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NOVEL REGENERATIVE MEDICINE APPROACHES WITH THE USE OF ADULT MESENCHYMAL STEM CELLS: *IN VITRO* AND *IN VIVO* EXPERIMENTAL PROCEDURES.

Coordinator of the School: Ch.mo Prof. Alessandro Zotti

Supervisor: Ch.mo Prof. Marco Patruno

Co-Advisor: Ch.ma Dr.ssa Tiziana Martinello

PhD Student: Dr.ssa Chiara Gomiero

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ABSTRACT

Tendon injuries are often associated with skeletal muscle lesions that can originate from a variety of events, including direct trauma, tendon and muscle lacerations and contusions, indirect insults and degenerative diseases as muscular dystrophies. Currently, a complete cure for musculoskeletal diseases is not present and the *restitutio ad integrum* is difficult to obtain.

In the last decade, adult MSCs gained general attention in both human and veterinary medicine and the understanding of MSC function is improved promoting the application of cell therapy and the development of powerful cell-derived therapeutics for regenerative medicine.

The first part of this research focused on the reprogramming of stromal cells derived from equine and sheep mesenchymal tissue towards tenogenic and myogenic fate *in vitro* using new non-viral transfection system.

1) Equine MSCs isolated from peripheral blood (PB-MSCs) can develop the tenogenic pathway using four specific growth factors such as TGFβ3 (transforming growth factor β3), EGF2 (epidermal growth factor 2), bFGF2 (fibroblast growth factor 2) and IGF1 (insulin-like growth factor 1) in presence or without Low Level Laser Technology (LLLT).
2) PB-MSCs were induced to differentiate towards myogenic fate using the complex TAT-MyoD in presence of a conditioned medium obtained from co-culturing PB-MSCs with C2C12 without a direct contact.
3) A novel surface-active maghemite nanoparticles (SAMNs) were tested as vectors for eukaryotic cell transfection of coding gene in PB-MSCs without the application of external magnetic fields.

The full characterization of these three techniques was achieved using molecular and immunohistochemistry analysis.

Real-time PCR (rt-PCR) was performed to study the expression level of the typical tenogenic genes markers Early Growth Response Protein-1 (EGR1), Tenascin C (TNC) and Decorin (DCN) to discover the best combination of GFs in presence or without LLLT.

To evaluate the myoblasts differentiation, rt-PCR analysis was executed to study Myf5 and Myogenin gene expression while immunofluorescence experiments was performed to estimate MyoD, Myf5 and Myogenin protein expression.

The cytotoxicity effects of SAMNs nanoparticles was observed with XTT cell proliferation assay and to evaluate SAMNs efficacy as vector for pDNA coding GFP, an immunofluorescence analysis was performed.

The second topic of this research project was on skin regeneration studied *in vivo*. Skin is a soft tissue and covers the entire surface area of body. It is a self-repairing, self-renewing organ that forms an important barrier from the outer environment to the inner environment. Therefore, damage to the skin leads to debilitating wounds that is an impairment of the anatomical structure and function of the skin. In the two papers of the second section, the capability of adult equine and ovine MSCs to regenerate skin injuries has been studied.

1) Wounds were induced in the gluteus region of six horses and treated with autologous epithelial stem cells (EpSCs), allogeneic EpSCs, vehicle treatment or untreated control.
2) Sheep allogeneic PB-MSCs were utilized to treat experimental lesions on the back of six sheep. This project is part of a large scheme where conventional treatments (Manuka Honey, Connettivina and Acemannane) were compared to innovative cures (MSCs and gas-ionized plasma). In this thesis, only the data about skin regeneration with PB-MSCs was reported.

In the first work of the second section, rt-PCR was performed on tissue biopsies collected after one and five weeks of treatment and IFN-y, IL-6, VEGF, EGF, IGF-1 and epidermal keratin (eKER) were analyzed to study cellular immune response, neovascularization and the epidermal keratinization.

In the second paper, clinical analysis have been performed to analyze the healing time, the presence, the color and the nature of exudate, the aspect of gauze, the hydration of the wound, the percentage of re-epithelization and contraction of the lesions. Tissue biopsies were collected after 15 and 42 days of treatments to conduct molecular analysis, histological and immunohistochemical staining.

Molecular analysis were performed to study the expression level of genes such as Collagen 1α1 (Col1α1) and Keratin of hair (hKER). Dermal and subcutaneous inflammation, granulation tissue and skin adnexa were evaluated using histological analysis while the expression of MHCII, von Willebrand factor (vWF) and a cellular proliferation marker (KI67) were estimated with immunohistochemical staining.

ACRONIMS

ADMSCs = Adipose derived MSCs

AEG = Apoeccrine Sweat Glands

bFGF2 = fibroblast growth factor

bHLH = basic helix-loop-helix

BM = Bone Marrow

BM-MSCs = MSCs isolated from Bone Marrow

BMPs = Bone Morphogenetic Proteins

CD = Cluster of differentiation

CFU-Fs = Fibroblasts like Colonies Forming Units

Col1α1 = Collagen 1α1

COMP = Cartilage Oligomeric Matrix Protein

Dcn = Decorin

DMEM = Dulbecco's Modified Eagle Medium

ECM = Extracellular Matrix

EGF= Epidermal Growth Factor

EGR1 = Growth Response Protein-1

EGF2 = epidermal growth factor-2

eKER = epidermal Keratin

EpSCs = Epithelial Stem Cells

ES = Embryonic Stem Cells

FBS = Fetal Bovine Serum

GFs = Growth Factors

GFP = Green Fluorescence Protein

hKER = Keratin of hair

HSCs = Hematopoietic Stem Cells

HSPCs = Hematopoietic Stem/Progenitor Cells

IFN-y = Interferon gamma

IGF-1 = insulin-like growth factor-1

IL-6 = Interleukin-6

KI67 = cellular proliferation

LLLT = Low Level Laser Technology

MDSCs = Muscle-derived Stem Cells

MHCII = Major Histocompatibility Complex II

MRFs= Muscle Regulatory Factors

MSCs = Mesenchymal Stem Cells

MYF5 = Myogenic Factor 5

PAX3-PAX7 = Paired Box Gene 3-7

PB-MSCs = Mesenchymal Stem Cells isolated from peripheral blood

PBS = Phosphate Saline Buffer

PRP = Platelet-rich Plasma

rt-PCR = real time-PCR

SAMNs = novel surface-active maghemite nanoparticles

SC = Stem Cells

SCF = Stem Cells Factor

Scx = Scleraxis

TeM = Tenomodulin

TNC = Tenascin C

TSC = Tendon derived Stem/Progenitor Cell

TGFβ3 = transforming growth factor-3

TNC = Tenascin C

VEGF = Vascular Endothelial Growth Factor

vWF = von Willebrand factor

GENERAL INTRODUCTION

Chapter 1: Stem cells

1.1 Adult stromal cells

Within recent decades, the focus of medical science shifted from repair to regeneration. Regenerative medicine including stem cell (SC)-based tissue engineering has become one of the most intensively researched medical fields, not only in human but also in veterinary medicine (Brehm W et al., 2012). Indeed this raising interest in the field of stem cells is ascribed to the great promise that adult stromal cells offer in treating previous incurable disease and because of the lack of ethical controversies like the one associated with embryonic stem cells (ES) (Fortier LA, 2005).

