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Wound Healing and Differentiation Potential among Human Vascular Wall Mesenchymal Stem Cells, Dermal Fibroblast and Myofibroblast Cell Lines

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LIST OF ABBREVIATIONS

AAA-MSCs: abdominal aortic aneurysm mesenchymal stem cells

ABPI/ABI: ankle brachial pressure index

ASC: Adult Stem Cell

BrdU: 5-Bromo-2'-Deoxyuridine

CD: Cluster of Differentiation

cDNA: copy of DNA

CLI: Chronic Lower Limb Ischemia

c-MET: receptor of Hepatocyte Growth Factor

COX: cyclooxygenase

CT: computerized tomography

DAPI: 4',6-diamidin-2-fenilindolo

DFU: Diabetic Foot Ulcer

DMEM: Dulbecco's Modified Eagle Medium

DNA: Deoxyribo Nucleic Acid

ECM: Extracellular Matrix

EC: Endothelial Cell

ECFC: Endothelial Colony Forming Cell

EGF: Epidermal Growth Factor

EGC: Embryonic Germ Stem Cell

ESC: Embryonic Stem Cell

ETM: Epithelial-Mesenchymal Transition

FBS: Fetal Bovin Serum

FGF: Fibroblast Growth Factor

FITC: Fluorescein Isothiocyanate

GAG: Glycosaminoglycan

GAPDH: Glyceraldehyde 3-Phosphate Dehydrogenase

GM-CSF: Granulocyte Macrophage-Colony Stimulating Factor

HGF: Heaptocyte Growth Factor

HIF-1: Hypoxia Inducible Factor-1

HLA: Human Leucocyte Antigen

HSC: Heamtopoietic Stem Cell

HUVEC: human umbilical vein endothelial cell

IL: Interleukin

ICM: Inner Cell Mass

IFN-gamma: Interferon-gamma

KDR: kinase insert domain receptor (or vascular endothelial growth factor receptor 2)

LM: light microscopy

MMP: Matrix Metalloproteinase

MRA: magnetic resonance angiography

MSC: Mesenchymal Stromal (Stem) Cell

***NSAIDs**: non-steroidal antinflammatory drugs*

***NG**: Neural Glial antigen*

PAD: Peripheral Arterial Disease

PAOD: Peripheral Arterial Obstructive Disease

PBMC: Peripheral Blood Mononuclear Cell

PBS: Phosphate Buffer Saline

PCR: Polymerase Chain Reaction

PDGF: Plateled-Derived Growth Factor

PDGFR: Plateled-Derived Growth Factor Receptor

PG: Prostaglandine

PRP: Plateled Rich Plasma

PTA: Percutaneous Transluminal Angioplasty

RNA: Ribo Nucleic Acid

RT: room temperature

RT-PCR: Reverse-Transcription-PC

SC: Stem cell

SDS-PAGE: Sodium Dodecyl Sulphate - PolyAcrylamide Gel Electrophoresis

SEM: Scanning electronic microscopy

SF: Scatter Factor

SMC: Smooth Muscle Cell

SSEA: Stage-specific Embryonic Antigen

TGB-beta: Transforming Growth Factor –beta

TNF-alfa: Tumor Necrosis Factor-alfa

TRA: transfer region

VE-cadherin: Vascular Endothelial cadherin

VEGF: Vascular Endothelial Growth Factor

VW: Vascular Wall

VWC: Vascular Wall Cell

VW-MSC: Vascular Wall Mesenchymal Stem Cell

vWF: Von Willebrand Factor

WHO: World Health Organization

WPMY: myofibroblasts stromal cell line

ABSTRACT

Background. Lower-limb ulcers represent a debilitating phenomenon with a prevalence of 3% in the people over 65 years old. The ulcers are particularly severe in diabetic patients where they tend to become chronic non-healing wounds, leading to a series of clinical complications. Nowadays, although surgical revascularization remains the gold standard therapeutical option for wound healing, many new therapeutic approaches are under development to facilitate and accelerate the recovery of the injured tissues. In this context, the use of growth factors, mesenchymal stem cells and autologous fibroblasts are acquiring increasingly importance. Based on these evidences, this study was aimed to test one of the proangiogenic factors, HGF, on hVW-MSCs isolated from human arteries and compare the differentiation potential between hVW-MSC and the stromal counterpart (dermal fibroblasts and myofibroblasts).

Materials and methods. HGF effect on hVW-MSCs was studied; proliferation, migration, motility, angiogenic induction and modulation of tissue remodeling and inflammation markers were specific areas investigated. HGF was also tested on MSCs recovered from abdominal aortic aneurysms (AAA-MSCs). Furthermore, assays of angiogenic and adipogenic differentiation potential were established on hVW-MSCs, dermal fibroblasts and myofibroblasts.

Results. HGF stimulates migration, motility and angiogenic differentiation of hVW-MSCs, but it has no effect on proliferation and tissue remodeling and inflammation markers. Results on AAA-MSCs show that HGF decreases the expression of inflammatory cytokines and positively modulate some markers involved in tissue remodeling. Finally, hVW-MSCs own a higher angiogenic and adipogenic commitment compared to dermal fibroblasts and myofibroblasts.

Discussion. The combined use of HGF and hVW-MSCs, especially because of their high differentiation potential, represents a promising therapeutic strategy, in order to facilitate the healing of unresponsive vascular ulcers.

Keywords: ulcers; wound healing; HGF; hVW-MSCs; fibroblasts; myofibroblasts; differentiation potential.

LIST OF ORIGINAL PUBLICATIONS

Some of the data presented in this thesis have been object of the following original articles:

Hepatocyte Growth Factor effects on Mesenchymal Stem Cells derived from human arteries: a novel strategy to accelerate vascular ulcer wound healing. S. Valente, C. Ciavarella, E. Pasanisi, F. Ricci, A. Stella and G. Pasquinelli. Hindawi Publishing Corporation. Stem Cell International 2016; 2016:3232859.

Differentiation Potential of Human Vascular Wall Mesenchymal Stem Cells, Dermal Fibroblasts and Myofibroblasts: A Critical Comparison E. Pasanisi, M. Buzzi, A. Stella and G. Pasquinelli (under submission to Karger – Cellular Physiology and Biochemistry).

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Introduction

Lower-extremity ulcerations

Lower-extremity ulceration is a debilitating phenomenon with prevalence from 1 to 3,6%, in patients over 65 years of age it is up to 5%, particularly in Western countries (N. J. M. London et R. Donnelly, 2000; G. Spentzouris et al, 2009). Lower limb ulcers affect not only the quality of life of the patients, but also the whole society, since the healthcare expenditures for preventing, treating and decelerating the development of lower limb ulcers are really great.

There are many different types of ulcers, according to several causes: venous, arterial, neurotrophic, lymphatic, malignant, infectious, medication induced, inflammatory, drug-induced and due to autoimmune diseases.

Most leg ulcers are caused by venous disease alone (72%) (D. J. Adam et al, 2003); these occur above the medial and the lateral malleoli, and the main mechanisms behind venous ulcers are reflux and/or venous outflow obstruction which causes stasis and venous hypertension (A. N. Nicolaides et al, 2008). It occurs an increased ambulatory venous pressure, which is transmitted to the capillaries of the subcutaneous tissues, which may result into a fluid passage in the extravasal environment and into an accumulation of macromolecules such hemoserine and fibrinogen; these last hinder the gas exchange, while the leucocytes are activated bringing to cytolysis, skin damage and ulcer development (A. N. Nicolaides et al, 1993; J.J. Bergan et al, 2006).

Ulcers due to arterial disease are the second most common (10-30%) (M. K. Lazarides et al, 2007; J. R. Mekkes et al, 2003; R. Renner et al, 2008). Many arterial pathologies can lead to arterial ulcers, but the main cause is arterial obstruction; peripheral arterial disease (PAD) is a major contributor, but also thrombotic events, aneurysm, plaques and hypercoagulable state (L. R. Leon et al,2005). Compensation mechanisms are activated in arterial ulcers, such as the blood viscosity, the aggregation of platelets and the erythrocyte deformability that leading to a condition of critical ischemia; also in this case, the endothelial damage increases the flow of liquids and fibrinogen in the interstitial area, resulting into tissue hypoxia and activating leucocytes and platelets, which obstruct capillaries and arterioles.

The next most common ulcers are neuropathic in origin (15-25%) (M. K. Lazarides et al, 2007; J. R. Mekkes et al, 2003; R. Renner et al, 2008). Usually, it is about patients with diabetes and the neuropathy is threefold: motor, sensory and autonomic; the

neuropathy coupled with diabetes creates the perfect environment for a non-healing ulcer.

Ulcerations may also be due to infectious causes (mostly bacteria, but also viruses, parasites and fungi), or mixed etiology causes (G. Spentzouris et al, 2009; D. J. Adam et al, 2003).

Rarely it is possible to develop ulceration due to lymphatic cause (G. Spentzouris et al, 2009).

Recently it has been shown that super-obese patients may get venous ulcers without having venous disease detected (F. Jr Padberg et al, 2003).

Finally each ulcer has different characteristics and clinical history, but surely an accurate diagnosis of etiology, often multifactorial, is essential before beginning treatment, in order to improve health of the patient and reduce costs and use of resources (Figure 1).

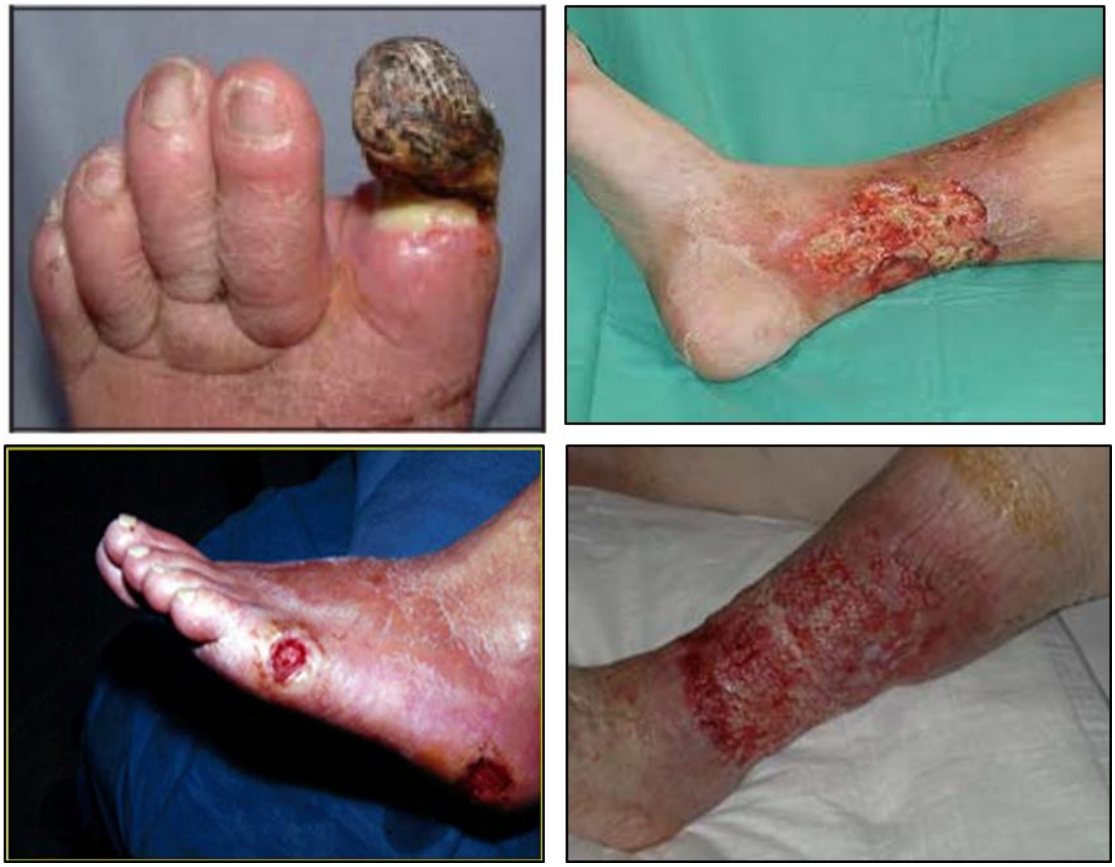


Figure 1: Examples of lower limb ulcers.

Peripheral arterial occlusive disease (PAOD)

Peripheral artery disease (PAD) or Peripheral arterial occlusive disease (PAOD) represents a shrinkage of the arteries that commonly affects the legs. PAOD affect 15-20% of people older 70 years of age, although 70-80% is asymptomatic (F. J. Serrano Hernando et A. M. Conejeroand, 2007). PAOD is a type of vascular disease in which the fatty deposits in the inner walls of arteries blocking the normal blood flow (Figure 2). The main development sites of PAOD are the iliac artery, the femoral artery, the popliteal artery and the tibial arteries; the disease leads to the obstruction of the arteries that come to the kidneys, stomach, arms, legs and feet, and, if untreated, can lead the patient to gangrene and amputation of limb. Besides, many patients affected with PAOD develop an higher risk to die because of heart attack and stroke (https://www.heart.org; retrieved 26 February 2015).

The main symptom is represented by leg pain during activity, known also as "intermittent claudication" that disappeared with the rest, but also by skin and foot ulcers, bluish skin, cold skin and poor nail and hair growth.

Among the risk factors of PAOD there are the same that leading to atherosclerosis (M. M. Joosten et al, 2012), including cigarette smoking, diabetes mellitus, hypertension, dyslipidemia, hyperhomocysteinemia, inflammation, male sex and growing age (F. G. Fowkes et al, 2013).

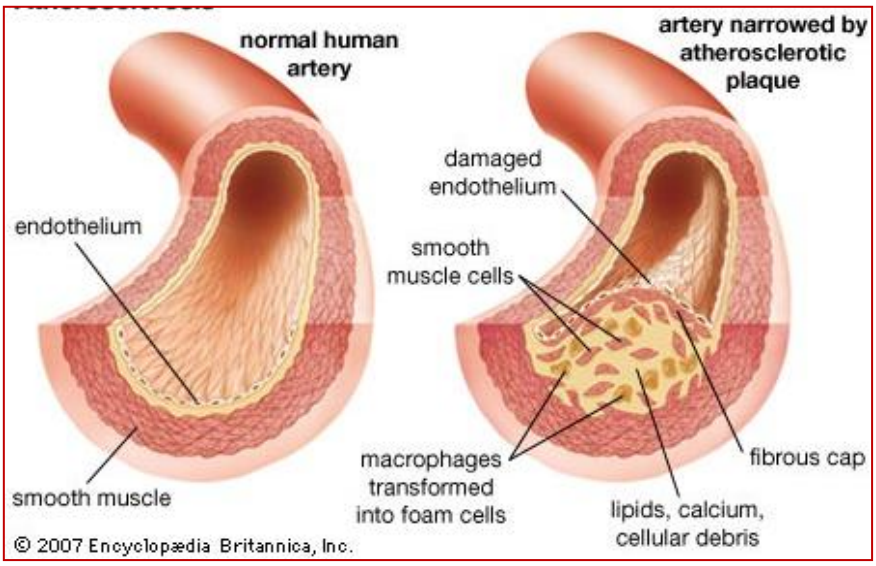


Figure 2: Comparison between normal and atherosclerotic arteries (www.britannica.com).

Pathogenesis and atherosclerotic process

The most important mechanism underlying the develop of PAOD is atherosclerosis, that affects the vascularization to the lower limb. The process begins at the birth and continues slowly with the advancement of the age; in the early phase of the disease the endothelium is damaged and leads to the fatty accumulation into the inner wall of the arteries (*tonaca intima*), forming plaques or atheroma which obstruct the blood flow. Atheroma is composed by material including macrophages, cholesterol crystals, calcified lesions and free radicals. The lipids accumulate in the artery endothelium binding the components of the extracellular matrix of the *intima*, which is activated to invoke immune system cells, as monocytes, T lymphocytes and macrophages; these last ingest the lipid molecules and stimulate an inflammatory response (A. Zernecke et C. Weber, 2010). The presence of calcium increases the rigidity of the plaque that can break, attracting in turn the platelets in the breaking site. The platelets, however, block the red blood cells and may form a clot, causing thrombosis that occludes the vascular lumen (Figure 3); furthermore, if this clot breaks, it can cause serious damages, as angina, myocardial infarctions or stroke.

The atherosclerosis treatment usually provides lifestyle changes, the use of ACE inhibitors and statins to decrease the blood pressure and blood cholesterol levels, and the angioplasty with stenting (V. Fuster, 2011).

