukey Alfa=0,05 DMS=3,50741

Error: 4,2788 gl: 25

Tratamiento	Medias	n	E.E.		
Trat 5	5,80	6	0,84	A	
Trat 1	14,89	6	0,84		B
Trat 4	14,99	6	0,84		B
Trat 2	15,12	6	0,84		B
Trat 3	19,45	6	0,84		C

Medias con una letra común no son significativamente diferentes (p > 0,05)

Before Embedding

Nueva tabla : 28/05/2016 - 01:12:09 a.m. - [Versión : 23/02/2016]

Análisis de la varianza

Variable	N	R²	R² Aj	CV
Resultado	30	0,77	0,73	21,09

Cuadro de Análisis de la Varianza (SC tipo III)

F.V.	SC	gl	CM	F	p-valor
Modelo.	770,83	4	192,71	20,90	<0,0001
Tratamiento	770,83	4	192,71	20,90	<0,0001
Error	230,56	25	9,22		
Total	1001,39	29			

Test:Tukey Alfa=0,05 DMS=5,14924

Error: 9,2223 gl: 25

Tratamiento	Medias	n	E.E.		
Trat 5	5,59	6	1,24	A	
Trat 4	13,64	6	1,24		B
Trat 1	14,27	6	1,24		B
Trat 2	18,02	6	1,24		B C
Trat 3	20,50	6	1,24		C

Medias con una letra común no son significativamente diferentes (p > 0,05)

Day 1

Nueva tabla : 28/05/2016 - 01:15:10 a.m. - [Versión : 23/02/2016]

Análisis de la varianza

Variable	N	R²	R² Aj	CV
Resultado	24	0,73	0,72	26,51

Cuadro de Análisis de la Varianza (SC tipo III)

F.V.	SC	gl	CM	F	p-valor
Modelo.	834,51	1	834,51	60,80	<0,0001
Tratamiento	834,51	1	834,51	60,80	<0,0001
Error	301,94	22	13,72		
Total	1136,46	23			

Test:Tukey Alfa=0,05 DMS=3,13657

Error: 13,7246 gl: 22

Tratamiento	Medias	n	E.E.		
Trat 2	8,08	12	1,07	A	
Trat 1	19,87	12	1,07		B

Medias con una letra común no son significativamente diferentes (p > 0,05)

Day 7

Nueva tabla : 28/05/2016 - 01:21:06 a.m. - [Versión : 23/02/2016]

Análisis de la varianza

Variable	N	R²	R² Aj	CV
Resultado	24	0,72	0,71	25,76

Cuadro de Análisis de la Varianza (SC tipo III)

F.V.	SC	gl	CM	F	p-valor
Modelo.	458,24	1	458,24	57,16	<0,0001
Tratamiento	458,24	1	458,24	57,16	<0,0001
Error	176,38	22	8,02		
Total	634,62	23			

Test:Tukey Alfa=0,05 DMS=2,39726

Error: 8,0171 gl: 22

Tratamiento	Medias	n	E.E.	
Trat 2	6,62	12	0,82	A
Trat 1	15,36	12	0,82	B

Medias con una letra común no son significativamente diferentes (p > 0,05)

Day 14

Nueva tabla : 28/05/2016 - 01:25:05 a.m. - [Versión : 23/02/2016]

Análisis de la varianza

Variable	N	R²	R² Aj	CV
Resultado	24	0,79	0,78	26,68

Cuadro de Análisis de la Varianza (SC tipo III)

F.V.	SC	gl	CM	F	p-valor
Modelo.	960,67	1	960,67	81,51	<0,0001
Tratamiento	960,67	1	960,67	81,51	<0,0001
Error	259,30	22	11,79		
Total	1219,98	23			

Test:Tukey Alfa=0,05 DMS=2,90669

Error: 11,7865 gl: 22

Tratamiento	Medias	n	E.E.	
Trat 2	6,54	12	0,99	A
Trat 1	19,19	12	0,99	B

Medias con una letra común no son significativamente diferentes (p > 0,05)

Exists differences significantly between treatment