Adult mesenchymal stem cells (MSC) are characterized by a fibroblast-like morphology, long term self-renewing capacity, give rise at least to one identical daughter cell, maintaining the stem cells pool, and an ability to generate many mature and specialized cell types (Chamberlain G et al., 2007).

Vice versa, ES are defined totipotent, because they are able to create an entire organism, a property retained by early progeny of the zigote up to the 8-cell stage of the morula. Most of the adult stem cells are multipotent since are able to differentiate into multiple cells type that are, however, restricted to a given tissue. Stromal cells have the potential to robustly restore a give tissue *in vivo*, which implies they are able to respond to specific needs to differentiate into cells type of that particular tissue.

Since the 1960s, the stromal compartment of bone marrow is the first source reported to contain multipotent progenitor cells able to restore the bone marrow functions (Fortier LA et al., 1998, Pittenger MF et al., 1999). For this reason, bone marrow is the best investigated origin of MSC. Several other tissue-specific stem cells have been defined, supporting the main hypothesis that each different tissue has a stem cells reservoir to guarantee the replenish of the tissue composed of mature cells with finite half-life. Example of identification of somatic stromal cells in adult tissue include adipose–derived MSC (Del Bue M et al., 2008) that seem to display a higher proliferation potential and less senescence compared to MSC from other tissue (Vidal MA et al., 2011). Another source is peripheral blood that appears to be a good alternative source compared to bone marrow and adipose tissue (Brehm W et al., 2012) and other tissues such as synovial membrane (Yoshimura H et al., 2007, Koga H et al., 2008), skin, hair follicle, gut, muscle, liver, testes, breast, central as well as peripheral nervous system. Indeed, adult stromal cells are divided by the tissue from which they originate into stromal cells derived from ectoderm, endoderm and mesoderm layer. Mesenchymal stromal cells are characterized by their common derivation from mesodermal layer (Spaas JH et al., 2016).

1.2 Mesenchymal stromal cells and their niches

Mesenchymal stromal cells (MSCs) are a class of adult stem cells, which have been firstly identified in bone marrow by Till and McCulloch in 1961. MSCs are described as adherent, clonogenic, non phagocytic cells capable to form fibroblast-like colonies (CFU-Fs) (da Silva Meirelles L, et al., 2009). In 2008, the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy proposed minimal criteria to define human MSCs: i) they must be plastic adherent when maintained in standard culture conditions, ii) they must be lineage negative and express CD105, CD73, and CD90 and lack expression of CD45, CD34, CD14 or CD11b, CD79a or CD19, and HLA-DR surface molecules, and iii) they must have the ability to differentiate to at least osteoblasts, adipocytes, and chondroblasts (Dominici M et al., 2006) and other mesodermal lineage cells, including cardiomyocytes, hepatocytes, endothelial cells, smooth muscle cells, and neuronal cells (Caplan AI 1989, 1991 and 2005; Parmar N et al., 2014) *in vitro* (Fig. 1) under usual culture conditions using suitable tissue culture media (Satija NK et al., 2013) and when implanted *in vivo* (da Silva Meirelles L, et al., 2009).

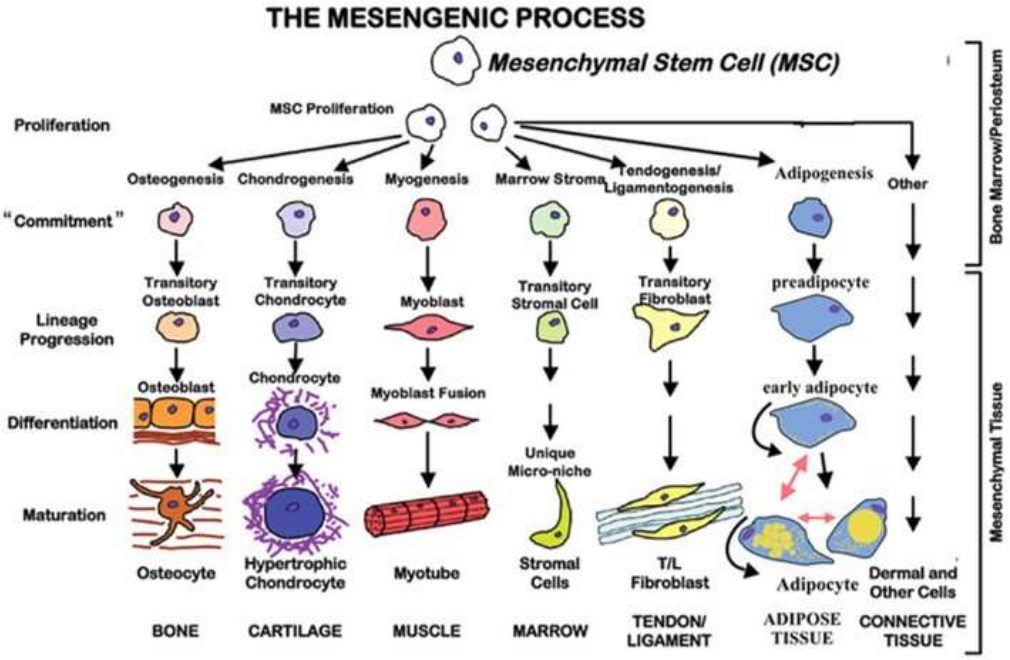

Fig.1. The mesengenic process. Mesenchymal progenitor cells entering different lineage pathways to contribute to formation of mature tissue such as bone, cartilage, muscle, bone marrow, tendon ligament, adipose and connective tissue (Caplan AI 2010).

On the other hand, even there are evidences that suggest that MSCs exist not only in the bone marrow but also virtually in all organs even if the exact localization of the MSCs *in vivo* remains poorly understood (Doherty MJ et al., 1998; Farrington-Rock C et al., 1998).

It then became clear that post-natal tissues have reservoir of specific stem cells, which contribute to maintenance and regeneration; examples include epithelial stem

cells in the epidermis (Chunmeng S et al., 2004, Spaas JH et al. 2016), in the intestinal crypts (Slack JM, 2000), neural stem cells in the central nervous system (McKay R, 1997), satellite cells in muscle (Chargé SB and Rudnicki MA, 2004); indeed MSCs were further isolated from adipose tissue (Zuk PA et al., 2002), tendon (Salingcarnboriboon R. et al., 2003), synovial membrane (De Bari C et al., 2003), synovial liquid (Jones E et al., 2008), periodontal ligament (da Silva Meirelles L et al., 2006) and lung (Sabatini F et al., 2005). Moreover MSCs populations were found also in blood and umbilical cord blood (Erices A et al., 2000), placentar villi (Igura K et al., 2004) and amniotic liquid (In't Anker PS et al., 2003). Indeed due to the etherology of these sources, different methodologies are required in order to isolate, cultivate and characterize MSCs related cells type: consequently, it is important to realize studies to characterize MSCs both histologically and phenotipically (Bianco P et al., 2008). Hence, these findings have led to the evaluation of MSCs potential for treating diseases and the birth of MSC-based therapy (Satija NK et al., 2013). Clinical trials for diseases, such as osteogenesis imperfecta, graft-versus-host disease, and myocardial infarction, have shown some promise, demonstrating the safe use of MSCs. Preclinical trials have exposed the successful use of MSCs for delivering therapeutic proteins and repairing defects in several disease models. However, lack of knowledge of MSCs behavior and responses *in vitro* and *in vivo* requires basic and animal studies before bringing these therapies to humans (Satija NK et al., 2013).