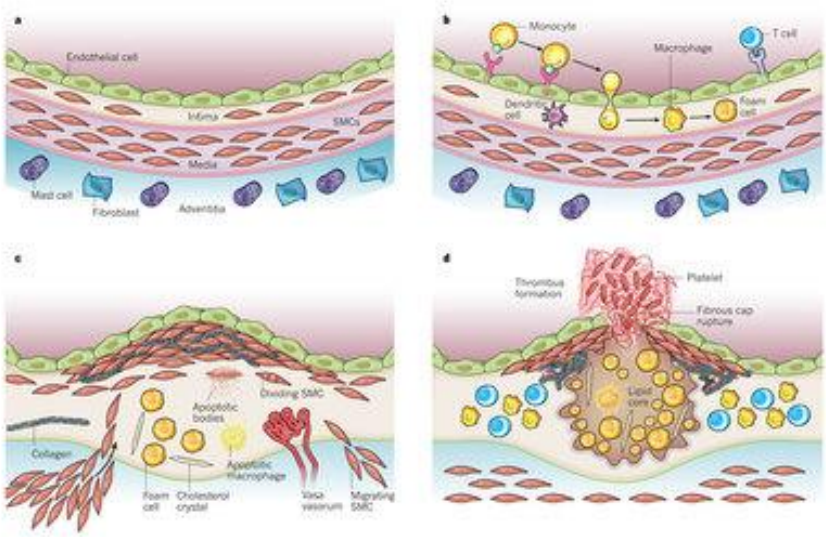


Figure 3: Plaque develop in the inner wall of the arteries, during the process of atherosclerosis (P. Libby et al, 2011).

Diagnosis and treatment of PAOD

The symptoms of the patients with suspected PAOD are usually classified according to the classification of Leriche-Fontaine, which identifies 4 stages depending on the severity of the arterial failure:

- Stage I: asymptomatic or incomplete blood vessel obstruction
- Stage II: claudication pain in limb when walking
- Stage III: rest pain, mainly in the feet
- Stage IV: necrosis and/or gangrene of the limb

Initially, the basic examination provides the evaluation of the possible pulses in the femoral, popliteal, pedal and posterior tibial arteries, the auscultation of the abdomen and the control of the temperature, the color and the tropism of the foot. A widely and early used method is represented by the ABPI/ABI, the ankle brachial pressure index (T.W. Rooke et al, 2013) and later by the Doppler ultrasounds to evaluate the level of the functional involvement and trying to locate the occlusive lesion; other widely used methods are angiography, an imaging technique, but also the computerized tomography (CT) scanners or magnetic resonance angiography (MRA), a noninvasive diagnostic procedure that provides pictures of the vessels (*Peripheral Arterial Disease* at Merck Manual of Diagnosis and Therapy, 2010; T. Leiner, 2005).

For the tretament of PAOD it is necessary to stop smoking and to monitor diabetes, hypertension and high cholesterol; besides, regular exercise should be practiced especially in the cases of claudication. Among the most commonly used drugs there are cilostazol, pentoxifylline, statins, prostanoids and others. Depending on the severity of ischemia, surgery also can be used: the methods most largely used are the percutaneous transluminal angioplasty (PTA), the atherectomy, the vascular bypass, thrombolysis, thrombectomy and in the most severe cases with gangrene, the amputation.

Diabetic foot ulcer

The diabetic foot ulcers (DFUs) are a common, costly and serious complication of diabetes mellitus (D. G. Amstrong et al, 2005; D. G. Amstrong et al, 2011) and they mainly affect the arterial system, where they tend to become chronic. In a diabetic patient and according to the International Working Group on the Diabetic Foot, DFU is

a deep wound that penetrates through the dermis up to the most internal vascular layer, below the ankle (W. J. Jeffcoate et al, 2004). The main mechanisms underlying DFUs develop are peripheral neuropathy and ischemia from peripheral arterial occlusive disease (PAOD). The causes of ischemic foot tissues can be the atherosclerosis or microvascular disease and in the presence of these situations, a moderate ischemia can lead to ulcer develop and impair healing (W. J. Jeffcoate et al, 2003; A Veves et al, 2002). Early studies on the DFUs ignored that the presence of PAOD could be a very important risk factor, while today it is officially recognized as a significant cause of DFUs, with an important increase in the developed countries (A. I. Adler et al, 1999; D. G. Amstrong et al, 2011). Furthermore, according to a study performed by Morbach and colleagues in 2003, it has been highlighted as the patients with neuroischemic or ischemic disease show a major risk to have amputations, as well as a higher mortality rate (P. K. Moulik et al, 2003).

Today, the main therapy for DFUs consists of restoring blood flow by angioplasty or bypass and the removal of the debridement and the necrotic tissue to accelerate the wound healing. Additional treatments have been recently introduced, such as biological dressing, compression therapy, physical therapy by the use of hyperbaric oxygen and negative pressure therapy, although the clinical results are still not satisfactory (G. Mulder et al, 2014). Nowadays, surgical revascularization is the gold standard for the tretament of arterial ulcer, but the blood flow restoration should be obviously combined with treatments aimed at reactivating the autologous healing processes, that resulted ineffective, moderating the inflammatory cell responses and promoting the local angiogenesis, in order to facilitate the wound healing processes (S. Valente et al, 2016).

Epidemiology and costs of chronic non-healing wounds

The prevalence rate for chronic non-healing wounds in the United States is 2% of the general population (C. K. Sen et al, 2009). The mean number of serious and complex conditions of comorbidity is 1,8/patient and among the most common there are diabetes mellitus (46,8%), obesity and overweight (71,3%), cardiovascular or peripheral vascular disease (51,3%). It is estimated that the costs of treating these wounds exceed 50 billion dollars per year (C. E. Fife et al, 2012) and these costs are 10 times more than the

annual budget of the World Health Organization (WHO). In this context, it is clear that the chronic non-healing wounds not only affect the quality of life of the patients, but they also have an important and great weight on the whole society, that is called to response to the costs of care. So, it is very important to focus the attention on new and promising therapeutic strategies aim to accelerate the wound healing process and to optimize costs and resources.

Wound healing

Acute wound healing consists of four phases and it is a dynamic process involving the coordinated actions of both resident and migratory cell populations within the extracellular matrix environment that leads to the repair of injured tissues. The first phase is hemostasis, caused by the rupture of the blood vessels; during this phase, the activation of thrombocytes and tissue factors lead to the formation of the clot, composed of a fibrin network. The second step is the inflammatory phase, during which bacteria, other pathogens, damaged or dead cells are removed by the phagocytosis process; in this phase, the clotting cascade is activated and the plateled-derived growth factor (PDGF) is released into the wound to induce cell migration and division during the next stage. The third phase is proliferation and growth of the new tissue: the angiogenesis promotes the formation of new blood vessels, the fibroblasts are activated to form a new extracellular matrix with deposition of collagen and fibronectin, the epithelial cells proliferate and form the new tissue in the injured site and myofibroblasts contract themselves (K. S. Midwood, 2003; H. G. Garg, 2000; S. Enoch et P. Price, 2004). The last phase is represented by the maturation and remodeling of the injured tissue, during which the cells not longer needed are removed by apoptosis (Figure 4); this phase can last from three weeks to months or years, because the healing process can be susceptible to interruption or failure. In fact, some wounds fail to heal in a timely and orderly manner, resulting in chronic non-healing wounds, especially in conditions complicated from the presence of venous and arterial diseases, diabetes mellitus, infections and metabolic deficiencies. In addition, the chronic wounds are more prevalent in older people due to the altered molecular and cellular characteristics of the aged skin and various associated co-morbidities. The frequent presence of chronic wounds does not

allow the complete recovery of the tissues, affecting on life quality of patients and healthcare costs.

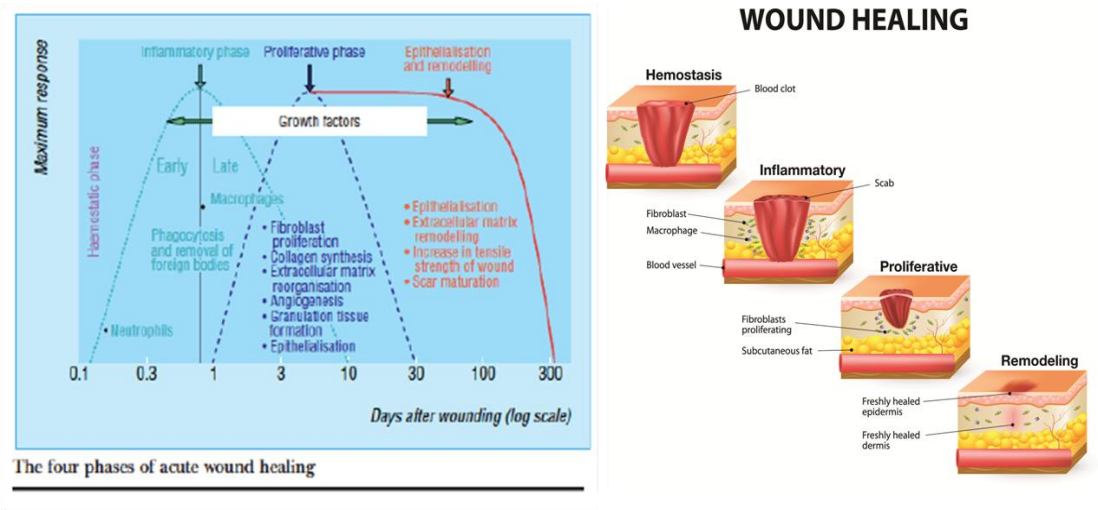


Figure 4: Schematic representation of the four phases of acute wound healing

New therapeutic approaches in the context of wound healing

For all reasons mentioned above, nowadays new therapeutic approaches or combined therapies are under development in order to facilitate the wound healing. It is good to remember that, for an efficient wound care, it is first of all essential revascularization, good wound care with debridement and exudates management, adequate rest, compression, pressure relief, skin care and new approaches and biological based treatments, among which there are the use of tissue engineered, the autologous fibroblasts, autologous adipose stromal vascular tissue, PRP gel treatments, cell therapy with multipotent stem cells and growth factors.

The use of gene and cell therapies (M. Shimamura et al, 2013), as well as the use of natural or synthetic engineered matrices (G. Mulder et al, 2014; T. Lazic and V. Falanga, 2011) appear very interesting in the scientific community. Some studies refer about the complete wound healing after transplantation of biomimetic tissue engineered dermis substitutes with allogeneic keratinocytes and fibroblasts cells inside ulcerated tissue (H.-J. You et al, 2012; W. A. Marston et al, 2003). Other works report about the use of the adult mesenchymal stem cells derived from several tissues to treatment non-healing wound thanks to their ability to differentiate in multiple mesengenic lineages, to

secrete several growth factors and to regulate the inflammatory processes (D. S. Kwon et al, 2008; Y. Wu et al, 2007; S.-K. Han et al, 2010; C. Nie, D. Yang et al, 2011; H. C. Lee et al, 2012).

Among the new therapeutic approaches, single or combined growth factors including Platelet-Derived Growth Factor (PDGF), Vascular Endothelial Growth Factor (VEGF), Transforming Growth Factor-beta (TGF-beta), Fibroblast Growth Factor (FGF), Epidermal Growth Factor (EGF), Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF) and Hepatocyte Growth Factor/Scatter Factor (HGF/SF) are largely studied (S. Enoch et al, 2006; S. Barrientos et al, 2014). These growth factors own multiple effects on tissue regeneration including cell proliferation and mobilization, extracellular matrix production and angiogenesis.

Hepatocyte Growth Factor (HGF)

HGF is the growth factor of the liver and it is a multifunctional and pleiotropic cytokine of mesenchymal origin involved in several biological responses including cell proliferation/survival, angiogenesis, morphogenesis, and motogenesis (K. Matsumoto and T. Nakamura, 1996), as well as inflammation and fibrosis inhibition (K. Okunishi et al, 2005; R. Gong, 2008).

HGF exerts its actions not only in the liver, but also into a wide spectrum of cells, through its autocrine and paracrine activities. HGF is an 84 KDa dimeric molecule, essential growth factor for liver regeneration (T. Nakamura et al, 2010), while the product of the c-Met pro-oncogene is the receptor of HGF (D. P. Bottaro et al, 1991), which carries out its role, once linked to HGF, by its tyrosine-kinase activity (T. Nakamura et al, 2010), (Figure 5).

c-Met is primarily expressed in epithelial cells, but few reports indicate that adult mesenchymal stem cells (MSCs) also express c-Met (S. Neuss et al, 2004; G. Forte et al, 2006; K. K. Aenlle et al, 2014).

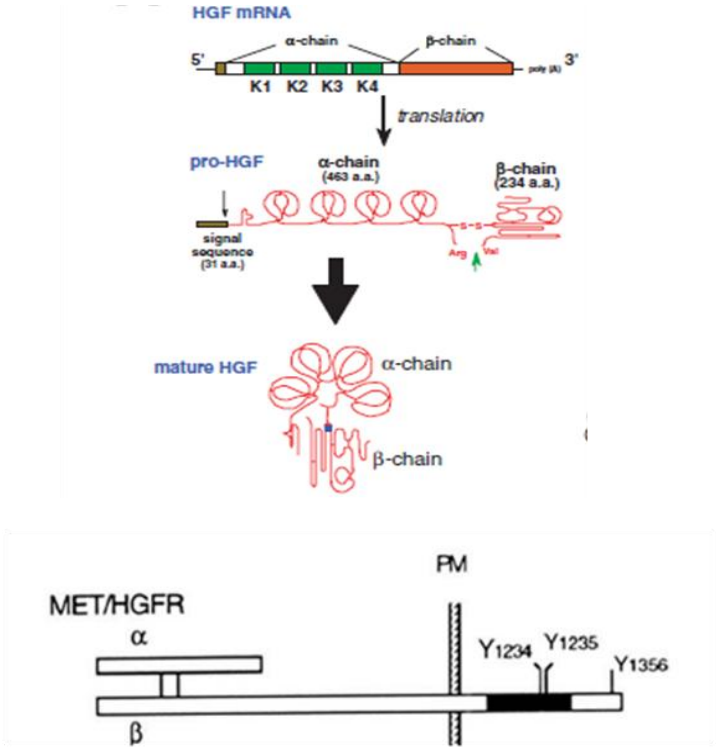


Figure 5: Schematic representation of molecular structure of HGF and its receptor c-Met (T. Nakamura and S. Mizuno, 2010; H. Zhu at al, 1994).

Recent data of our research group have shown that patients with ulcers have an alteration of HGF/Met pathway (F. Vasuri et al, 2013).

Another molecule of great interest, indirectly connected to HGF/Met pathway, is HIF-1 (Hypoxia Inducible Factor-1). During hypoxia, HIF-1 drives the expression of several genes, otherwise repressed, especially growth factors, among which Met receptor itself (G. L. Semenza et al, 2001); HIF-1 could therefore regulate HGF/Met pathway, by influencing the amount of Met receptor on the cell surface (Figure 6).

The results of this work (F. Vasuri et al, 2013) indicate that, in ulcers and condition of hyperglycemia and/or hypoxia, there is a general down-regulation of the HGF/Met pathways, with a repression of the local HGF production; in addition, also HIF-1 stability decreases (S. B. Catrina et al, 2004). Since HGF/Met pathway plays key roles, among which promotion of cell growth, tissue homeostasis, neoangiogenesis, in conjunction with anti-inflammatory and anti-fibrogenic activities (T. Nakamura et al, 2005), it supposes that the local or systemic application of HGF could reactive Met receptor, stabilizing the normal wound healing process. According to these evidences,

growth factor's secretion, as HGF, could play an important role in tissue regeneration of ulcers, as well as HGF/Met axis could be the new target of future therapies.

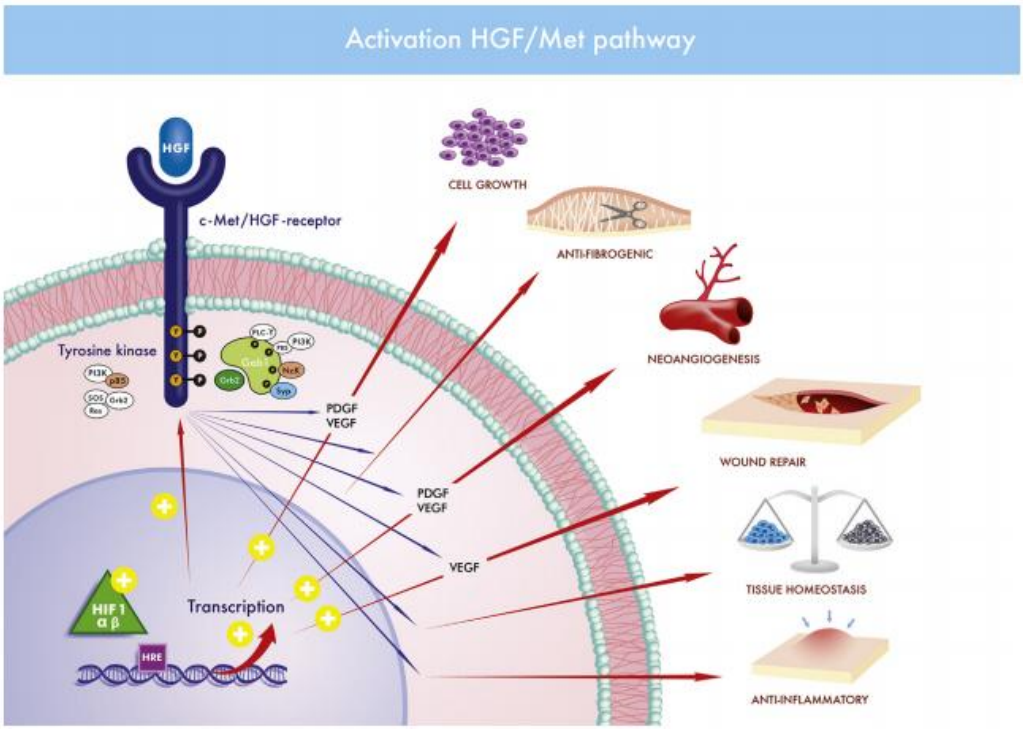


Figure 6: Pathway HGF/Met and its effects on cell processes (F. Vasuri et al, 2013).