The ability of stem cells to both self-renew and produce daughters cells able to initiate the process of differentiation, is the key for tissue homeostasis, providing a continuous supply of new cells to replace short-lived but highly differentiated cells type. The decision between stem cells self-renew or differentiation must be tightly controlled, and now is known that the stem cells niche provide the integration of intrinsic factor and extrinsic cues to regulate the stem cells number, division, self-renew, and differentiation.

Schofield, who defined the ***niche*** a “stable micro-environment that might control hematopoietic stem cells (HSCs) behavior”, introduced the concept of “niches” in 1978 (Schofield R., 1978). In brief, the precise spatial organization of the stem cells respect to surrounding support cells plays an important role in the ability of the niche to adequately provide proliferative and anti-apoptotic signals and to exclude factors that promote differentiation (Jones DL and Fuller MT, 2006). Adult and tissue specific stem cells are found in specialized niches in their corresponding tissues of origin. Specialized niches for different types of adult stem cells are characterized by the complex interactions between surrounding cells, extracellular matrix molecules, and soluble factors.

Recent studies have shown the existence of two types of niches in the bone marrow compartment: an “endosteal” and a “perivascular” niche which are closed to each other or interdigitated.

Osteolineage cells, such osteoblast and fibroblast, lining endosteal surfaces located at the endosteum of the trabecular bone were the first functional niche cells to be discovered. Imaging approaches have demonstrated that transplanted primitive HSCs localize closer to the endosteum than more mature progenitors (Lo Celso C

et al., 2009). Increasing osteoblast number has been shown to expand the HSCs pool (Arai F et al., 2004) whereas deletion of osteoblasts leads to bone marrow HSCs depletion (Ferraro F et al., 2011). Osteolineage cells secrete large amounts of proteins that affect HSCs, including granulocyte colony- stimulating factor (G-CSF), and express surface molecules that retain HSCs in the niche (Taichman RS et al., 1996).
In addition to the endosteum, hematopoietic stem/progenitor cells (HSPCs) localize adjacent to bone marrow sinusoids (Lo Celso C et al., 2009). The importance of endothelial cells for HSCs traces back to the embryonic life, because HSCs first emerge in the aorta-gonad mesonephric region from a common hemangioblast. Dinget al. (Dinget L et al., 2012) demonstrated that en- dothelial cells regulate stem cell factor (SCF) production to retain HSCs in the niche.
Perivascular niche cells in close contact to endothelial cells have also been described as influencing HSPC biology in humans and mice (Dinget L et al., 2012; Sacchetti P et al., 2009). CD146+ adventitial perisinusoidal cells have bone-forming properties and sustain hematopoiesis (Sacchetti P et al., 2009). Depletion of perivascular cells leads to HSPC mobilization (Omatsu Y et al., 2010). Nestin+ cells are enriched in niche and retention genes (Angpt1,Vcam1,Cxcl12, andScf), have skeletal stem cell properties, and colocalize perivascularly with sympathetic nerve terminal. Nestin+ cells express the b3-adrenergic receptor, and following nor-adrenergic signaling or administration of G-CSF (which ultimately activates BM sympathetic activity), they downregulate retention signals, allowing HSPC mobilization (Mendez-Ferrer S et al., 2010).

1.3 Mesenchymal stromal cells plasticity, interconversion potentials and differentiation

Adult stem cells have great potential and their main feature is the ability of self-renewal and of differentiating into a number of different cell types. Generally, stem cells have been classified as being totipotent, pluripotent, multipotent, oligopotent, and unipotent, depending on all their differentiation potential (Wagers AJ and Weissman IL, 2004). Totipotent cells are able to give rise to all embryonic and extra-embryonic cells type while pluripotent cells give rise to all cells of the embryonic proper. The multipotent cells originate a subset to cells lineages and oligopotent cells are able to give rise to a more restricted cells subset respect to the multipotent cells. Finally, unipotent cells create only one mature cells type (Wagner W et al., 2010). Traditionally, adult MSCs are defined as multipotent cells committed to a particular cells fate to produce cells from the tissue of origin and not cells of non-related tissue. Currently there are increasing evidences that suggest their ability to differentiate into ectodermal and endodermal lineages under certain microenvironment conditions (Lakshmipathy U and Verfaillie C, 2005; da Silva Meirelles L et al., 2006). These findings lead to the concept of “stem cells plasticity” the ability of adult stem cells to acquire mature phenotypes that are different from their tissue of origin (Groove JE et al., 2004). The stem cells plasticity concept is

crucial and can be rigorously defined and experimentally proven. In fact, until now most studies has not shown that multi lineage differentiation is derived from the single cells that differentiate into the expected cells type, and even when this happens it is a very low frequency (Lakshmipathy U and Verfaillie C, 2005). Much of the problem regarding the plasticity of stem cells is derived from the lack of established parameters that help to uniformly define plasticity. Lakshimpaty et al. (2005) suggested three main criteria based on which stem cells plasticity had to be examined:

- A single cell differentiate into multiple cell lineages;
- Differentiated cells are functional *in vitro* and *in vivo*;
- Engraftment is robust and persistent.

Wagers and Weissman (2004) suggest some possible explanations of the mechanisms that underlie the stem cells plasticity that could would allow stem cells to transdifferentiate that is the ability of adult stem cells to contribute to cells type of different lineages (Wagers AJ and Weissman IL, 2004). This theory emerged when first papers were published suggesting evidence of BM-MSCs contribution to non-hematopoietic tissue (Ferrari G et al., 1998) or of neural stem cells to blood lineages (Bjornsonn CRR et al., 1999). This lineage conversion was proposed to occur directly, by activation of an otherwise silent differentiation program to change the commitment of the cells. Brockes and Kumar in 2002 suggested that lineage differentiation could also theoretically occur via dedifferentiation of tissue specific cells into a more primitive, multipotent cells, and subsequent re-differentiation along new lineage pathway. The dedifferentiation mechanism has been described in amphibians; in adult mammals (Tosh D and Slack J, 2002) is possible to find the conversion of pancreatic exocrine cells to hepatocytes *in vivo* under conditions of copper deficiency, as well as *in vitro*. Other examples include the transition between smooth and skeletal muscle in the developing oesophagus, and the conversion of myoblasts to adipocytes. There has been much recent interest in transdifferentiation of stem cells, for example, the ability of haematopoietic stem cells or MSCs to give rise to neural and other epithelial derivatives after transplantation. However, transdifferentiation remains an important area for understanding cell plasticity (Tsai RY et al., 2002).

Studies from a number of groups have shown that bone marrow cells can be plated onto tissue culture plastic and the initial adherent bone marrow-derived stromal colonies are derived from a single MSCs (Bianco P et al., 2001). These colonies are multipotent and can be induced to form bone, cartilage, and fat by simple manipulation of culture conditions (Pittenger MF et al., 1999, Park SR et al., 1999, Kuznetsov SA et al., 2001). Culture in the presence of dexamethasone, methyl isobutylxanthine, insulin, and indomethacin has been found to favor adipogenesis of human bone marrow cells that present an accumulation of lipid rich vacuoles within cells, and they express peroxisome proliferation-activated receptor γ2, lipoprotein lipase, and the fatty acid-binding protein aP2 (Pittenger MF et al., 1999). Accumulation of lipid in the vacuoles is assayed histologically by oil red O staining (Zhu M et al., 2013). The nuclear hormone receptor peroxisome proliferation

activated receptor γ (PPAR γ) is a critical adipogenic regulator promoting MSCs adipogenesis while repressing osteogenesis (Nuttal ME and Gimble JM, 2004).
The culture in presence of serum with transforming growth factor-β (TGF-β) favors chondrogenesis (Mackay AM et al., 1998). The cells develop a multilayered, matrix-rich morphology, and histological analysis shows strong staining with toluidine blue, indicating an abundance of glycosaminoglycans within the extracellular matrix (Kopen CG et al., 1999) such as type II collagen and aggrecan. Sections are also stained with Safranin O to detect the accumulation of proteoglycans (Park SR et al., 1999, Pittenger MF et al., 1999). Expression markers associated with chondrogenesis have been positively characterizated in MSC-derived chondrocytes, including transcription factors (Sox-9 and Scleraxis) and extracellular matrix (ECM) genes (Collagen type II and IX, aggrecan, biglycan, decorin, and cartilage oligomeric matrix protein COMP) (Baksh D et al., 2004, Tuan RS et al., 2003). In contrast, it is well established that marrow cells cultured in serum with dexamethasone, β-glycerophosphate and ascorbic acid favor osteo-progenitor differentiation with enhanced alkaline phosphatase expression, matrix production, nodule formation, and deposition of calcium, confirming the presence of osteoprogenitor cells. Calcium deposition is examined by the von Kossa stain and through a quantitative measurement of calcium deposition (Park SR et al., 1999, Triffitt JT et al., 1998, Zhu M et al., 2013). With the understanding of defined conditions required to modulate cell phenotype, the potential for cells to “switch” or differentiate among different phenotypes after considerable differentiation has led to the concept of plasticity of phenotype. It has long been known that an association exists between an increase in marrow adipose tissue and osteopenia (reduction in skeletal bone mass) with increasing age and in a variety of experimental and pathological conditions, such as disuse osteoporosis and glucocorticoid-induced osteoporosis (Nuttall ME and Gimble JM, 2000). This suggests plasticity or interconversion potential among the lineages and confirms that the adipocytic and osteogenic cells share a common lineage.
Most investigations of myogenesis in adult stem cells are based on a small population of skeletal muscle-derived stem cells, or satellite cells. *In vitro, s*tudies demonstrated a successful induction of myogenesis from adult stromal MSCs after transfection with activated Notch1 (Dezawa M et al., 2005). Moreover, it has been shown that MSCs may differentiate into skeletal muscle cells with conditioned medium as well as in co-culture with a fusion between MSCs and myoblasts (Dezawa M et al., 2005; Dugan JM et al., 2014; Sung MS et al., 2013). Specific signaling molecules, such as dexamethasone together with insulin and EGF (epidermal growth factor) (Jalali et al., 2014), are able to induce the differentiation into skeletal muscle. Furthermore, MSCs isolated from bone marrow and treated with FGF (Fibroblast Growth Factors), forskolin, PDGF (Platelet-Derived Growth Factor) and transfected with an NICD plasmid were able to express MyoD (Dezawa M et al., 2005).
Finally, GDF proteins, members of TGF-β superfamily, promote the formation of tendons *in vivo* (Wolfman NM et al., 1997). Differentiation of MSC into tenocytes *in*

vitro requires mechanical loading (Altman GH et al., 2002) which is critical to tendon fiber alignment during development.

1.4 Cluster of differentiation (CD) expression on mesenchymal stromal cells

Several studies aimed at clarify the mechanism underlying the potential of MSCs to differentiate into multiple lineage have encountered difficulties. MSCs are usually harvested as plastic adherent multipotent cells, capable of differentiating into bone, cartilage and fat cells (among others), can be isolated from many adult tissue type. However, even if isolated by density-gradient fractionation, they remain a heterogeneous mixture of cells with varying proliferation and differentiation potentials (Kolf CM et al., 2007). For example, bone marrow contains various types of adherent cells including mesenchymal stromal cells, endothelial cells, osteogenic cells, phagocytotic cells, and others (Wagers AJ and Weissman IL, 2004). Although acceptable for cell-based therapeutic applications, a rigorous understanding of MSCs requires a better definition of what an MSCs is. For this reason Pittinger MF et al., (1999) was one of the first to analyze the surface antigens profile in human MSCs in detail. They described that MSCs are uniformly positive for the following markers: SH2 that is an antibody which recognize an epitope on endogline or CD105 (Mackay AM et al., 1998); SH3 antibody that recognize the epitope CD73; CD29 or integrin beta-1, CD44 (involved in cells-cells interactions, cells adhesion and migration), CD71, CD90 or Thy-1, CD106, CD120a, and CD124. Unfortunately, there are no articles bringing together and summarizing the cell surface markers of MSCs, but the review of Mafi et al. (2011) is useful to systematic summarize and provide a good basis for collect the published literature in this regard so far. What clearly emerged from different studies is that CD105, CD90, CD44, CD73, CD29, CD13, CD34, CD146, CD106, CD54 and CD166 rank the among the most commonly reported positive cell surface markers on mesenchymal cells. In addition to these, a number of other cell surface markers have been further identified: STRO-1, SH2, SH3, SH4, HLA-A, HLA-B, HLA-c, HLA-DR, HLA-I, DP, EMA, DQ (MHC Class II), CDIO5, Oct4, Oct4A, Nanog Sox-2, TERT, Stat-3, fibroblast surface antigen, smooth muscle alpha-actin, vimentin, integrin subunits alpha4, alpha5, beta1, integrins alphavbeta3 and alphavbeta5 and ICAM-1. Interestingly, several studies have reported conflicting information about some of the cell surface markers including CD10, CD34, CD44, CD45, CD49d, and CD106 or VCAM-1. Moreover, STRO-1 is considered one of the most important MSCs markers (Simmons PJ and Torok-Storb B, 1991): the cell population negative for STRO-1 is not capable of forming colonies. STRO-1 positive cells can become HSC-supporting fibroblasts, smooth muscle cells, adipocytes, osteoblasts, and chondrocytes (Dennis JE et al., 2002). Another factor accounting for the variability in the expression of adult MSCs surface markers seems to be the different stages during cell proliferation and culture where the markers have been accessed.

On the other hand, MSCs were found negative for hematopoietic lineage markers expression, like CD14 (lipopolysaccharide receptor), CD34, and the common leukocyte antigens CD45 (Kuroda Y et al., 2011). Moreover there are a number of cell surface markers that have been reported as being absent in MSCs: among them the most frequently reported are CD11b, CD49d, CD106, CD10, and glycophorin-A (Kolf CM et al., 2007). MSCs also do not express the costimulatory molecules CD80, CD86, or CD40 or the adhesion molecules CD31 (platelet/endothelial cell adhesion molecule PECAM-1), CD18 (leukocyte function-associated anti- gen-1 LFA-1), or CD56 (neuronal cell adhesion molecule-1) (Haynesworth SE et al., 1992, Galmiche MC et al., 1993, Pittenger MF et al., 1999, Sordi V et al., 2005, Le Blanc K et al., 2003).