Platelet-rich plasma (PRP)

Human derived autologous platelet-rich plasma (PRP) is also a promising wound healing treatment (K. M. Lacci et al, 2001), due to the mitogenic and chemoattracting effects exerted by growth factors released at high concentrations after platelet α-granules degranulation (K. M. Lacci and A. Dardik, 2010). Some researches and clinical trials refer about a positive effect of PRP on re-epithelialization of non-healing wounds (V. R. Driver et al, 2006; H. S. Setta et al, 2011; T. Slesaczeck et al, 2012). PRP is defined as a portion of the plasma fraction of autologous blood having a platelet concentration above baseline (R. E. Marx, 2001), and it is used in several surgical field to facilitate tissue reparation by releasing locally acting growth factors, especially in the context of maxillofacial surgery, periodontal and oral surgery, heart bypass surgery, spinal surgery, etc. (P.A. Everts et al, 2006). PRP, in fact, may suppress cytokine release and limit inflammation, to improve tissue healing and regeneration, promoting

new capillary growth and accelerating epithelialization; finally, PRP has antimicrobial activity against several pathogens (Y.Q. Tang et al, 2002).

PRP can also be got from umbilical cord blood unsuitable, on a quantity level, to conservation of Hematopoietic Stem Cells (HSCs): in this case it is about hemo-component at topic use. In particular, recent studies have demonstrated that PRP exerts its anti-inflammatory effects through HGF (J. Zhang et al, 2013). PRP and HGF treatments suppress the IL-1β-induced gene expression of COX-1, COX-2 and mPGES-1, and consequently protein expression of COX-1, COX-2 and PGE_2. In this regard, the anti-inflammatory effects of PRP and HGF have mechanisms similar to those of NSAIDs; but, while these drugs have serious side effects, PRP is thought to be intrinsically safe for its autologous nature, feature to its advantage.

In conclusion, in order to guarantee an efficient tissue regeneration in ulcers of lower-limb, PRP derived from umbilical cord blood can also be used in replacement to FBS (Fetal Bovine Serum) in the culture medium, from which depends the *in vitro* expansion of ECFCs (Endothelial Colony Forming Cells). ECFCs are, indeed, an attractive target for new vascular regenerative therapies and an animal serum-free culture medium, for their *in vitro* isolation and expansion is greatly needed (J. Zhang et al, 2013). In this context and for the above-mentioned characteristics, PRP results more appropriate, if compared with FBS, for future use of ECFC.

Stem Cells (SCs)

The stem cells are undifferentiated cells provided with self-renewal for an undefined time. Under appropriate conditions and stimuli, the stem cells are able to give rise to several mature cellular types, through a process called multilineage differentiation. The stem cells can replicate them by two different mechanisms: the asymmetric and symmetric division. The asymmetric division lead to the origin of two daughter cells: one of which conserves the staminal characteristic, while the other daughter cell (the progenitor cell) differentiates in a mature cell. The symmetric division give rise to two identical daughter cells, which may be two stem cells or two progenitor cells (Figure 7).

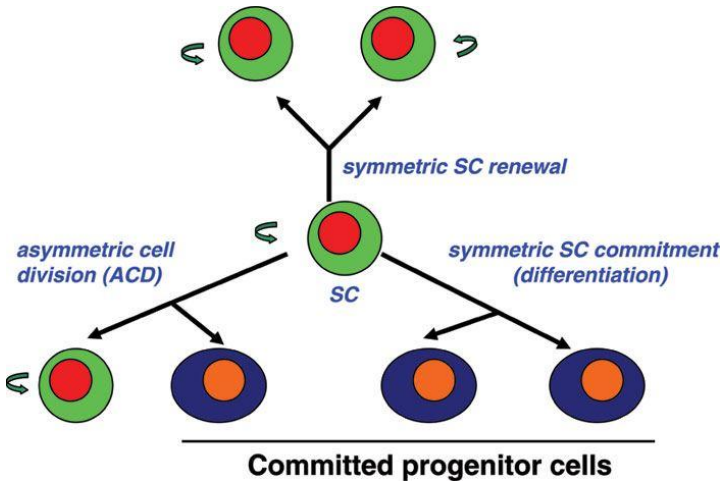


Figure 7: Schematic representation of the mechanism of asymmetric and symmetric division.

The stem cells can be classified as totipotent, pluripotent, multipotent and unipotent, based on their differentiation potential (A. J. Wagers et I. L. Weissman, 2004), (Figure 8). Totipotent stem cells are able to give rise to embryonic and extra-embryonic tissues; the zygote is the totipotent stem cell, from which the three germ layers origin. Pluripotent stem cells are instead able to generate all cells derived from endoderm, mesoderm and ectoderm, but not the cells of extra-embryonic tissues; the embryonic stem cells (ESCs) and embryonic germ cells (EGCs) are pluripotent stem cells. Again, multipotent stem cells are able to differentiate into a specific subset of mature cell types, while unipotent stem cells differentiate into a unique specialized cell type. Finally, stem cells can be classified in two more important categories: the embryonic stem cells and the adult stem cells (O'Connor, 2008).

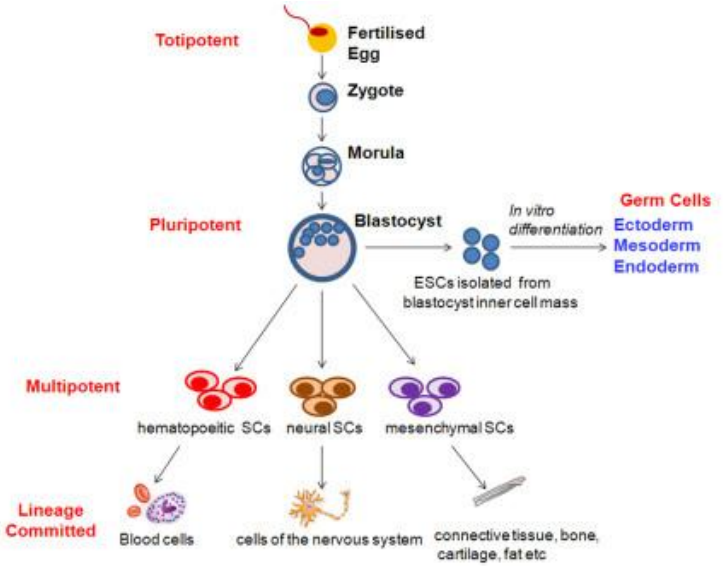


Figure 8: Stem cells hierarchy.

Embryonic Stem Cells (ESCs)

The ESCs are pluripotent cells which derive from embryo during the stage of blastocyst, 5 days after fecundation. The blastocyst is a structure consisting of about 100 cells and it is composed of two parts: the external layer of cells called trophectoderm that will form the placenta and the inner cell mass (ICM), composed of pluripotent stem cells which will give rise to all cells of the organism. ESCs are immortal cells, they have unlimited proliferation potential, a normal karyotype and they are able to differentiate in a derivates of all three embryonic germ layers (Figure 9), including gut epithelium, cartilage, bone, smooth muscle, and striated muscle, neural epithelium, embryonic ganglia and stratified squamous epithelium. (J. A. Thomson, 1998). The ESCs express moreover high telomerase levels: telomerase is a ribonucleoprotein involved in maintaining telomere length and it is directly related to immortality of ESCs; compared to ESCs, telomerase is less expressed in diploid human somatic cells and telomeres reduce themselves during replicative life-span (C. B. Harley et al, 1991; C. M. Counter et al, 1996). Among the cell surface markers expressed by ESCs there are the Stage Specific Associated Antigen (SSEA–3 and SSEA-4), keratan sulfate antigens (TRA-l-60 and TRA-1-81) and alkaline phosphatase (J. A. Thomson et al, 1995; J. A. Thomson et al, 1998). Furthermore, ESCs show a core transcriptional regulatory circuitry composed of the transcription factors OCT-4, SOX-2 and NANOG, essential for the

maintenance of pluripotency and self-renewal in early development of ESCs. These transcription factors are localized in the same portion of their target genes, where they regulate their expression (L. A. Boyer et al, 2005).

ESCs were derived for the first time in 1981 from mouse embryo (M. Evans et al, 1981; M. Kaufman et al, 1981), and some year later J. Thomson and his research group developed a technique to isolate and grow *in vitro* human embryonic stem cells (J. A. Thomson et al, 1998). The usefulness of ESCs has been proposed in many contexts, from regenerative medicine to the tissue replacement after injury and disease; however, many ethical issues persist and limit their clinical applications.

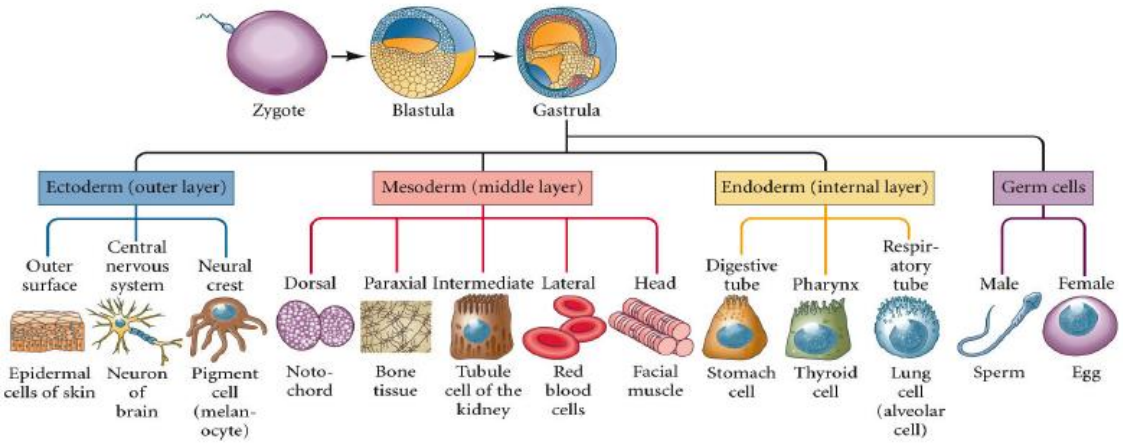


Figure 9: Embryonic stem cells and differentiation in a derivates of the three germ layers.

Adult Stem Cells (Somatic Stem Cells)

Adult stem cells or somatic stem cells (ASCs) are undifferentiated multipotent cells localized in the adult organism; they are provided of self-renewal and capability to differentiate in the specialized cells within the organ where they reside and they have the function of precursor cells. The presence of ASCs was discovered since 1960, when two populations of stem cells were identified in the bone marrow: the hematopoietic stem cells (HSCs) which give rise to all blood cells and the mesenchymal stem cells (MSCs) or bone marrow stromal cells that give rise to osteocytes, chondrocytes and

cells of connective tissue (A. J. Becker et al, 1963; J. Till et al, 1961). The main ASC function is replacing cells that die in the tissues after injury or disease (N. Schultz, 2008). Later, ASCs have been found in several adult tissues including not only bone marrow, but also brain, spinal cord, peripheral blood, dental pulp, blood vessels, cornea, liver, pancreas and epithelia. Little is known about ASC origin and characterization, and the most of information come from studies with the mice. Two are the properties considered fundamental to the definition of ASCs: self- renewal, the ability to divide themselves through numerous cell cycles maintaining the undifferentiated state, and the multipotency or multidifferentiation potential, the ability to specialize themselves in mature cells of several tissue districts; this two properties may be characterized *in vitro* by clonogenic assays and the mechanism underlying this two properties get involved three molecular stem cell regulators: Notch, Wnt and TGF-β (G. Dontu et al, 2004; P. A. Beachy et al, 2004; M. Sakaki-Yumoto et al, 2012). Recent studies have also highlighted how the ASCs own some plasticity or stem cell trans-differentiation, referred to the ability to differentiate into cells from distinct germ layers (Figure 10). In fact, although it was initially thought that ASC capacity to differentiate was rather limited to the organ where they reside, it was subsequently discovered that ASCs from brain are able to differentiate in liver, cardiac and skeletal muscle cells and that stem cells from blood can generate stem cells of brain (G. Ferrari et al, 1998; F. Lagasse et al, 2000; R. Galli et al, 2000). This phenomenon is visible *in vitro* when it is possible to induce stem cells isolated from a tissue into mature stem cells of other tissues, modifying the growth medium. ASCs reside in particular micro-environments called niches within the organs and here they are in a quiescent state until they are activated to proliferate; in the event of a disease or tissue injury, ASCs migrate outside of the niche and go for differentiation (E. Fuchs et al., 2004; K. Urbanek et al., 2006).

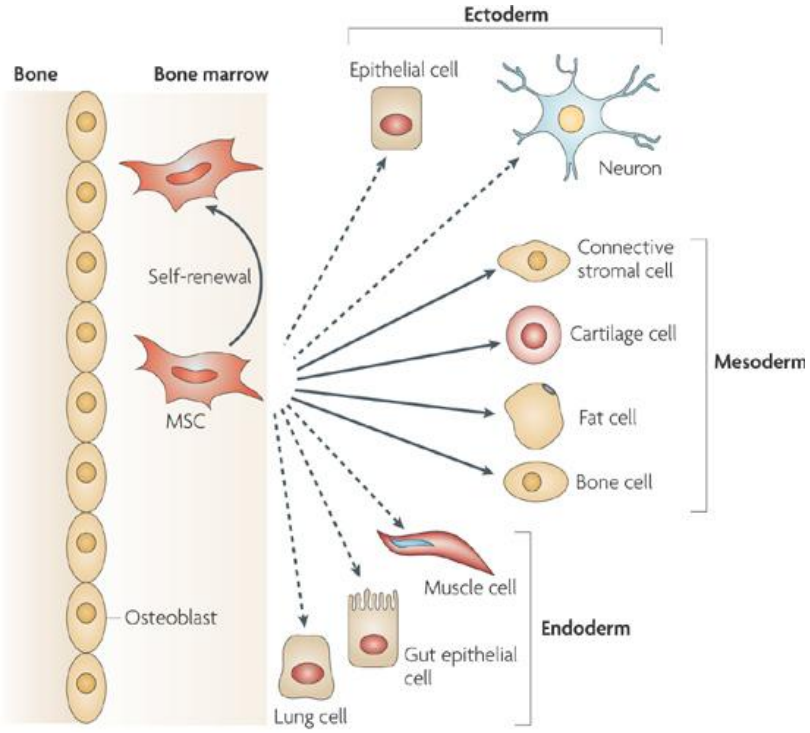


Figure 10: The adult stem cells (A. Uccelli et al, 2008).

Mesenchymal stem cells (MSCs)

Mesenchymal stem cells are multipotent stromal cells (A. J. Friedenstein, 1968) able to differentiate in several lineages including osteoblasts, chondrocytes, adipocytes and myocytes, but also into other cell types from endodermic and ectodermic lineages (M. F. Pittenger et al, 1999). MSCs are cells derived from mesenchyme, an embryonic connective tissue generated from mesoderm, which differentiates into hematopoietic and connective tissues. MSCs are so cells belonging to mesenchyme, the connective tissue composed of a great amount of extracellular matrix (ECM) and the MSCs; the mesenchyme has an important role as support of the functional cells.

MSCs were initially isolated from bone marrow at the end of 1800 but, as years go by, they were also isolated from a large variety of other tissues as placenta, cartilages, adipose tissue, adult muscle, bone tissues, umbilical cord blood, corneal stroma etc. (S. Gronthos et al, 2001; A. Asakura et al, 2001; A. Erices et al, 2000)

Under normal culture conditions, these cells adhere to plastic, show a fibroblast morphology and own an high proliferative potential (E. M. Horwitz et al, 2005; D. J. Prockop et al, 1997); *in vitro*, MSCs can be characterized because they express

mesenchymal (CD44, CD73, CD90, CD105, HLA-G, Stro-1) and stemness markers (Oct-4, Nanog, Notch-1, Sox-2, KDR, c-Kit), pericyte (CD146, PDGFR-β, NG2) and neuronal markers (Nestin), while they show negativity for hematopoietic markers (CD14, CD34, CD45) (M. Dominici et al, 2006). Finally, MSCs which secrete several growth factors and play an important role in the regulation of inflammatory processes, inhibiting T cell proliferation by IFN gamma secretion (Z. Selmani et al, 2008; R. Maccario et al, 2005; E. Zappia et al, 2005; I. Rasmusson et al, 2005; G. Ren et al, 2008).