In 2008, the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (ISCT) introduced a set of statement whit the aim to define human MSCs for both laboratory-based scientific research, as well as pre-clinical studies. ISCT stated that MSCs must be plastic adherent in standard culture conditions, have a specific surface antigen expression and the ability to differentiate in different lineage *in vitro* (Horwitz EM et al., 2005; Dominici M et al., 2006). They stated that human MSCs are defined by their expression of CD105, CD73 and CD90, and lack the expression of CD45, CD34, CD14, CD11b, CD79a, or CD19 and HLA-DR surface molecules (Horwitz EM et al., 2005; Dominici M et al., 2006). they concluded that convincing data for defining “stemness” of un-fractionated plastic-adherent cells was lacking, and proposed that plastic adherent cells described as mesenchymal stem cells be termed as mesenchymal “stromal” cells. For several reasons ISCT decided to maintain the acronym MSCs to avoid any confusion in the scientific community, since this term has been extensively used in the literature for at least two decades than it has been designed to eliminate the term “stem” from the nomenclature, as this word has specific connotation (Horwitz EM et al., 2005).

1.5 Clinical application of mesenchymal stromal cells

Despite the challenges of isolating, expanding and defining stem cells populations, they hold great promise for tissue regeneration at clinical useful level (Fortier LA., 2005). Recent progress especially on the tissue-resident adult stem cells biology has suspired great optimism and given new hopes in offering the possibility to use these undifferentiated cells or their further differentiated progenies for cell replacement in regenerative medicine. Cellular therapies have emerged as leading candidates for regenerative treatment of a variety of diseases; in particular, MSCs have shown great promise in numerous clinical trials (Kraus KH and Kirker-Head C, 2006).

Depending on the disease process and wound, different strategies involving specific cell delivery systems, genetic modification, and the use of scaffolds have been developed. Different strategies for MSCs delivery such direct topical/spray, scaffold loaded, subcutaneous injection, or systemic delivery (Ennis WJ et al., 2013).

Freshly harvested bone marrow has been used for years by surgeons to augment local skeletal tissue healing, and methods to concentrate and enrich the marrow are been introduced commercially as well as scaffold used to concentrate or deliver the cells to the repair site. One of the first and most obvious uses of MSCs is in the area of bone regeneration in sites where the body cannot organize this activity, i.e. in non-unions fractures. Critical size defects in non-union models, have clearly demonstrated that culture expanded marrow MSCs in a porous, calcium phosphate, ceramic delivery vehicle are capable of regenerating structurally bone, where whole marrow of the vehicle alone cannot accomplish satisfactory this repair. Bone repair is an example for regeneration of tissue ad integrum to its original quality, exhibiting similar biochemical and biomechanical properties (Kraus KH and Kirker-Head C, 2006).

MSCs have been used also in the cartilage regeneration, since cartilage is an avascular and a highly specialized tissue incapable of regeneration or repair of even small defects in adults. Although chondrocytes have been used in attempt to repair large cartilage defects, it is difficult to integrate neo tissue with that of the host. Inability to repair or even regenerate cartilage defects results in pain and mobility impairment. Current standard of care is similar in all species and comprises a combination of physical therapy, reduced exercise and medical (systemic and local) and surgical modalities (i.e. arthroscopy) (Brehm W et al., 2012). In the last decade, several types of scaffold have been used in combination with MSCs in order to provide an inductive microenvironment for MSCs to enter the chondrogenic lineage and facilitate the integration of the neo tissue in the lesion site (Zscharnack M et al., 2010).

Moreover, MSCs could be injected into a specific muscle of the muscular dystrophy mouse to cure it by providing newly synthesized dystrophin to the affected myotubes. The donor MSCs dedifferentiated into skeletal myoblast, fused with the host myotubes, and caused the synthesis and distribution of the dystrophin. Labelled MSCs injected into injured rat or pig heart appear to differentiate into cardiac myocytes (Toma C et al., 2002). Several studies have focused on the use of autologous MSCs for tendon repair, as well as the efficacy of these cells in acute graft-versus-host disease (GvHD) (Le Blanc K et al, 2008). Falanga V et al. (2007) showed an acceleration of wound closure in both human and diabetic mouse models by topical delivery of MSCs with a modified fibrin spray system (Falanga V et al., 2007). This work, for the first time, supported the concept of MSC wound engraftment. Javazon et al. (2007) (Javazon EH et al., 2007) demonstrated improvement in wound healing in a diabetic mouse model by using a topical application of stem cells. Dash et al (2009) treated ischemic and diabetic ulcers and noted both accelerating wound healing and decreased pain. Those experiments used intramuscular injections of MSCs and showed an increase in immature cells, blood vessels, and reticulin fibers (Dash NR et al., 2009).

Only in the last years, it is becoming accepted that MSCs stimulate host recovery and regeneration through the secretion of numerous pro-regenerative factors. *In vitro* studies have documented the secretion of multiple anti-inflammatory, angiogenic, neutrotrophic, immunomodulatory and antifibrotic factor from MSCs. In

addition to the potential clinical use of MSCs, it should be taken into account that diverse poorly differentiated adult stem cells types have been identified in the most mammalian tissues and organs (Fig.2).

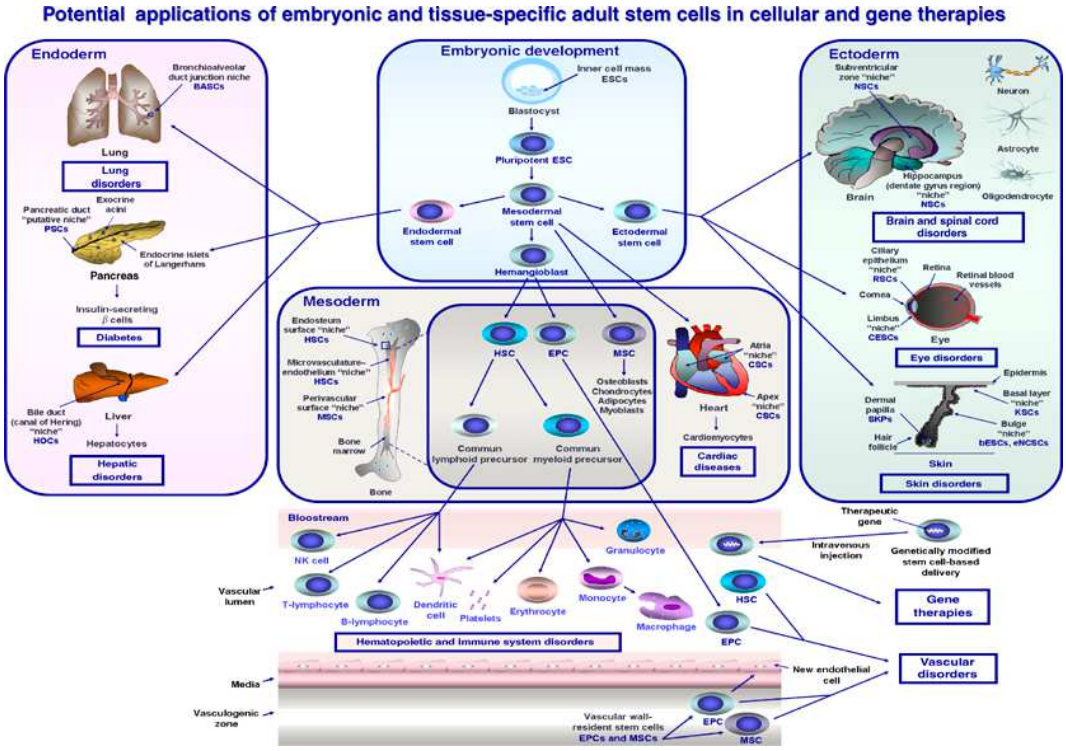

Fig.2. Scheme showing the potential therapeutic applications of embryonic and tissue-specific adult stem cells in cellular and gene therapies. The pluripotent ESC types derived from blastocyst stage during embryonic development and multipotent tissue-resident adult stem cells arising from endodermal, mesodermal, and ectodermal germ layers are shown (http://www.ebioworld.com).

Hematopoietic stem cells transplantation: the bone marrow hematopoietic stem cells (BM-HSCs) provide a critical role by continually renewing all of the new mature and differentiated hematopoietic cell lineages in peripheral circulation including leucocytes, erythrocytes and thrombocytes along lifespan of an individual. The immature and quiescent multipotent HSCs which are characterized by the expression of specific biomarkers including CD34- or CD34+/CD38-/low, Thy1+ C-Kit-/low, CD133+ are localized with the osteoblast in a specialized niche within a bone marrow region designed as endosteum (Mimeault M. et al., 2007). Marrow derived hematopoietic stem cells transplantation had been successfully established as a method to restore marrow function in patients whose bone marrows have been obliterated by disease, or by administration of marrow ablative therapies. After the first series of patients reported to receive hematopoietic stem cells transplant in Grain Britain between 1977 and 1983, these source of cells have become the most commonly used autograft product. (McCarthy DM and Goldmann JM, 1984).
The BM-derived MSCs can generate diverse mesodermal cells lineages involved in osteogenesis, adipogenesis, cartilage and, muscle formation under appropriate culturing condition ex vivo and in vivo. MSCs may also be induced to differentiate into fibroblast, neuronal cells, pulmonary cells, pancreatic islet cells, corneal

epithelial cells and cardiomyocytes ex vivo and/or in vivo using specific growth factor and cytokines (Chang YJ et al., 2006; Bobis S et al., 2006). Moreover, it has been found that BM-derived and tissue-resident MSCs are little immunogenic and display immunomodulatory effect in host in vivo. Indeed, MSCs can prolong skin allograft survival and reverse severe acute graft-versus-host disease *in vivo* supporting their use in treating skin disease as well as in the maxillofacial surgery (Shanti, RM et al., 2007).

Adipose tissue-derived stem cells: adipose tissue is a highly specialized, complex and active metabolic and endocrine structure that contributed to the energy storage under form of fat. In mammals, adipose tissue is present in diverse anatomic compartment and designed as subcutaneous adipose tissue, internal organ-surrounding adipose tissue and interstitial adipose tissue (Shen W et al., 2003). Like BM is of mesenchymal origin and contain a stromal vascular fraction. Mature adipocytes, connective tissue matrix, nerve tissue and stromal host cells including immature MSC like cells, fibroblast, vascular smooth muscle cells, endothelial cells, and immature cells such as the resident hematopoietic progenitor cells and macrophages compose specifically adipose tissue. A putative adult stem/progenitor cells population has been identify within the human adipose compartment and termed as processed lipoaspirate (PLA) or adipose tissue derived stem cells (ADSC) (Zuk PA et al., 2002; Lin Y et al., 2007). The stromal cells isolated from the lipoaspirates express the CD29, CD44, CD71, CD90, CD105/SH2 and SH3 (Zuk PA et al., 2002); they could be distinguished from BM-derived stromal MSCs by its unique expression of antigen CD49d (a4-integrin) and CD106 (VCAM). Indeed, it has been demonstrated that ADSCs may be differentiated into functional cells expressing the specific markers of mesodermal or ectodermal tissue origin *in vitro* and *in vivo* under well definite culture conditions. The most advantageous property of ADSCs is that they can easily obtained by surgical resection, lipoaspiration, or ultrasound assisted lipoaspiration, and this characteristic constitutes another promising source enriched in immature cells for cellular therapy. Among them, there are the clinical management of diverse bone, cartilage and musculoskeletal disorders (Niemela SM et al., 2007; Liu Y et al., 2007).

Muscle-Derived stem cells: adult skeletal muscle contain two distinct stem/progenitor cells, the muscle-derived stem cells (MDSCs) and satellite cell population that may actively participate to myofiber regenerative process and repair of diseased musculoskeletal tissues (Usas A and Huard J, 2007).

Muscle-committed satellite cells expressing the marker such as M-cadherin, myogenic factor5 (MYF5) and paired box gene 7 (PAX7) transcription factor, and neuronal cell adhesion molecule-1, are quiescent progenitor cells located at the periphery of skeletal myofibers under homeostatic conditions. The satellite cells endowed with self-renew ability may be activated and trigger a migration and differentiation into myogenic cells in vitro and after muscle injuries *in vivo* (Rouger K et al., 2007). The multipotent MDSCs, which may correspond to the more immature progenitor cells, if compared with satellite cells, can give rise to satellite cells and more committed progenies such as musculoskeletal, osteogenic, chondrogenic, vascular, cardiac and peripheral nerve cells lineages *in vitro* under

specific conditions, and induce new myofiber formation in animal models *in vivo*. Muscle stem/progenitor cell-based therapy and orthopaedic tissue engineering using *ex vivo* gene therapy, are promising approaches for the treatment of muscle atrophy with aging, muscle wasting (cachexia) and various musculoskeletal and neuromuscular degenerative disorders such as muscular Duchenne and Becker dystrophies and amyotrophic lateral sclerosis (Peault B et al., 2007). At present time, no curative treatment for DMS exist and the current therapies principally consist to delay its progression and provide palliative cares that will result to the death of young patient. Importantly, the results obtained from phase I trial have revealed that the autologous transplantation of CD133+ MDSCs was safe, without secondary systemic effects and improved the symptoms of DMS in treated patients. Recently, MDSC or ADSC injection based-therapies have also emerging as a valid alternative therapeutic option for the remedial treatment of deficient urethral functions such as the repair of the damaged urethral sphincter associated with the stress urinary incontinence (Torrente Y et al., 2007). The genetic and/or epigenetic alteration and changes in the microenviroment “niche” of adult MDSCs and/or satellite cells or the embryonic muscle precursor may however lead to defective skeletal muscle differentiation and rabdomyosarcoma development. The metastatic forms of rabdomyosarcomas have a poor clinical management and prognosis.