The vascular niche of mesenchymal stem cells

Until not long ago, it was believed that the vessel wall was relatively quiescent. But the discovery of mesenchymal stem cells and progenitor cells in many tissues of the organism led to a re-evaluation of the vascular system and its biology. Briefly, the vascular wall is composed of cells of mesodermal origin, including endothelial cells (ECs), smooth muscle cells (SMCs) and stromal fibroblasts resident in the adventitia. The vascular wall consists of 3 layers: the intima, a layer in which ECS resides; the media, composed of many layers of SMCs and the adventitia, consisting of fibrous and adipose tissue, small capillaries usually named "vasa" and nerves, also called "vasa vasorum" (P.J. Gallagher, 1992; B. Hinz et al, 2007; E.L. Ritman et al, 2007; W. Warwich et al, 1989). The contribute of ECs and SMCs in the responses to injury was well investigated (P. Libby, 2005; R. Ross, 1999), but less it is known about of the progenitors from which vascular wall cells (VWCs) originate. The vessels are in fact exposed to multiple stresses and the VWCs can growth and participate to repair processes during the life in response to injuries. But also in pathological conditions, as atherosclerosis or abdominal aortic aneurysm, VWCs and their progenitors are actively involved in the disease develop. Furthermore, some differentiation commitment was obtained from human vascular wall resident mesenchymal stem cells (G. Pasquinelli et al, 2007; A. Hoshino et al, 2008; D.T. Covas et al, 2005). It is supposed that the vasculogenic niche, where these progenitors reside, is located in the vascular wall between the media and adventitia layers (Figure 11). This area probably contains stem cells able to differentiate into ECs, hematopoietic cells, mesenchymal stem cells and

immune cells (E. Zengin et al, 2006). A pivotal study of Tintut et al, demonstrated that mesenchymal stem cells are also present in adult animal vessels, while Pasquinelli et al, investigated about the presence of human vascular wall mesenchymal stem cells (hVW-MSCs) in human thoracic aorta and other many arteries cryopreserved from healthy multiorgan donors (Y. Tintut et al, 2003; G. Pasquinelli et al, 2006). These studies described in addition the heterogeneity of the vasculogenic niche composition, in which different populations with stem cell characteristics are located., among which ECs, progenitors of SMCs, MSCs and progenitor cells of vascular wall. Finally recent evidences about the study of the perivascular ultrastructure identify two possible targets associated with MSCs, that means the pericyte and the perivascular fibroblasts, cells without contractile filaments and with long cytoplasmic processes. This evidence could demonstrate why some MSC express pericyte markers and smooth muscle actin, while other MSCs maintain a specific mesenchymal phenotype (M. Crisan et al, 2008; I. Joris et al, 1974).

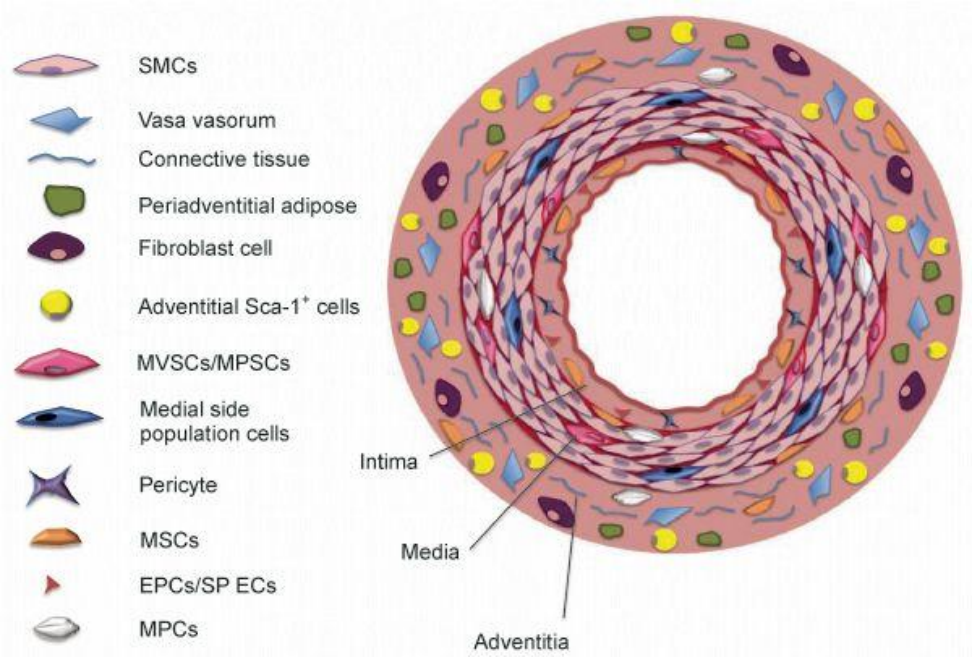


Figure 11: Structure of a vessel and heterogeneous composition of the vascular niche (D. F. Leach et al, 2015).

Fibroblasts and myofibroblasts

The fibroblasts are spindle-shaped cells that reside in the connective tissues of several organs. The phenotype of these cells can vary from a non-contractile fibroblast to a contractile myofibroblast with a range of intermediate morphologies (Mc Anulty, 2007; B, Eyden, 2005). Unfortunately, a specific marker to identify fibroblast does not exist and this represents the major limit to investigate and study these cells. Regarding their origin different hypothesis have been sustained (A.E. Postlethwaite, 2004); some Authors believe that fibroblasts would originate from epithelial-mesenchymal transition (ETM) (J. Zavadil and E.P. Bottinger, 2005; K. K. Kim et al, 2006), after the loss of epithelial markers, as example E-cadherin. Other studies support the theory that fibroblasts would arise from resident fibroblast division or from circulating bone marrow-derived fibrocytes or, yet, from differentiating monocytes (Y. Chang et al, 2014); others still include the origin of fibroblast from resident stem cells, such as hMSCs or pericytes. Fibroblasts are responsible for the maintenance of extracellular matrix (ECM), secreting ECM component, that means collagens, proteoglycans, fibronectin, tenascin and laminin. These cells also produce matrix metalloproteinases (MMPs) and their inhibitors, and regulate the interstitial fluid volume and pressure. Recently, even these cell typologies were introduced in the new therapeutic approaches to treat wound healing. When a tissue is injured, resident fibroblasts differentiate into myofibroblasts in particular in the early phase, when a granulation tissue is formed (Figure 12). Myofibroblasts proliferate during the proliferation phase and disappear in the last phase of wound healing, through a programmed cell death mechanism, also called apoptosis. These cells show abundant cell-matrix adhesions, intercellular junctions, gap junctions and contractile microfilaments, necessary in wound repair. In this regard, both fibroblasts and myofibroblasts play a central and critical role in wound healing and repair processes, since they generate traction and contractile forces necessary to promote wound contraction (B. Li et al, 2009).

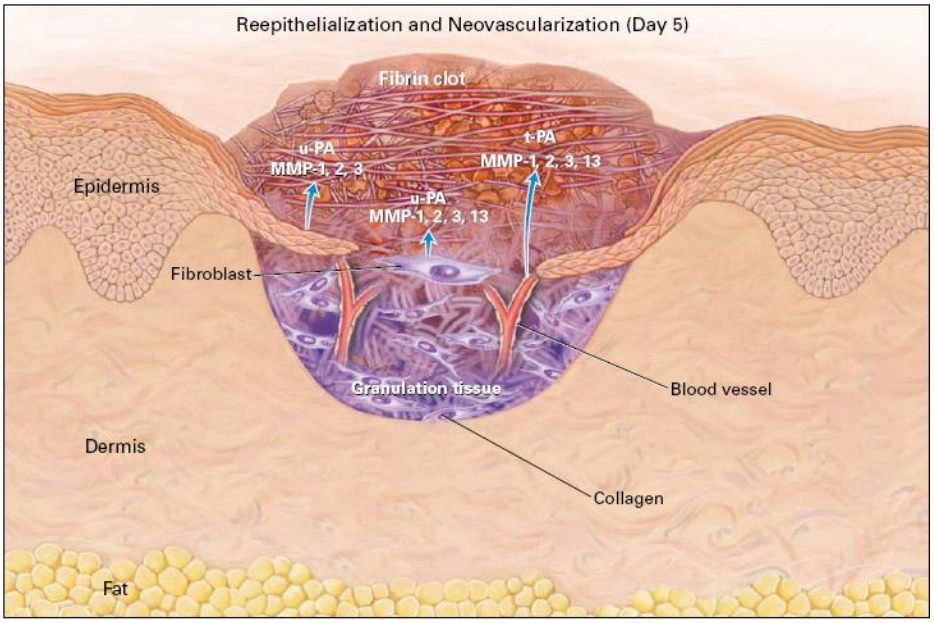


Figure 12: Cutaneous wound after injury (A. J. Singer and R. A. F. Clark, 1999)

Aim of the thesis

The study of this thesis is contextualized within the lower limb ulcers and wound healing issue. The lower extremity ulcerations represent a debilitating clinical situation that affects many people, especially over 65-year-old individuals, where the disease is much more prevalent (about 5%). Mostly in the presence of peripheral arterial occlusive disease, these patients own a higher probability to develop foot ulcers and chronic non-healing wounds that fail to heal and the recovery of the tissues is not complete, affecting not only on life quality of the patients but also on the healthcare costs. The situation is furthermore complicated by conditions as diabetes mellitus, infections or metabolic deficiencies. Nowadays, the gold standard to treat diabetic foot ulcers and the associated chronic wounds is the surgical revascularization, even if new therapeutic approaches are tested in conjunction with the conventional treatment, in order to facilitate the wound healing process.

Among the most promising prospects there is the use of gene and cell therapy or natural and synthetic engineered matrices (M. Shimamura et al, 2013; G. Mulder et al, 2014; T. Lazic and V. Falanga, 2011). Furthermore, many studies report about the use of adult mesenchymal stem cells derived from several tissues, thanks to their ability to differentiate in multiple lineages, secreting several growth factors and mediating the inflammatory processes (D. S. Kwon et al, 2008; Y. Wu et al, 2007; S.-K. Han et al, 2010; C. Nie, D. Yang et al, 2011; H. C. Lee et al, 2012).

Finally, many growth factors are widely experimented in the context of the lower-limb ulcers, as example PDGF, VEGF, TGF-beta, FGF etc, for their effects on cell proliferation, cell migration, extracellular matrix production and angiogenesis. One of the factors that is gaining relevant importance is the hepatocyte growth factor, the growth factor of liver; HGF is a pleiotropic and multifunctional cytokine, expressed in cells mainly of epithelial origin, with a several biological activities among which the promotion of cell growth and motility, the maintenance of tissue homeostasis, the promotion of self-repair of injured livers, kidneys and lungs, the induction of angiogenesis, tissue regeneration and protective. A work of our team reported interesting results about patients with chronic lower limb ischemia (CLI) in which there seems to be an alteration of the molecular pathway HGF/c-MET (F. Vasuri et al, 2013).

In addition, our research group developed over the years a detailed protocol to isolate the human mesenchymal stem cells from vascular wall of several segments of arteries, where it is present a vascular niche; these arteries come from healthy donors and they have been cryopreserved into nitrogen liquid for at least 5 years; arteries were kindly

provided from Cardiovascular Tissue Bank facility, University – Hospital Sant'Orsola Malpighi, Bologna (S. Valente et al, 2014). Furthermore, it has been also developed a protocol to isolate a population of MSCs from the abdominal aortic aneurysm, where, the presence of mesenchymal stem cells (AAA-MSCs) was never discovered before (C. Ciavarella et al, 2015). These cells are altered since over-express MMP-9 and own an ineffective immunomodulatory capacity.

We decided at first to focus our attention on vascular wall derived MSCs (hVW-MSCs) since they are potentially usable in the context of ulcers and wound healing; initially we tested HGF on healthy hVW-MSCs and then we functionally altered AAA-MSCs. In the last phase, we tried to characterize the differentiation potential of different cell types involved in the mechanism of tissue repair.

The aim of the thesis is structured as follows.

PART 1: study of HGF in vitro effect on healthy hVW-MSCs.

During this phase, HGF was tested at different times on healthy hVW-MSCs to study its effects in terms of proliferation, migration, motility, angiogenesis and tissue remodeling.

PART 2: *HGF in vitro effect on tissue remodeling and inflammation in healthy hVW-MSCs and in functional altered AAA-MSCs.*

HGF was tested in a pathological setting represented by AAA-MSCs isolated from abdominal aortic aneurysm, in particular in the issue of the tissue remodeling and inflammation.

PART 3: Comparison of differentiation potential among different cell types involved in wound healing process.

In the second phase of the study we decided to compare the behavior of hVW-MSCs with the one of normal human dermal fibroblasts and myofibroblasts, as cells involved in wound healing processes and tissue repair, in order to clarify the differences especially about their differentiation potential. For this aim we performed some functional assays to study angiogenic and adipogenic potential of these cells.

Materials and methods

Human Vascular Wall – Mesenchymal Stem Cells (hVW-MSCs), Abdominal Aortic Aneurysm – Mesenchymal Stem Cells (AAA-MSCs), Dermal Fibroblasts, Myofibroblasts (WPMY_1), human umbilical vein endothelial cell (HUVEC) and culture conditions

Human Vascular Wall Mesenchymal Stem Cells (hVW-MSCs) and Abdominal Aortic Aneurysm Mesenchymal Stem Cells (AAA-MSCs) utilized in this study have been isolated from human arteries upon consent informed and according to the Ethic Protocol (APP-13-01) approved by the Local Ethics Committee of University Hospital St. Orsola-Malpighi of Bologna (Italy). Aneurysm tissues for AAA-MSCs isolation were provided by the Vascular Surgery Unit of University Hospital St. Orsola-Malpighi of Bologna (Italy), (C. Ciavarella et al, 2015). To realize the experiments, segments from carotid, thoracic and femoral arteries were used to isolate hVW-MSCs. The human artery segments were enzymatically digested overnight at 37°C, in rotation and within a solution of serum-free DMEM culture medium and 0,3 mg/mL Liberase type II (Roche, Milan, Italy). After digestion, a filtration with 40-70-100 μm nylon mesh cell strainer was carried out (Becton Dickinson; Franklin Lakes, NJ) and the cells obtained were seeded and cultured in complete DMEM 20% FBS in a humidified atmosphere of 5% CO2 for 2-3 days, while at following passages they were cultured in complete DMEM 10% FBS. The cells were expanded *in vitro* and characterized to prove their positivity to mesenchymal and stemness markers (S. Valente et al, 2014).

In this study, experiments were performed using cells taken at passages 3 and 4 and cultured in DMEM plus 10% of FBS with or without Hepatocyte Growth Factor/Scatter Factor (HGF/SF) (WHO Reference Reagent, HGF/SF, NIBSC code: 96/564, National Institute for Biological Standards and Control, Potter Bar, Hertfordshire, ENG 3QG) at different time (from 6 hrs to 7 days) and concentrations (from 2.5 to 70 ng/mL). Cell starvation with low percentage of serum (0.5% FBS) for 12 hrs was performed to induce cell cycle synchronization before pretreatment with 0.2 μM PHA-665752 inhibitor (Tocris Bioscience, Bristol, UK) for 12 hrs, followed by HGF/SF addition.

Normal human dermal fibroblasts (kindly provided by Professor Francesco Alviano, acquired by Lonza) and a cell line of WPMY-1 (ATCC® CRL-2854™), were also used, both cultered in complete DMEM with 10% FBS.

Human umbilical vein endothelial cells (HUVEC) were used in the *in vitro* tube formation assay as positive control of the angiogenesis process.
All experiments were executed in triplicate.

Proliferation assay

Different methods were used to evaluate cell proliferation.

Cell viability was investigated using **Alamar Blue fluorescence assay** (Invitrogen, Milan, Italy). The hVW-MSCs were seeded in a 12-multiwell plate at the density of 3×10^4 and cultured in complete DMEM with or without HGF/SF (2.5, 5, and 10 ng/mL) for 1, 3, and 7 days. The same experiment was also conducted by the pretreatment of PHA-665752 inhibitor at 0,2 μM. Alamar Blue solution (10% v/v in cultured medium) was added to each well at the end of treatments and incubated for 4 hrs at 37°C according to the manufacturer's instruction. Alamar Blue fluorescence (Ex/Em = 540/590 nm) of three replicates in each well was measured in a Wallac VICTOR2 multiplate reader (Perkin Elmer, Milan, Italy). In addition, a standard polystyrene well was used to measure the background fluorescence; this fluorescence was subtracted from the reading of each well (Figure 13).

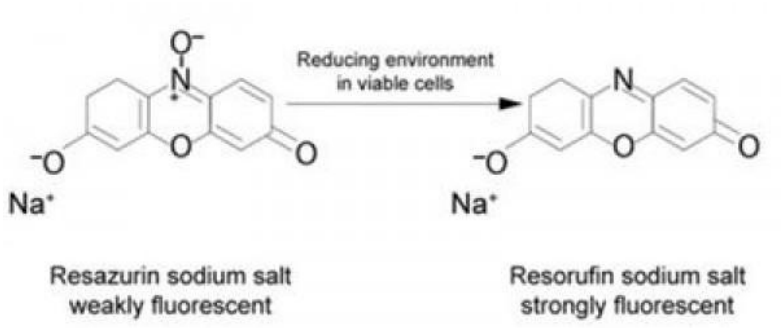


Figure 13: Chemical structure of Resazurin, the blue dye of Alamar Blue. The dye is a redox indicator that yields a colorimetric change and a fluorescent signal in response to metabolic activity.

Immunofluorescence staining was also used to assess cell proliferation, in particular for cycling cells expressing Ki-67 protein. hVW-MSCs were plated at a density of 6 × 10^5 on coverslip in 6-well plates in DMEM overnight to allow the cell confluence and treated with or without HGF/SF (2.5, 5, 10, and 70 ng/mL) for 6 and 24 hrs. In parallel experiments, additional cell-seeded glass was starved in 0.5% FBS for 12 hrs, pretreated with 0.2 μM PHA-665752 inhibitor, and subjected to HGF/SF exposure at the same time and concentrations. Untreated cells were used as a control.

To develop the immunofluorescence staining, the cells were seeded upon the slides at density of 30x10^3 in DMEM 10% FBS and cultered O/N at 37°C in incubator. The following day cells were washed with PBS, fixed in 2% paraformaldehyde in PBS with 1% Tryton X-100 for 4 minutes at RT and treated for 30 minutes at RT with 1% bovine serum albumin (BSA), a blocking reagent for nonspecific binding. Later, the slides were labeled with monoclonal antibodies against nuclear transcription factor Ki-67 (1 : 100, Novocastra, Leica Microsystems, Wetzlar, Germany) and the intermediate filament Vimentin (1 : 100, Dako Cytomation, Glostrup, Denmark) for 1 hr at 37°C in a wet chamber; both antibodies were prepared in 1% BSA in PBS, while for negative control the primary antibody was not use and no fluorescence was detected. A series of washing were conducted and then the cells were stained with AlexaFluor-488 (1 : 250, Life Technology, Carlsbad, CA, USA) secondary antibody for 1 hr at 37°C in the dark and counterstained with Pro Long antifade reagent with DAPI (Molecular Probes, Milan, Italy). Finally, the slides were observed and photographed with a Leica Leica DMI6000 B inverted fluorescence microscope (Leica Micro-systems, Wetzlar, Germany). For each experimental condition, the number of Ki-67 intensely stained cells as well as DAPI stained nuclei was manually counted on ten random fields and their values were expressed in percentage as ratio of Ki-67 stained cells on total cells number (Figure 14).

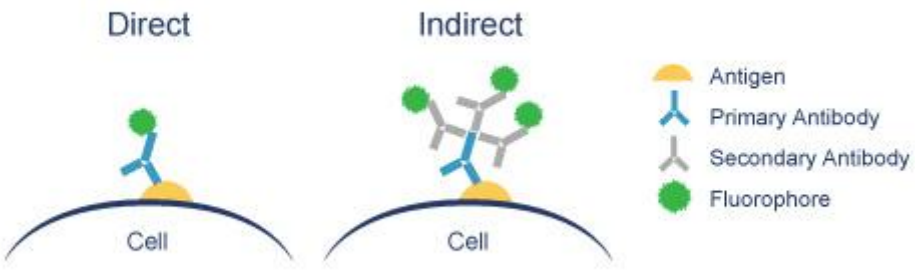


Figure 14: Comparison between direct and indirect Immunofluorescence.

The cell proliferation was also evaluated with "**Cell Proliferation ELISA, BrdU (colorimetric)**" (Roche, Milan, Italy), a colorimetric immunoassay based on the measurement of BrdU incorporation during DNA synthesis: a non-radioactive alternative to the [3H]-thymidine incorporation assay (Figure 15). The cells (hVW-MSCs, dermal fibroblasts and WPMY_1) were seeded in a concentration of 1000 cells/well, in a 96-well plate in an incubator at 37°C, 5% CO_2 and with 90% humidity for 7 days. In a second step, BrdU was added to the cells and the cells were reincubated for 4hrs, to permit BrdU to be incorporated in place of thymidine into the DNA during its synthesis. Later, the cells were fixed, DNA was denatured and the anti-BrdU-POD was added to bind the BrdU incorporated in the neo-synthetized DNA: the immune complexes were detected by the substrate reaction and the result was quantified by measuring absorbance at the respective wavelength using a scanning multiwall spectrophotometer. The absorbance values were directly correlated to the amount of DNA synthesis and so to the respective proliferating cells.

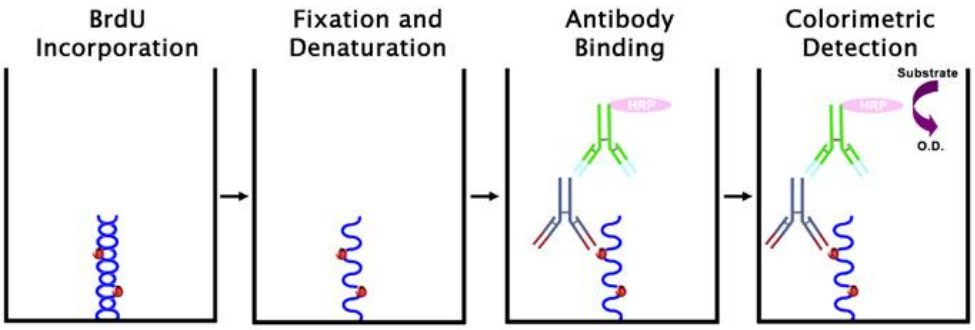


Figure 15: Schematic representation of BrdU colorimetric assay.

Multilineage differentiation assays

In vitro Tube formation assay

To evaluate angiogenic potential, hVW-MSCs (isolated from carotid, thoracic and femoral arteries), dermal fibroblasts and WPMY_1 were seeded at confluence, starved with DMEM+2%FBS and treated with HGF/SF (0, 5, 10, 25, 50 ng/mL) and Vascular Endothelial Growth Factor (VEGF) at concentration of 50 ng/mL for 7 days in DMEM 2% FBS. Untreated cells, cultered in DMEM 10% FBS, were used as negative control.

At the end of induction, the cells were seeded at concentration of $15x10^3$ in a 96-well plate, previously coated with a semisolid matrix (Matrigel, BD Bioscences) for 1 hr at 37°C in an incubator. Capillary-like structure formation was observed at inverted light microscopy after 2, 4 and 6 hrs and photographed. Two different methods were used to quantify the *in vitro* angiogenesis, within the two parts in which the thesis is structured.
To quantify the angiogenesis, the number of capillary-like structures was manually counted on digitalized images taken at 4x magnification for each experimental condition. In parallel experiments, flow cytometry was performed to detect the expression of vWF, KDR, and CD31 mature endothelial cell markers in the controls, as well as the growth factors-treated cells. For surface antigen, the treated cells were rinsed in PBS, labeled with primary antibodies against KDR-APC and CD31-PE. To reveal vWF expression, the cells were fixed and permeabilized with the IntraPep Kit (Beckman-Coulter), incubated with von Willebrand Factor (vWF; Santa Cruz Biotechnology, Santa Cruz, CA, USA), and subsequently incubated with anti-mouse IgG-FITC (Dako) secondary antibody. The endothelial lineage commitment was quantified in all experimental conditions attributing a score from 0 to 4, according to the flow cytometry values of each mature endothelial cell marker. The final values are reported as mean.

Adipogenic assay

For adipogenic assay, hWV-MSCs, dermal fibroblasts and WPMY_1 were seeded at the density of $40x10^3$ on 6-well plates. The cells were exposed to specific induction media according to the manufacturer's instructions (StemPro Adipogenesis differentiation kit, Life Technologies) for 14 days while the control cells were cultered in 6-well plates with DMEM 10% FBS. The assay was carry out in double for the analysis with Oil Red O staining. After induction, the cells were phormalin-fixed and stained with Oil Red O to underline cytoplasmic lipidic droplet formation. The staining was observed at inverted light microscopy and photographed.

RNA extraction, Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) and Real Time- PCR

Total RNA was extracted from hVW-MSCs, AAA-MSCs, dermal fibroblasts and WPMY_1 using TRIreagent according to the manufacturer's instructions (TRIzol reagent; Invitrogen). Also for hVW-MSCs and AAA-MSCs treated with HGF/SF at different concentration (from 2.5 to 70 ng/mL) the total RNA was extracted by the same method. Reverse transcription of 1 µg of total RNA was carried out by a thermocycler in a 20 µL volume of reaction using a High Capacity Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA, USA). RNA amount was measured by using ND-1000 spectrophotometer (NanoDrop, Fischer Thermo Wilmington, DE, USA). Polymerase Chain Reaction (PCR) products were analyzed by electrophoresis on a 2% agarose gel, stained with ethidium bromide and photographed under ultraviolet light. All PCR product sizes were identified loading a 100-base-pair (bp) DNA ladder and normalized to GAPDH, used as housekeeping gene.

Real Time PCR performed in a Gene Amp 7000 Sequence Detection System (Applied Biosystems); the reaction was leaded in triplicate, in 20 µL volume using the SYBR Green mix (Life Technologies) and specific couples of primers designed by NCBI BLAST tool and acquired from Sigma Aldrich (Table 1). The gene expression was normalized to Glyceraldehyde 3-phospate dehydrogenase (GAPDH) and expressed as fold changes relative to control (the comparative 2^-ΔΔCt method was used).

The PCR primers were purchased from Sigma-Aldrich. Genes and respective primers are presented in Table 1 and Table 2, respectively for qualitative PCR and for Real Time PCR.

Gene	Primer sequence	Product size (bp)	*T* (°C)
GAPDH	FWD 5′-ACCACAGTCCATGCCATCAC-3′ REV 5′-TCCACCACCCTGTTGCTGTA-3′	452	61
c-MET	FWD 5′-AGAAATTCATCAGGCTGTGAAGCGCG-3′ REV 5′-TTCCTCCGATCGCACACATTTGTCG-3′	440	68
HGF	FWD 5′-TTTGCCTTCGAGCTATCGGG-3′ REV 5′-GCAAGAATTTGTGCCGGTGT-3′	254	62

Table 1: Couple of primers used for qualitative PCR (S. Valente et al, 2016).

Gene	Primer sequence
GAPDH	FWD AATGGGCAGCCGTTAGGAAA REV AGGAGAAATCGGGCCAGCTA
c-MET	FWD GTGCCAAGCTACCAGT REV CTTCGTACAAGGCGTCT
MMP-2	FWD CCCCAAAACGGACAAAGAG REV CTTCAGCACAAACAGGTTGC
MMP-9	FWD GTACTCGACCTGTACCAGCG REV AGAAGCCCCACTTCTTGTCG
MT1-MMP	FWD CGCTGCCATGCAGAAGTTTT REV TGTCTGGAACACCACATCGG
IL-10	FWD GGGGCTTCCTAACTGCTACA REV TAGGGGAATCCCTCCGAGAC
TNF-alfa	FWD GGATCATCTTCTCGAACCCCG REV CCTAGCCCTCCAAGTTCCAAG
VE-cadherin	FWD TCTTCACCCAGACCAAGTACA REV GGCTCATGTATCGGAGGTCG

Table 2: Couple of primers utilized for Real Time PCR. Abbreviations: c-MET, hepatocyte growth factor receptor; GAPDH, glyceraldehyde 3 phosphate dehydrogenase; IL, interleukin; LPT: leptin; MMP, matrix metalloproteinases; TNF-alpha, tumor necrosis factor alpha; VE-cadherin: vascular endothelial-cadherin.

In Vitro Wound Healing Assay – Scratch assay

Cell migration was investigated using a scratch assay. hVW-MSCs were seeded in 12-multiwell plate at a density of 1×10^5/well and grown until to confluence. Cell monolayers were wounded with a p200 pipette tip, washed with PBS to remove cell debris, and treated with complete DMEM containing HGF/SF (2.5, 5, 10, and 70 ng/mL) for 24 hrs. In further experiments, cells were starved, inhibited with 0.2 μM PHA-665752, and cultured in presence of HGF/SF at the same time and concentrations. At the end of HGF/SF treatment for both experiments, cells were fixed in absolute methanol for 10 minutes, washed in PBS, stained with 0.1% Crystal Violet in 25% methanol for 30 minutes, and air-dried; all steps were performed at RT. The wound closure was observed under a phase-contrast light microscope (LM) equipped with a

digital camera (Nikon), acquiring images for each sample at time 0 and 24 hrs, respectively, using Software NIS-elements D3.2 Nikon (Tokyo, Japan). Computer-assisted image analysis (Image-Pro Plus software, Media Cybernetics, http://www.mediacy.com) was employed to perform the quantification of the area of cells migrated into scratched area as well as the total wounded area in three different fields. Values were expressed in percentage as ratio of migrated cell area on total scratched area. Additional cell-seeded glasses were used for Vimentin intermediate filaments immunofluorescence staining.

Transwell Migration Assay

The cellular ability to migrate through a porous membrane under the influence of a chemoattractant factor was evaluated using transwell chambers (Costar, Corning Incorporated, NY, USA). Briefly, 500 μL of DMEM with 10% FBS with or without chemoattractant HGF/SF (10 ng/mL) was placed below the polycarbonate membrane with 8 μm pores, while a quantity of 300 μL of cellular suspension containing 2.5×10^4 hVW-MSCs in growth medium was plated on the upper layer of the membrane for 24 hrs at 37°C in incubator (Figure 16). After the manual removal of the non-migrated cells from the upper side of the membranes, the inserts were detached from the plastic support using a scalpel, fixed, stained with 0.1% Crystal Violet for 30 minutes at RT, and mounted on glass slide. The cells migrated in the lower layer were observed using a Leitz Diaplan LM (Wetzlar, Germany), equipped with a video camera (JVC 3CCD video camera, KY-F55B, Jokohama, Japan). Digital images were acquired at 10x of magnification using Image-Pro Plus 6 software (Media Cybernetics). Additional membranes were processed for SEM analysis.

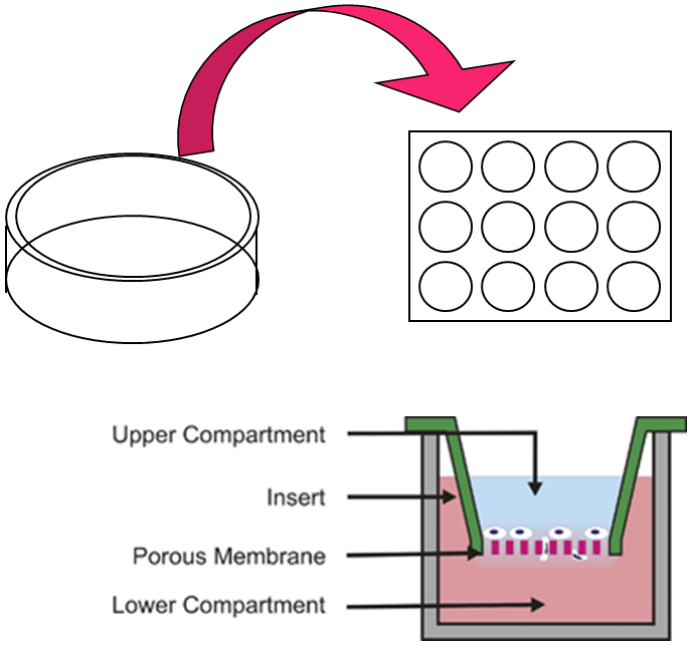


Figure 16: Schematic representation of transwell migration assay.

Scanning Electron Microscopy (SEM)

For SEM, samples were rinsed in 0.15 M phosphate buffer to remove the culture medium, fixed in 2.5% buffered glutaraldehyde (TAAB Laboratories, UK) overnight at 4°C, washed again in phosphate buffer, postfixed in 1% osmium tetroxide in 0.1 M phosphate buffer, wet in distilled water, and dehydrated with increasing ethanol concentrations (70–100%). Each step was performed at RT for 15 minutes. For drying the samples, they were immersed before in a solution of 50% absolute ethanol/50% hexamethyldisilazane (HMDS, Fluka Analytical, Sigma, Steinheim, Germany) and after in pure HMDS for 30 minutes each passage at RT and finally air-dried. Before observation, the samples were mounted on aluminum supports (Multilab type stub pin 1/2, Surrey, UK) using a silver paste maintaining the cell-seeding surface, coated with gold in a sputtering device (Quorum Q150RS, Technologies Ltd., Laughton, UK), and observed at 5–10 kV with a Quanta 250 (FEI Company, Milan, Italy) scanning electron microscope.

Western Blot Analysis

Total cellular proteins were extracted by untreated and treated hVW-MSCs using lysis buffer (KH2PO4 0.1 M pH 7.5, NP-40 1%, and 0.1 mM α-glycerol phosphate, added with complete protease inhibitors cocktail, Roche Diagnostics) and quantified spectrophometrically with the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hempstead, UK). Thirty μg proteins were subjected to 8% SDS-PAGE and transferred to nitrocellulose membrane (GE Healthcare Life Sciences, Amersham) at 30 mA for 2 hrs and 30 minutes. The membrane was blocked with 5% nonfat dry milk in TBS-tween for 1 hour at room temperature (RT), incubated with primary antibodies against c-Met (1: 500, Santa Cruz Biotechnologies), HGF (1 : 500, Santa Cruz Biotechnologies), and anti-α-actin (clone AC-74, Sigma-Aldrich) at 4°C overnight. Secondary antibodies (human anti-rabbit/mouse horseradish peroxidase-conjugated (GE Healthcare, Milan, Italy) were used at 1: 10000 dilutions for 1 h at room temperature (RT). Protein signal was detected using Westar ηC chemiluminescent substrate (Cyanagen); (Figure 17).