1.6 Mesenchymal stromal cells in veterinary medicine

Regenerative medicine is one of the most intensively researched medical branches with enormous progress every year. Contextually to the interest that over the past few years, MSCs have risen in human medicine, also in the veterinary field there has been an increased interest in understanding the biology and potential clinical application of MSCs. Indeed MSCs research in veterinary medicine has been performed not only in order to find a potential clinical treatment for previous incurable veterinary disease, but also to develop animal models, which could be useful to elucidate the MSCs in vitro and in vivo behavior, and use the obtained results as a template for human MSCs research. While widespread clinical use of human adult stem cells is largely restricted to the use of hematopoietic stem cells derived from adult peripheral blood, adult bone marrow, or umbilical cord blood (Koch TG et al., 2009), MSCs in veterinary field are mainly utilized for animal patients like the treatment of equine tendinopathies (Dudhia J et al., 2015, Garvican ER et al., 2014, Smith RK et al 2013) or cartilage degeneration in dogs (Zhang Y et al, 2015).

However, in some pathological conditions, including non-union fractures, osteoporosis, osteoarthritis and infection the normal repair and remodelling process are often impaired. Furthermore, other associated connective tissues such as cartilage, tendon and ligament demonstrate a limited capacity for regeneration in response to damage caused by trauma or disease. For these reasons, MSCs offer promise as novel cell-based therapies to ameliorate the healing process of damaged bones, tendon and ligament (Wang X et al., 2013). *In vivo* studies employing rabbit

as animal model have demonstrated that MSCs transplantation increased bone production and the stiffness of the regenerated structures using mechanical strength testing and animals models where the sight of injury was in weight-bearing location (Tatebe M et al., 2005). Subsequently in canine experiments, critical bone loss has successfully been tackled with the use of MSC in combination with other concepts like bone substitutes called scaffold or platelet-rich plasma (PRP). Allogeneic MSC loaded on hydroxyapatite-tricalcium phosphate implants improved the regeneration of a critical-sized segmental defect in the canine femur (Arinzeh TL et al., 2003). Recently, Adamzyk et al. (2015) studied the viability, growth and osteogenic differentiation of bone marrow-derived human and sheep MSCs in combination with a 3D scaffold made of polyetherketoneketone (PEKK) a high performance thermoplastic polymer that is FDA-approved for cranium- and maxillofacial as well as spinal surgery (PEKK). The results show that the 3D PEKK scaffolds were cyto- and bio-compatible, allowed for adherence, growth and osteogenic differentiation of human and ovine MSCs.

Animal models have been used also to study the vertebral disk regeneration: Muschler GF et al. (2003) demonstrated that, in a canine model of spinal fusion, an enriched bone matrix containing a bone marrow clot have a greatest union, stiffness and number of osteogenic cells compared to bone marrow alone. Recently, Cavallo C et al 2016 used a new approach that is represented by the use of bone marrow concentrate (BMC) that could allow the delivery of cells surrounded by their microenvironment in injured tissue. This study is focused on the potentiality of BMC seeded onto a hyaluronan-based scaffold (Hyaff-11) to differentiate into osteogenic lineage. This process depends on the specific interaction between cells derived from bone marrow (surrounded by their niche) and scaffold that create an environment able to support the regeneration of damaged tissue. The data obtained demonstrate that BMC grown onto Hyaff-11 are able to differentiate toward osteogenic sense, producing specific osteogenic genes and matrix proteins.

Regarding the cartilage regeneration, research conducted using rabbits demonstrated that MSCs in combination with scaffold or fibrin gels produced hyaline-like cartilage that integrated with the surrounding cartilage and improved the repair of the osteochondral defects created in the knee (Chang F et al., 2008).

Song K et al. (2016) demonstrated the cancellous bone and hydrogel composite scaffold are a promising biomaterial that shows an essential physical performance and strength with excellent osteochondral tissue interaction *in situ*. Moreover, the bi-layered scaffold significantly enhanced cell proliferation compared to the cells seeded on either single scaffold.

For tendon and ligament related injuries, researchers have started to investigate the therapeutic potential of MSCs. The majority of the studies relating to the repair of tendons and ligament by MSCs are limited to *in vitro* assay, rat, rabbit, pig, and horse animal models; from them it appears that MSCs therapy in combination with either a collagen, laminin or fibrin scaffold may be effective in the initial stages of tendon repair (Hirfild-Stein M et al., 2007; Kajikawa Y et al., 2007).

Undoubtedly, horse is established as an animal model for focal cartilage injuries and osteoarthritis (Goodrich LR et al., 2007; Frisbie DD et al., 2001). Advantages of

horse joint models compared with those of the of the other animals, are their sheer size, which allows for easy manipulation and exploration, and their cartilage thickness and composition, which most closely resemble those of human articular cartilage among the current animal models (Fisbie DD and McIlwraith CW, 2001). For these reasons, spontaneous injuries seen in the horses are similar to those seen in human athletes (Smith RK et al., 2005). Surgical therapies are invasive, expensive and sometimes dangerous for the patient because of complications due to induction and recovery from general anesthesia and the risk of infection. Actually, regenerative medicine is defined as innovative medical therapies that enable the body to repair, replace, restore and regenerate damaged or diseased cells, tissues and organs (Spaas JH et l., 2012). Smith et al. (2003), studied for the first time the implantation of cultured-expanded autologous bone marrow derived MSCs into a spontaneously occurring core lesion of the superficial flexor tendon. This case demonstrated the feasibility of using culture-expanded MSCs therapeutically and more important the absence of adverse reaction at 10 days or 6 weeks post injection.

Crovace A et al. in 2007 created core lesions in the superficial digital flexor tendon by injecting collagenase in three horses. The lesions were subsequently treated with either culture-expanded BM-MSCs suspended in fibrinogen, freshly isolated mononuclear cells from the bone marrow aspirates suspended in fibrinogen, or a placebo treatment with as unknown substance.

Cell based therapy using MSCs are increasingly been used in races horses like human athletes often suffer of musculoskeletal disease which currently are not curable. In equine medicine, the therapeutic use of MSCs derived from bone marrow has been reported (Guest DJ et al., 2008). Recently, Komatsu I et al 2016 tested the hypothesis that introduction of a tendon derived stem/progenitor cell (TSC) sheet accelerates tendon healing and tendon regeneration in a rat model. TSC sheets were produced on temperature-responsive culture dishes and grafted on unwounded Achilles tendons and at sites of a 3mm of Achilles tendon defect. The results showed that the implanted TSC sheet remained stably attached on the tendon surface at 4 weeks after implantation. While in the tendon defect model, tendon defect area where TSC sheet was implanted was well regenerated and had better organized collagen fibers with elongated spindle shaped cells, compared to relatively disorganized collagen fibers and round shaped cells in the control group.

Lastly, MSCs derived from either the bone marrow or adipose tissue have been demonstrated to accelerate cutaneous wound healing (Otero-Vinas M and Falanga V, 2016). Huang SP et al, (2012) in an animal study, demonstrate that the use of adipose MSCs seeded on an acellular dermal matrix enhanced wound healing, promoted angiogenesis, and contributed to newly formed vasculature in murine mouse models (Huang SP et al., 2012), reduces inflammation and promotes granulation tissue (Phillips T et al., 1994).