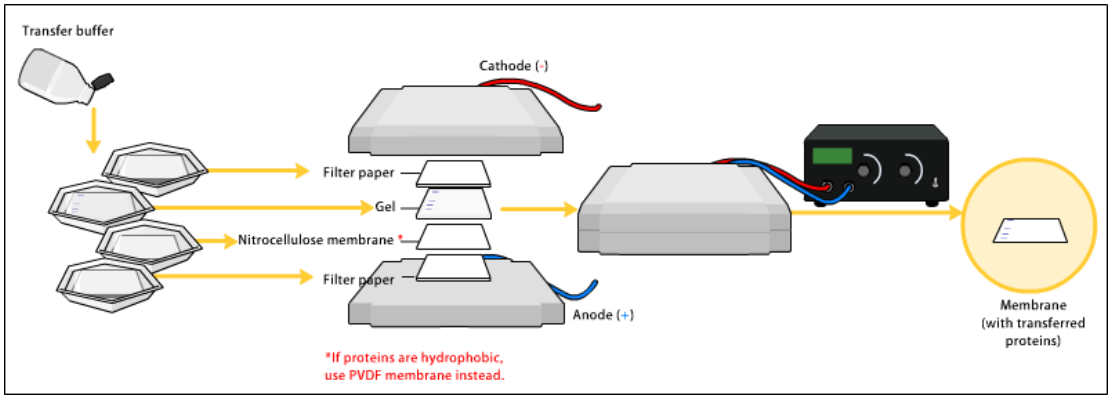


Figure 17: Schematic representation of western blotting assay.

Statistical analysis

To analyze the results, GraphPad Prism 5.0 software (GraphPad Prism software, San Diego, CA) was used and the data were expressed as the means ± SEM. Statistical analysis among samples was determinate by Student's t-test (for two groups of

samples), one-way ANOVA test and Bonferroni post-test (for more than two groups of samples). The value < 0.05 was considered to be statistically significant, with 95% as confidence intervals. Each experiment was performed in triplicate.

Results

PART 1: study of HGF in vitro effect on healthy hVW-MSCs

hVW-MSC Isolation and Stemness Property

hVW-MSCs isolated from human arteries showed a spindle-shaped morphology and a marked adhesion growth. hVW-MSCs expressed mesenchymal (CD44, CD73, CD90, CD105, HLA-G), stemness (Stro-1, Oct-4, and Notch-1), pericyte (CD146, PDGFR-*β*, and NG2), and neuronal (nestin) markers, together with the plasticity to differentiate in multiple mesengenic lineages, clonogenicity, and immunomodulatory functions; details on their morphology, immunophenotype, and molecular and functional features are reported elsewhere (S. Valente et al, 2014).

HGF and *c-MET* mRNA and Protein Expression in hVW-MSCs

In a previous study, Neuss et al. demonstrated that hMSCs derived from bone marrow expressed HGF and its receptor, c-Met (S. Neuss et al, 2004). In this study, we verified whether hVW-MSCs, recovered from human arteries, also may constitutively possess these genes. Gene expression analysis showed that hVW-MSCs express HGF and *c-MET* receptor mRNA (Figure 18). Western Blot confirmed the expression of the c-Met receptor in hVW-MSCs and showed increased protein levels in hVW-MSCs exposed to HGF/SF, especially at 10 ng/mL (24 hrs). Meanwhile, the expression of the HGF protein did not show evident differences under HGF/SF stimulation (Figure 18).

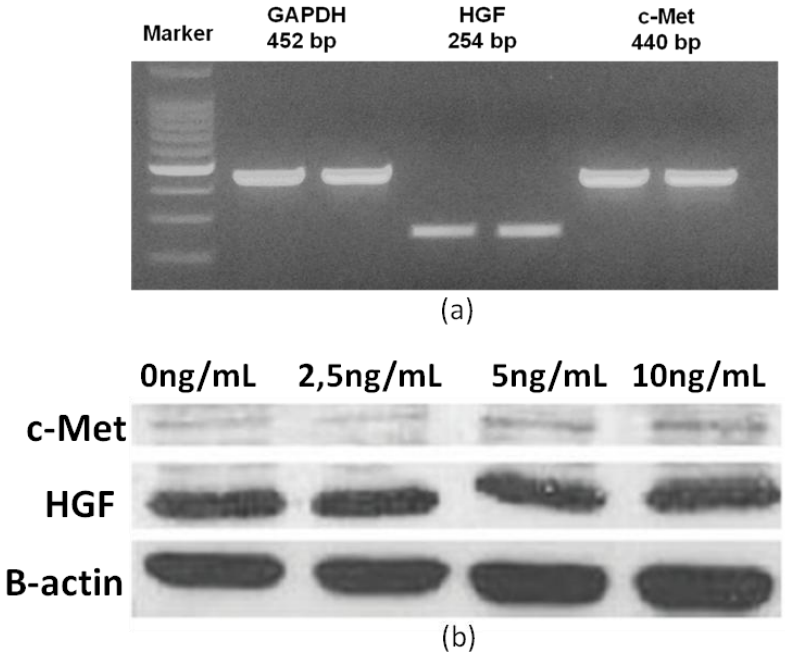


Figure 18: Basal expression of HGF and *c-MET* in our cell modell. (a) hVW-MSCs were enzymatically isolated from human arteries and, at passage 3. Basal expression of HGF (252 bp) and c-MET (440 bp) mRNA in hVW-MSCs, detected by qualitative PCR. (b) c-Met and HGF protein detection on cell lysates of hVW-MSCs exposed to HGF/SF (0, 2.5, 5, and 10 ng/mL) for 24 hrs (S. Valente et al, 2016).

HGF/SF effect on hVW-MSC proliferation

hVW-MSCs were exposed to 0, 2.5, 5, and 10 ng/mL of HGF/SF for 24 hrs. The cell proliferation was assayed by two methods: immunofluorescence for Ki-67 staining and Alamar Blue assay.

Immunostaining analysis showed a decrease of hVW-MSCs exposed to HGF/SF for 24 hrs, compared to untreated hVW-MSCs after 24 hrs of incubation. In particular, the percentage of cycling cells positive to Ki-67 on total DAPI cells was decreased in all the concentrations of HGF/SF tested.

The fluorescence with Alamar Blue assay did not show differences between the control cells and hVW-MSCs treated with HGF/SF for 24 hrs in all conditions (Figure 19).

The experiment was performed also in the presence of the c-Met inhibitor; hVW-MSCs were pretreated with PHA-665752 and the proliferation was evaluated with the same methods after the HGF/SF addition. From the obtained results, we can see that the

HGF/SF addition to hVW-MSCs pretreated with PHA restored the normal proliferation and the results overlapping with those of the first series (Figure 20).
These data confirmed that the treatment with HGF/SF has no effect on hVW-MSCs.

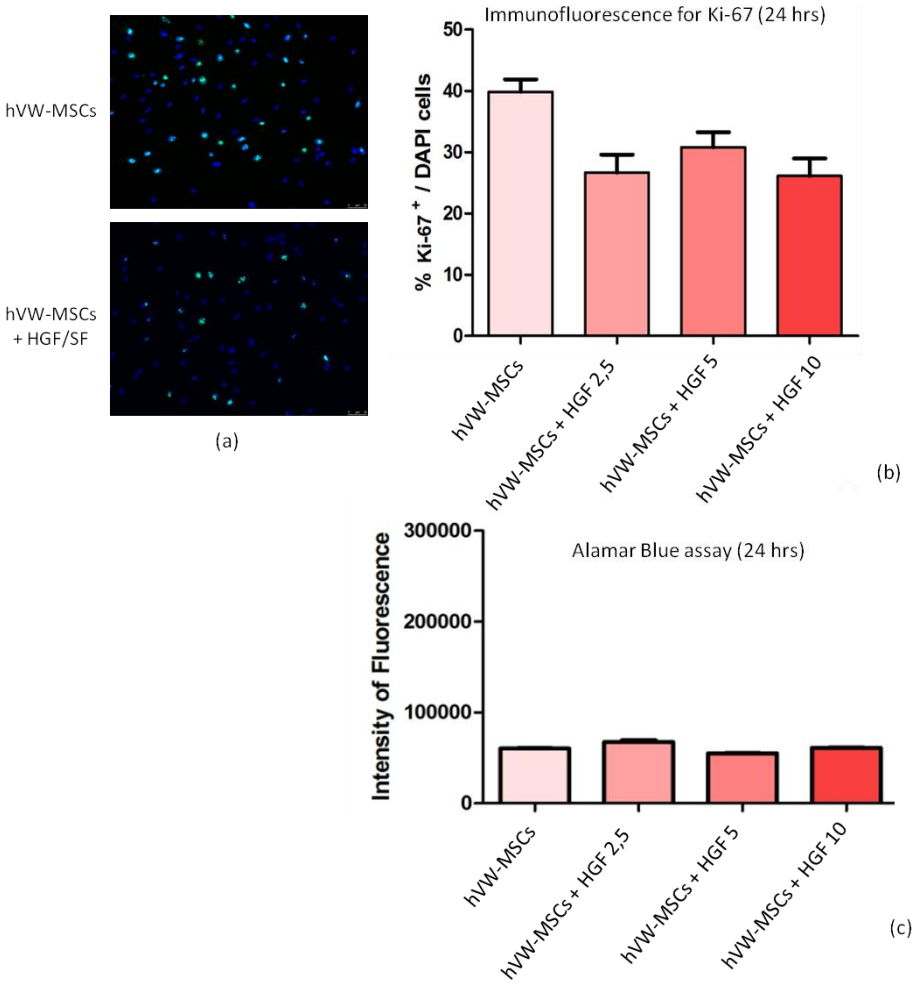


Figure 19: Proliferative effect of HGF/SF on hVW-MSCs. (a) Representative pictures of cycling cells without and with HGF/SF treatment. Digital images were taken at 10X of magnification and nuclei were labeled with DAPI in blue (S. Valente et al, 2016). (b) *In vitro* proliferation of hVW-MSCs exposed to HGF/SF assessed by immunofluorescence staining for Ki-67 up to 24 hrs. For each experimental condition, the number of ki-67 intensely stained cells and DAPI stained nuclei were manually counted on ten randomly field. Their values expressed as ratio of ki-67-stained cells to total cells (c) Evaluation of cell proliferation by Alamar Blue assay for 24 hrs.

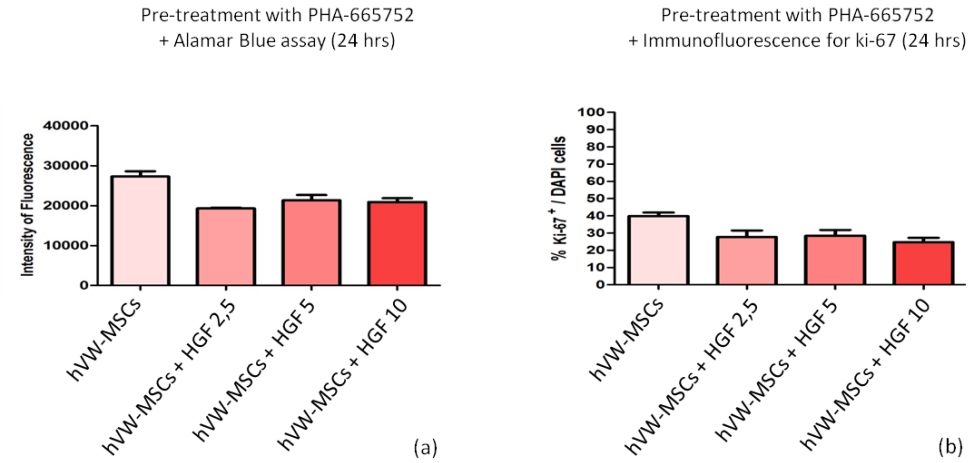


Figure 20: Proliferative effect of HGF/SF on PHA-665752 pretreated hVW-MSCs. (a) The graph shows the percentage of cycling cells Ki-67 positive, pretreated with PHA and then exposed to HGF/SF at different concentrations. (b) Cell viability evaluated by Alamar Blue assay on PHA-pretreated and HGF/SF-treated hVW-MSCs

HGF/SF effect on hVW-MSC migration

To investigate the *in vitro* HGF/SF effect on cell migration, we used a wounded confluent monolayer of hVW-MSCs, cultured for 24 hrs in presence and not of HGF/SF. hVW-MSCs exposed to HGF/SF at different concentrations (2.5, 5 and 10 ng/mL) covered the wounded area more efficiently than untreated controls. The experiment was performed also with the addition of 0.2 μM of the c-Met inhibitor, PHA-665752, before the HGF/SF treatment. Nevertheless, in absence of HGF/SF stimuli, hVW-MSCs displayed a spontaneous capacity to migrate into the cell-free wounded area; the spontaneous migration was probably related to an autocrine mechanism due to endogenous HGF release. The addition of HGF/SF to PHA-pretreated hVW-MSCs did not completely restore the hVW-MSCs migration. To validate the light microscopy results, SEM investigation was performed at the same concentrations and time of HGF/SF treatment. SEM confirmed the high efficacy of HGF/SF to promote hVW-MSC migration into wounded area restoring the cell monolayer scratched; similar results were obtained using single immunofluorescence staining for Vimentin. The Vimentin was normally expressed in all hVW-MSCs, but the

labeling fluorescence intensity was higher in hVW-MSCs migrated in the scratched area and this is probably due to a cytoskeleton remodeling process inducted by HGF/SF (Figure 21).

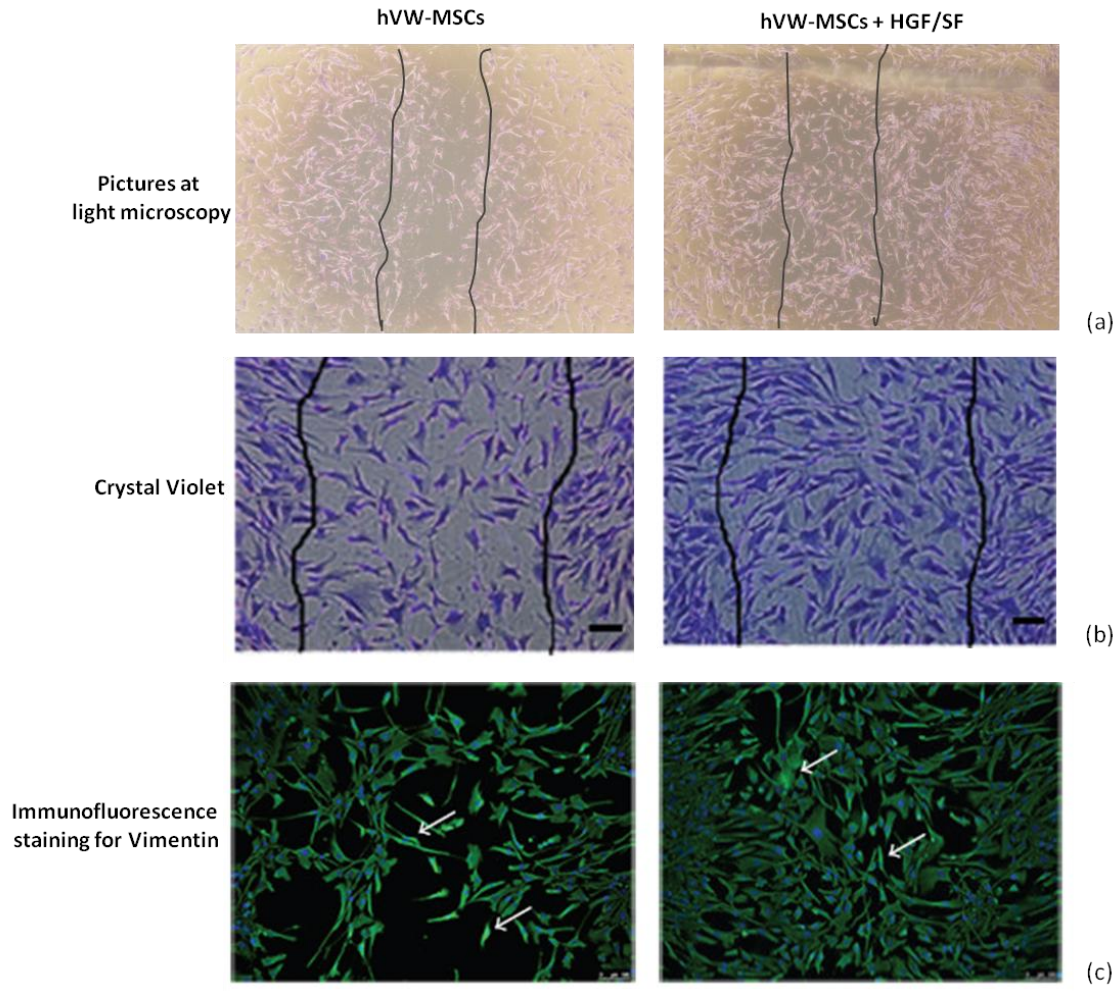


Figure 21: Migratory effect of HGF/SF on hVW-MSC. (a) Representative pictures at light microscopy show that hVW-MSC exposed to HGF/SF revealed a higher capacity to migrate in the wounded area, compared to untreated cells. Wounded area is delimited by black lines. (b) Crystal Violet staining of hVW-MSCs migrated into the scratch area after 24 hrs of addition of HGF/SF, compared to the untreated hVW-MSCs. Wounded area is delimited by black lines. Scale bars = 50 μm. (c) Immunofluorescence staining for Vimentin revealed that the intermediate filaments was markedly stained in migrated cells, compared to un-migrated cells. Scale bars = 100 μm. The reported images are representative of three independent experiments (S. Valente et al, 2016).

HGF/SF effect on hVW-MSC motility

The HGF/SF ability to mobilize and chemoattract hVW-MSCs was tested through a transwell migration assay. Crystal Violet dye showed that HGF/SF at concentration of 10 ng/mL enhanced hWV-MSC motility when compared to spontaneous hVW-MSC migration. At SEM, the main of hVW-MSCs rested on the seeding surface if un-treated with HGF/SF; conversely, hVW-MSCs efficiently populated the migration surface after the addition of 10 ng/mL of HGF/SF (Figures 22).

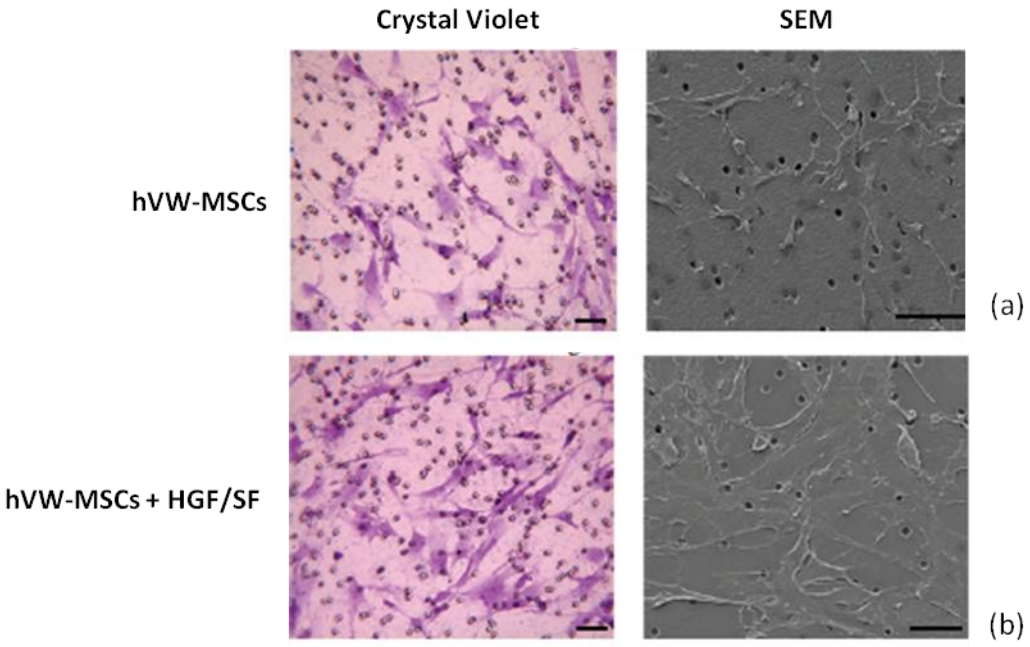


Figure 22: Motility effect of HGF/SF on hVW-MSCs. Representative Crystal Violet and SEM images of (a) untreated (hVW-MSCs) and (b) HGF/SF- treated hVW-MSCs. The cells migrated through an 8 μm porous membrane adhering to the migration surface. The HGF/SF ability as chemoattractant facilitated the migration of hVW-MSCs. Histological and SEM images: scale bars = 50 μm (S. Valente et al, 2016).

HGF/SF effect on hVW-MSC angiogenic differentiation

The HGF/SF effect on hVW-MSCs angiogenic differentiation was evaluated through the *in vitro* tube formation assay. hVW-MSCs were induced for 7 days with HGF/SF at 10 ng/mL or VEGF at 50 ng/mL and at the end of induction the cells were seeded in a

3D semisolid matrix. The capillary-like structures were photographed at light microscopy at 2, 6 and 24 hrs; at 2 hrs, the cells aligned from the cell periphery forming tube structures, while at 6 hrs the density of capillary-like network was more evident. Furthermore, at 24 hrs the capillary structures decreased in all samples but they remained still visible in hVW-MSCs induced with HGF/SF. HUVEC were used as positive control, because they spontaneously formed a capillary-like network. These structure were more evident in the induced samples, compared to untreated hVW-MSCs and in in addition, they were more pronounced in HGF/SF-treated cells rather than VEGF-treated cells (Figure 23). A further experiment was conducted by flow cytometry and it revealed that the mature endothelial markers (vWF, KDR and CD31) were more expressed after HGF/SF and VEGF stimulation. HGF/SF particularly induced an higher expression of vWF in confront to VEGF, while KDR and CD31 were similar in both conditions. Finally, hVW-MSCs treated with HGF/SF and VEGF showed a high endothelial commitment, overlapping to HUVEC, while the untreated hVW-MSCs preserved the mesenchymal identity (Figure 24).

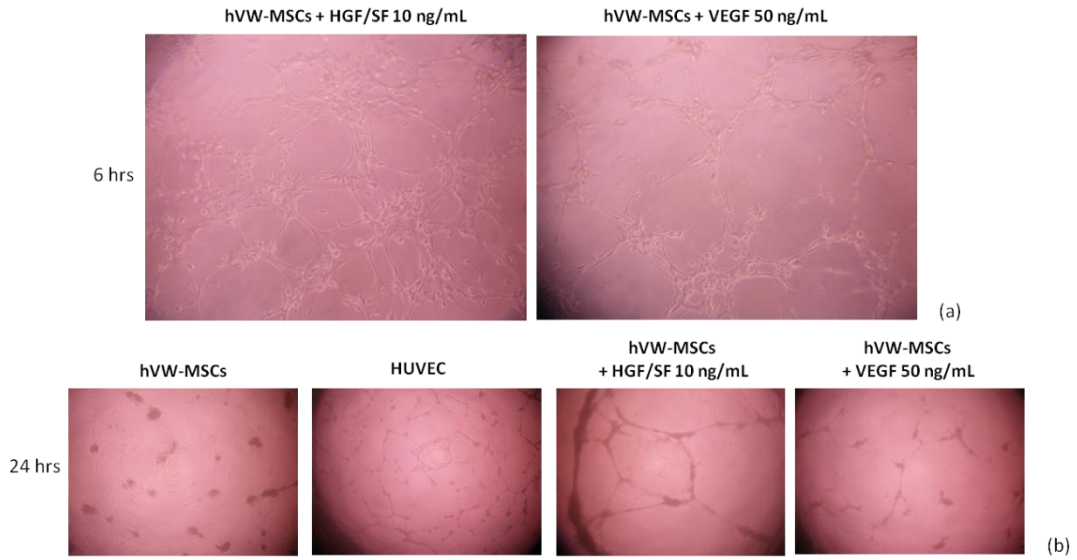
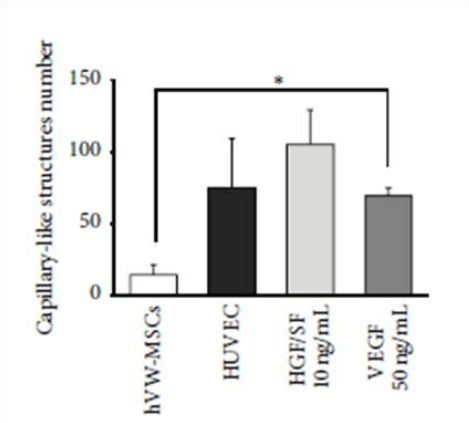


Figure 23: HGF/SF effect on hVW-MSCs angiogenic differentiation. (a) Representative pictures of HGF-treated hVW-MSCs when seeded in a semisolid matrix of matrigel. HGF/SF at 10 ng/ml and VEGF at 50 ng/ml were able to induce the formation of a high quantity of capillary-like structured. (b) At 24 hrs after the seeding on matrigel, hVW-MSCs treated with HGF/SF at 10 ng/ml still showed the capillary-like network, in confront of the positive control (HUVEC) and VEGF-treated hVW-MSCs. Scale bars = 100 μm (S. Valente et al, 2016).



(a)

	vWF (%)	KDR (%)	CD31 (%)
hVW-MSCs	14.9	0	0
hVW-MSCs + HGF/SF 10 ng/mL	95,6	57,1	93
hVW-MSCs + VEGF 50 ng/mL	73,1	68	93,9
HUVEC	95,7	22,9	95,8

(b)

Figure 24: Angiogenic effect of HGF/SF on hVW-MSCs. (b) The quantitative analysis of the capillary-like structures revealed a higher number of tubes in hVW-MSCs treated with HGF/SF rather than VEGF-treated cells. * p value < 0.05; Student's *t*-test. (c) Flow cytometry analysis of vWF, KDR, and CD31 expression on untreated hVW-MSCs, HGF/SF- and VEGF-treated cells, and HUVEC (S. Valente et al, 2016).

Angiogenic induction enhances VE-cadherin expression

A further experiment was performed on hVW-MSCs treated with increasing concentrations of HGF/SF (0, 10, 25 and 50 ng/mL) for 7 days. Total RNA was extracted after the induction for the analysis of the expression of VE-cadherin, a typical marker of endothelial cells. A Real Time PCR assay was conducted and the results are reported in the Figure. The data show that the angiogenic induction enhanced VE-cadherin expression until the concentrations of 25 ng/ml. At 50 ng/mL of HGF/SF, the expression of this marker decreases (Figure 25).

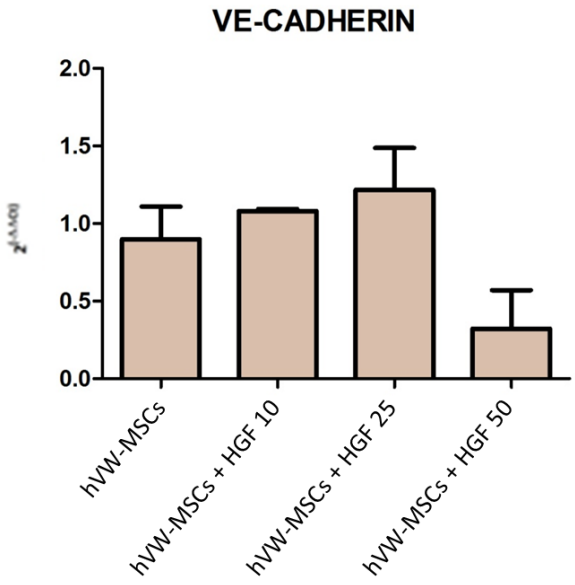


Figure 25: VE-cadherin expression in hVW-MSCs treated with HGF/SF at different concentration (0, 10, 25 and 50 ng/mL). The expression of VE-cadherin increases until 25 ng/mL of HGF/SF induction.

PART 2: HGF in vitro effect on tissue remodeling and inflammation in healthy hVW-MSCs and in pathological AAA-MSCs

HGF/SF effect was later investigated in the context of tissue remodeling. Based on the evidences in the literature, we wanted to test HGF on our cell model (hVW-MSCs) to verify if the exogenous stimulus with the growth factor could influence the expression of some molecular markers involved in tissue remodeling and if its receptor could be modulated after the stimulation. hVW-MSCs were so treated with HGF/SF at different concentrations (10, 25 and 50 ng/ml) for 24 hrs and then, total RNA was extracted to analyze, by using Real Time PCR, the mRNA expression of c-MET and matrix metalloproteinases: MMP-2, MMP-9 and MT1-MMP. From the obtained results, we can observe that HGF/SF has no influence on the molecular expression of these markers (Figure 26).

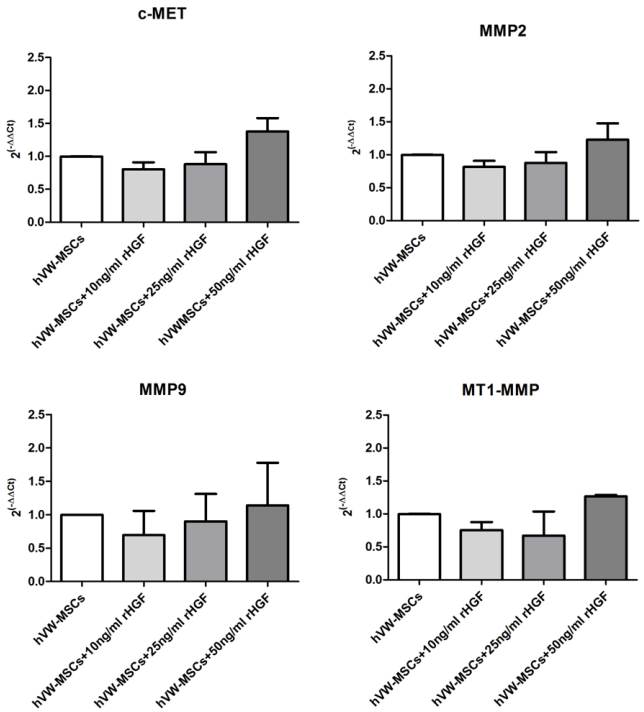


Figure 26: mRNA relative expression by Real Time PCR of some tissue repair molecular markers: c-Met, MMP-2, MMP-9 and MT1-MMP. The results are expressed as fold changes relative to the control (hVW-MSCs).

At this point of the study, we decided to test HGF/SF in a context different from healthy hVW-MSCs, testing its action in a pathological model. AAA-MSCs isolated from

abdominal aortic aneurysm according to the protocol developed by C. Ciavarella et al. (2015) represented a good and available model to test HGF/SF. Surprisingly, in pathological environment, HGF is able to influence the expression of the same markers evaluated in healthy context. On these pathological cells, we analyzed the same molecular markers evaluated in the healthy hVW-MSCs, after rHGF treatment. From the obtained results, significant differences can be observed in the c-MET and MMP2 expression, above at 50 ng/ml of HGF/SF (Figure 27).

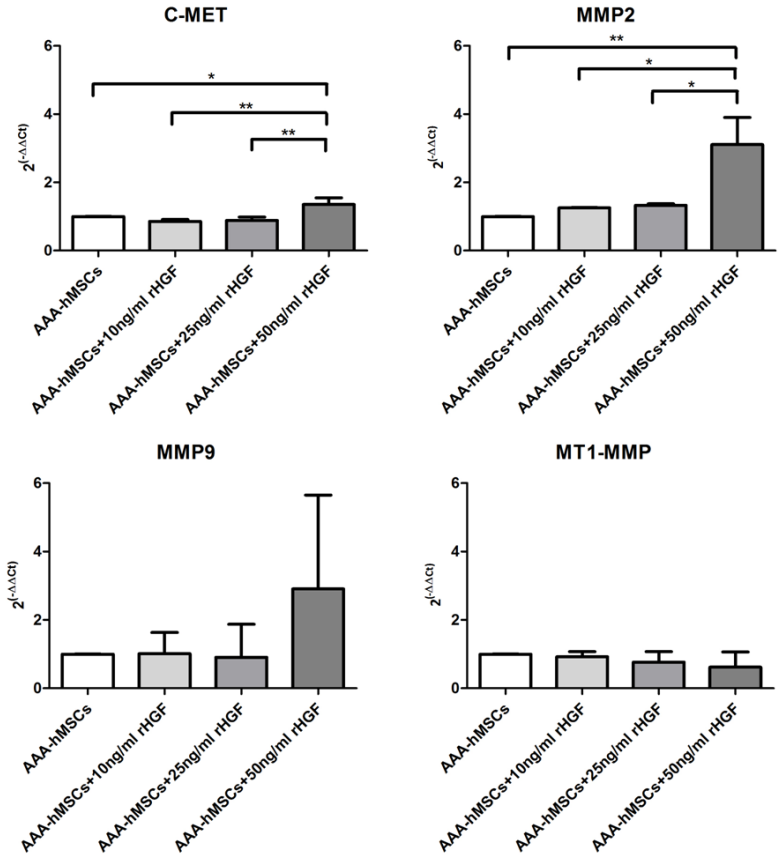


Figure 27: mRNA relative expression of c-MET, MMP-2, MMP-9 and MT1-MMP in AAA-MSCs treated for 24 hrs with HGF/SF at different concentration. The results are expressed as fold changes relative to the control (AAA-MSCs). The statistical differences were obtained by one-way ANOVA with multiple comparison among all the experimental conditions. *p<0.05, **p<; 0.01.

Finally, HGF/SF was tested on healthy hVW-MSCs and AAA-MSCs relatively to its influence on two cytokines involved in the inflammation processes: IL-10 and TNF-

alfa. hVW-MSCs were treated with HGF/SF at different concentration (5, 10, 50 ng/mL), but from the molecular analysis on the cytokine expression, no difference was observed in the mRNA relative expression. Conversely, in AAA-MSCs HGF/SF seems to reduce IL-10 and TNF-alfa expression until the concentration of 25 ng/mL; upon this concentration HGF/SF strongly induced the cytokine expression (Figure 28).

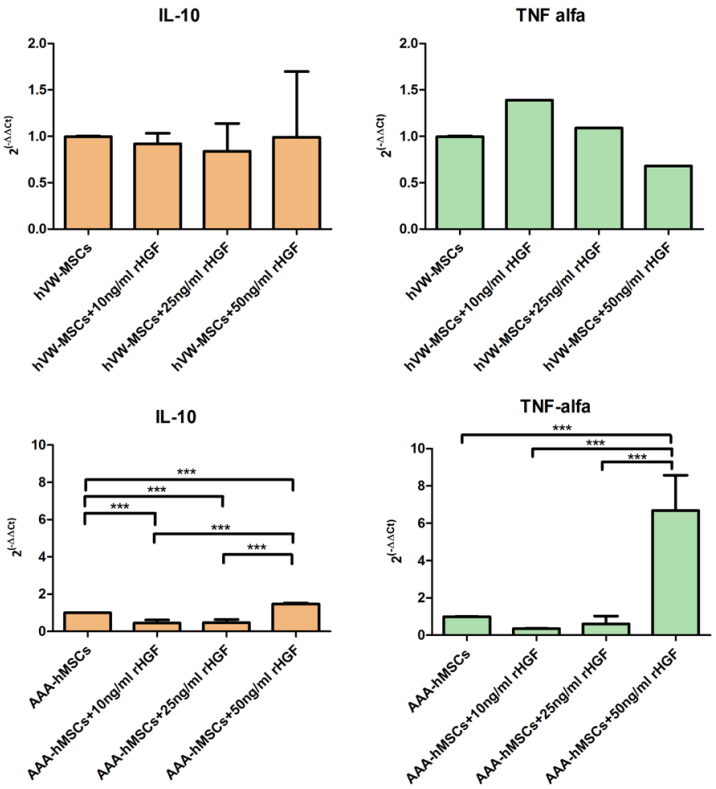


Figure 28: comparison of HGF/SF effect between healthy hVW-MSCs (upper part of the graph) and pathological AAA-MSCs (lower part of the graph). The expression of IL-10 and TNF-alfa is not influence by HGF/SF treatment in hVW-MSCs, while it is reduced in AAA-MSCs until 25 ng/mL. The results are expressed as fold changes relative to the control (hVW-MSCs and AAA-MSCs). The statistical differences were obtained by one-way ANOVA with multiple comparison among all the experimental conditions. ***$p<0.001$.

PART 3: Comparison of differentiation potential among different cell types involved in wound healing process

Cell proliferation

hVW-MSCs isolated from different segments of human arteries, dermal fibroblasts and WPMY_1 represent promising candidates for wound healing and tissue repair.
We cultered these cells for 7 days and the BrdU incorporation revealed some differences in the cell proliferation of these cells. hVW-MSCs characterization has been previously described (S. Valente et al, 2014). No difference between thoracic and femoral hVW-MSCs was showed, while a very significant increase in dermal fibroblast and WPMY_1 proliferation was visible (Figure 29).

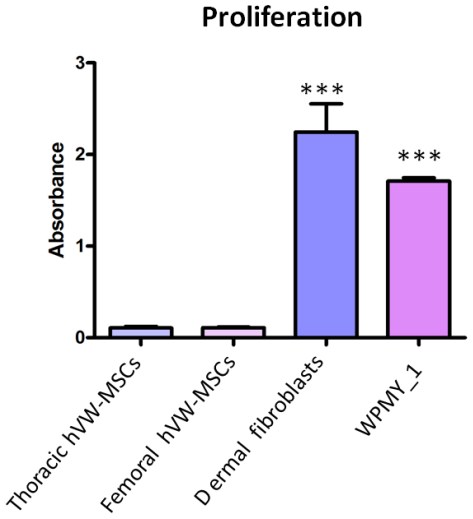


Figure 29: Evaluation of cell proliferation among hVW-MSCs derived from thoracic and femoral arteries, dermal fibroblasts and WPMY_1. BrdU incorporation during DNA synthesis; the proliferation was much higher for dermal fibroblasts and WPMY_1 in comparison to thoracic and femoral hVW-MSCs. Results are expressed as mean ± standard deviation and they are representative of at least three independent experiments (ordinary one-way ANOVA test and Bonferroni post-test; ***, $p<0.001$).

Angiogenic and adipogenic potential among thoracic hVW-MSCs, femoral hVW-MSCs, dermal fibroblasts and WPMY_1

Our preliminary results also show that hVW-MSCs isolated from thoracic and femoral arteries, human dermal fibroblasts and WPMY own a different differentiation potential.
To analyze angiogenic potential we induced these cells with 50 ng/mL for 7 days, similarly to the experiments conducted for the study of HGF/SF effects. After the induction, the cells were seeded on semisolid matrix of matrigel and photographed at 2, 4 and 6 hrs. From the pictures, it is possible to observe that the angiogenic potential of hVW-MSCs is much higher rather than the stromal counterpart. The formed capillary-like network is more prominent in mesenchymal stem cells, while the VEGF induction has no effect on the formation of tube-like structures. Conversely, on WPMY_1, VEGF induction has a slight angiogenic effect.
Likewise, thoracic and femoral hVW-MSCs, human dermal fibroblast and WPMY_1 were inducted for adipogenic commitment with a specific medium for 21 days. At the end of induction, the cells were staining with Oil Red O and observed at the light microscopy. Also in this case, hVW-MSCs revealed an higher adipogenic potential when compared to dermal fibroblasts and WPMY. The first type is able to form lipid droplets under specific induction, while in the second type of cells, the induction for adipogenic commitment has no effect (Figure 30).

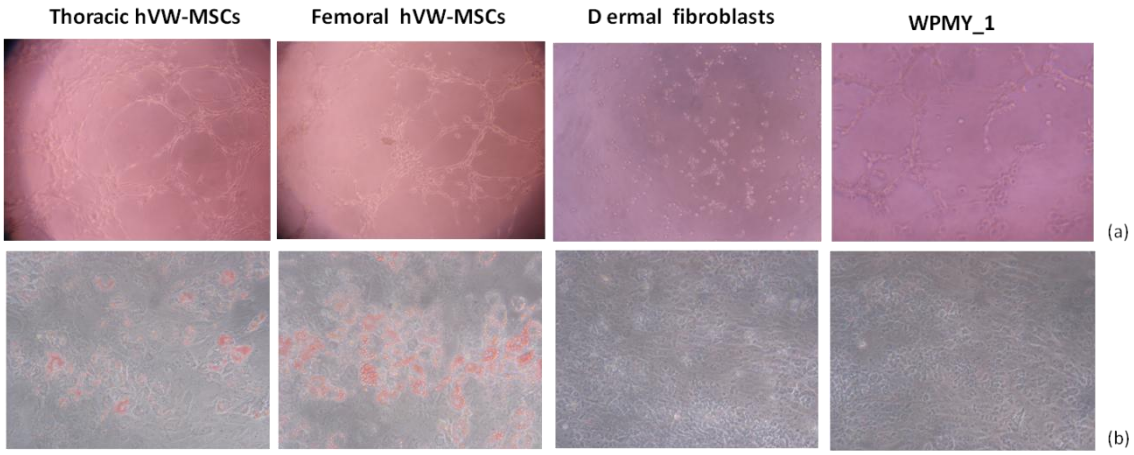


Figure 30: Representative pictures of angiogenic and adipogenic potential among hVW-MSCs, human dermal fibroblasts and WPMY_1.

Discussion

Lower limb ulcers associated to peripheral arterial occlusive disease represents a debilitating clinical situation that affect predominantly the older population. In concomitance with the presence of other co-morbidity, as diabetes mellitus, infections or metabolic deficiencies, these patients show an higher probability to develop foot ulcers and more generally chronic non healing wounds that fail to heal. The recovery of the tissues is very difficult and nowadays the gold standard in the treatment of the disease remains the surgical revascularization. Nevertheless, many studies refer about the use of new and promising therapeutic approaches and strategies, among which the gene and cell therapy, engineered dermis substitute implantation (M. Shimamura et al, 2013; G. Mulder et al, 2014), stem cells transplantation (S.-K. Han et al, 2010; C. Nie et al, 2011; H. C. Lee et al, 2012; E. V. Badiavas and V. Falanga, 2003), topical application of human platelet-derived products (V. R. Driver et al, 2006; H. S. Setta et al, 2011; T. Slesaczeck et al, 2012) and growth factors such as PDGF, VEGF, TGB-beta, FGF, EGF, and GM-CFS (S. Enoch et al, 2006; S. Barrientos et al, 2014). Among these growth factors, we decided to focus our attention on HGF/SF, a multifunctional protein involved in the wound healing (K. Conway et al, 2006), explicating its action on cell mitogenesis, motogenesis, morphogenesis, repair and regeneration via c-Met receptor. Furthermore, HGF/SF is a very promising factor also for its antiapoptotic, anti-inflammatory, antifibrotic and proangiogenesis properties (T. Nakamura and S. Mizuno, 2010; F. Bussolino et al, 1992; S.-I. Hayashi et al, 1999). At last, a study of our research group reported that the patients critical limb ischemia have an alteration in the axis HGF/c-Met (F. Vasuri et al, 2013). Based on these evidences, we supposed that the exogenous administration of HGF could reactivate the c-Met receptor, facilitating the tissues healing.

So, in our study, we explored the HGF/SF effects on human mesenchymal stem cells isolated in our laboratory from human artery vascular wall, to investiagte a possible mechanism able to improve the wound healing and to understand if HGF can contribute to the mobilization of hVW-MSCs.

The first data showed that HGF and its c-Met receptor are constitutively expressed in our cell model, both at the mRNA and protein level. A slight increase of c-Met is in particular revealed after HGF exposure for 24 hrs, especially at 10 ng/mL.

Regarding HGF effect on cell proliferation, we observed that HGF slightly reduced hVW-MSCs proliferation especially at 24 hrs after the treatment with 10ng/mL. The experiment was also conducted with PHA-65752, an antagonist of c-Met receptor,

demonstrating that this effect was independent from the activation of the HGF/SF axis. So, we can say that, unlike what originally thought, HGF does not exert a significant influence on hVW-MSCs proliferation via c-Met receptor. This is in part demonstrated in literature, where HGF would seem to be cell and tissue dependent (S. Neuss et al, 2004; F. Bussolino et al, 1992; J.-M. Zahm et al, 2000; R. Ramanujum et al, 2013; R. Joplin et al, 1992) and where some studies refer about the inhibition of MSCs proliferation through the cell cycle arrest after HGF treatment (G. Forte et al, 2006; K. Chen et al, 2011).

The analysis of migration by scratch assay was conducted through the evaluation of the repopulation of the damage area. This effect on hVW-MSCs motogenesis is reported and studied in a wide range of cell types (S. Neuss et al, 2004; G. Forte et al, 2006; F. Bussolino et al, 1992; J.-M. Zahm et al, 2000). The HGF effect on wounded confluent monolayers of hVW-MSCs was also analyzed by SEM and Immunofluorescence. The results indicated that HGF increased the hVW-MSCs ability to migrate in the cell-free area restoring the wounded cell monolayers and the migrating cells expressed more intensely Vimentin, an intermediate filament of cytoskeleton, suggesting the HGF effect on cytoskeleton remodeling. The experiment was conducted in parallel with PHA-665752 to demonstrate that hVW-MSCs was reduced in the presence of c-Met receptor inhibitor. Surprisingly, from the data hVW-MSCs revealed a spontaneous migration, probably related to the autocrine secretion of endogenous HGF. The transwell migration assays confirmed in addition the chemoattractive property of HGF. The histological and ultrastructural observations showed that hVW-MSCs crossed the porous membrane, use the cells were attracted by HGF placed at underlying bottom. This result is very interesting especially in the clinical context, as HGF could recruit endogenous stem cells. The angiogenic differentiation induced by HGF/SF stimulation, a key point in wound healing and tissue repair, was also investigated and compared to the proangiogenic factor VEGF which owns a big effect in promoting the endothelial commitment of hVW-MSCs (K. E. Johnson and T. A. Wilgus, 2014; G. Pasquinelli et al, 2007; S. Valente et al, 2014). In our study, HGF/SF was used as angiogenic promoter, as already mentioned in numerous studies (F. Bussolino et al, 1992; S.-I. Hayashi et al, 1999; S. Ding et al, 2003), in order to induce the alignment and formation of vessel-like structures. The assay was assessed on hVW-MSCs by an in vitro matrigel assay and it showed that the cells treated with HGF/SF were able to form a large capillary-like network. This effect was compared to the positive control (HUVEC) and

with the treatment with VEGF and surprisingly, the stimulation with HGF/SF at 10 ng/mL was able to promote the formation of tube-like structure that persisted even at 24 hrs. This result suggested that HGF/SF could act though integrins and adhesion molecules stabilizing the junctions among the cells. In support of this hypothesis, we carried out an experiment on the total RNA extracted from hVW-MSCs induced with HGF/SF at different concentrations. From this preliminary data, we can observe that HGF/SF stimulated in a dose-dependent manner the expression of one endothelial marker, VE-cadherin; this effect is visible until the stimulation of 25ng/mL of HGF/SF, concentration over which the VE-cadherin expression decreases. Furthermore, both HGF/SF and VEGF stimulated the expression of vWF, KDR and CD31, the typical markers of endothelial commitment.

Therefore, we decided to investigate HGF/SF role on tissue repair and remodeling processes, analyzing the modulation of gene expression of c-Met and some matrix metalloproteinases (MMP-2, MMP-9 and MT1-MMP) after the stimulation with HGF/SF. The results indicate that the growth factor have no effect on these molecular markers, mainly in healthy context. The evaluation of HGF/SF influence of two cytokines involved in the inflammation process (IL-10 and TNF-alfa) shows similarly behavior. At this point of the study we wanted to investigate HGF/SF effect in a pathological context. We have chosen a particular context, that means the abdominal aortic aneurysm, because a MSC niche is present in this tissue. The AAA-MSCs are pathological mesenchymal stem cells which over-express MMP-9 and own an ineffective immunomodulatory capacity, as demonstrated by C. Ciavarella et al (2015). AAA-MSCs were exposed to HGF/SF stimulation and then the same markers involved in remodeling were analyzed; as initially supposed, in the pathological context some differences can be observed, in particular in c-Met and MMP-2 expression, above 50 ng/mL of HGF/SF comparing with the expression in the control. Instead, the expression of inflammatory cytokines resulted decreased in the presence of HGF/SF stimulation; this effect is visible until 25 ng/mL of HGF/SF, suggesting a possible anti-fibrotic and anti-inflammatory role. Although preliminary, this data is very promising especially in the clinical context, where HGF/SF could be used for their properties and anti-inflammatory applications.

Finally, we focused our attention on other cell types, that means human dermal fibroblasts and myofibroblasts, in order to evaluate their differentiation potential in confront of hVW-MSCs isolated from different human arteries. The MSCs together

with the stromal counterpart including fibroblasts and myofibroblasts represent in fact the most promising candidates among the new therapeutic approaches to facilitate and treatment of foot ulcers and wound healing. From the assays on cell proliferation, we can observe that the proliferative capacity is more pronounced in dermal fibroblasts and myofibroblasts rather than in hVW-MSCs, while the data on differentation potential appear very interesting. We assessed two assays to evaluate the differentiation potential: the cells were induced for angiogenic and adipogenic commitment respectively with proangiogenic factor and specific medium. The obtained results show the hVW-MSCs own a more higher angiogenic potential compared to fibroblasts and myofibroblasts. Only myofibroblasts displayed a light capacity to form tube.like structures when seeded on the matrigel, while in dermal fibroblasts the angiogenic induction has no effect. Similarly, only hVW-MSCs are able to form lipid droplets when induced with a specific medium, suggesting their possible use in the reconstitution of the fatty tissue, always in the context of wound healing.

According to our data, the healthy hVW-MSCs isolated from the vascular niche of human arteries constitutively expresses HGF and its receptor c-Met. Interestingly, the addition of exogenous HGF/SF enhances hVW-MSC migration, motility and capillary-like structures formation. Furthermore, HGF/SF could also be used in different pathological contexts where it seems to have some anti-fibrotic and anti-inflammatory properties, acting on the tissue repair and remodeling processes. Finally, a preliminary data on the evaluation of differentiation potential put on evidence the higher hVW-MSC potential in terms of angiogenesis and adipogenesis, rather than in the stromal counterpart represented by dermal fibroblasts and myofibroblasts.
These data could suggest a novel possible therapeutic strategy, based on the use of hVW-MSCs, in the light of their high differentiation potential, and on the local delivery of exogenous HGF/SF, which could accelerate and facilitate the healing of unresponsive vascular ulcers.

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