Chapter 2: Musculoskeletal tissue and regeneration

2.1 Muscle anatomy and physiology

Skeletal muscle tissue is the largest organ in the animals, representing up to 50% of body mass in some athletic species such as the dog and the horse (Gunn HM, 1989). Each muscle is composed of hundreds to hundreds of thousands of individual, elongated, multinucleated cells named fibers. Each fiber is constituted of many parallel myofibrils that consist of a repetitive series of an identical banded unit called sarcomere (Fig. 3). A sarcomere is formed by overlapping arrays of thick filaments and thin filaments (constituted of the contractile proteins myosin and actin, respectively), which represent the contractile machinery (Brooks SV, 2003; Schiaffino S and Reggiani C, 2011).

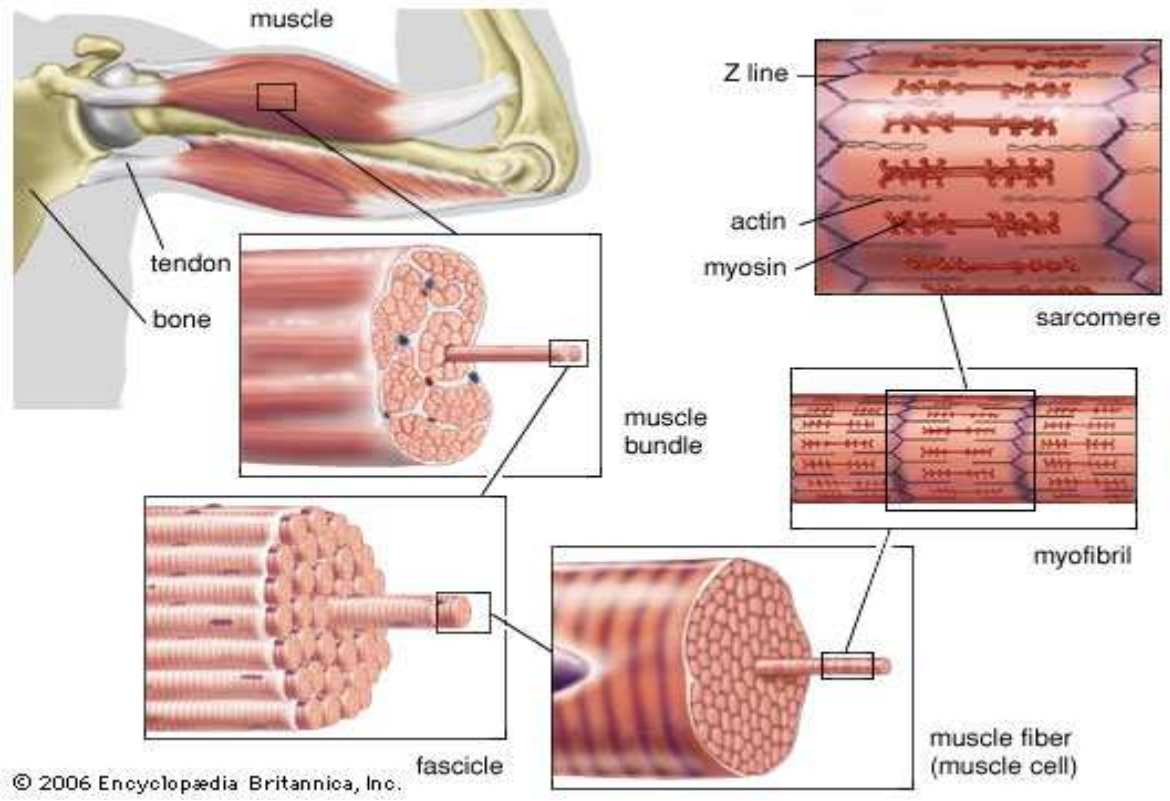

Fig.3. Macroscopic and microscopic muscle structural organization (Encyclopedia Britannica).

Myosin is a hexamer consisting of two heavy chains (MHC) and two pairs of light chains (MLC). The two heavy chains have the COOH-terminal ends that form a coiled spiral of two α-helices creating the body of the thick filaments. The other end of the heavy chains projects outward from the thick filament forming the cross-bridge portion of the molecule that connect the light chains with a non-covalently bound (Brooks SV, 2003). The heavy chains are organized into three structurally and functionally different domains. The globular head domain (catalytic domain) contains both actin-binding site and ATP-binding site and is responsible for generating force (conserved region of myosin). The long α -helical neck domain lies

adjacent to the head domain and extends toward the tail of the molecule. The tail domain is the third and longer domain. It contains the binding sites that determine the specific activities of a particular myosin (Vale R, 2000; Rüegg C et al., 2002). The final product is the thick filament decorated with hundreds of myosin heads responsible for force generation and filaments sliding movement (Schiaffino S and Reggiani C, 2011).

Actin filaments constitute the thin filaments of sarcomere. They are helical polymers that have 13 actin molecules arranged on six left-handed turns. The actin monomer consists of two similar domains each of which contains a 5-stranded β-sheet and associated α-helices. One of the domains carries a sub-domain involved in actin-actin interactions while the other is involved in the formation of the nucleotide-binding pocket. Moreover, the thin filaments contain the regulatory proteins tropomyosin and troponin that are involved in the interaction between myosin and actin (Geeves MA and Holmes KC, 1999). Actin is the main structural component of the thin filaments that form the trail along which myosin motors work. Two isoforms of actin can be expressed in mammalian skeletal muscle fibers: α-skeletal and α-cardiac isoforms, which differ by only four amino acids and are encoded by two different genes (Schiaffino S and Reggiani C, 2011).

In mammals, the number of fibers in a muscle is determined at birth and it is barely modified during the life except in case of injury or disease. Whereas, the number of myofibrils and fiber diameter can radically change depending on external factors. For example, training exercise can induce hypertrophy while immobilization, inactivity, disease, or old age may lead to atrophy that means decrease in number of myofibrils and volume of fibers (Brooks SV, 2003). Skeletal muscle injuries may be caused from a variety of events, including direct trauma (as muscle lacerations and contusions), indirect insults (strains) and from degenerative diseases (muscular dystrophies) (Cossu G and Sampaolesi M, 2007). Skeletal muscle can regenerate completely and spontaneously in response to minor injuries, such as strain. In contrast, after severe injuries, muscle healing is incomplete, often resulting in the formation of fibrotic tissue that impairs muscle function. Although researchers have extensively investigated various approaches to improve muscle healing, there is still no gold standard treatment (Laumonier T and Menetrey J, 2016).

2.2 Muscle healing process

Skeletal muscle has a robust innate capability for repair after injury through the presence of adult muscle stem cells known as satellite cells (SC) (Mauro A, 1961). They reside in a niche between the sarcolemma and the basal lamina, normally in a quiescent state. *In vitro* cultures of satellite cells have shown the ability to differentiate not only into muscle cells but also to other mesenchymal-originated tissues such as bone and cartilage and therefore can be classified as stem cells (Boonen KJM & Post MJ, 2008). During muscle regeneration and under the control of extrinsic signals from the surrounding tissue, the satellite cells can become active coming out of their state of quiescent and start to differentiate into more specialized

cells that can then fuse with other near cells and create new multinucleated muscle cells (Grounds MD et al., 2002; Buckingham M et al., 2003).
Briefly, the disruption of muscle tissue homeostasis, caused by injury, generates sequential involvement of various players around three main phases: i) degeneration/inflammation phase characterized by rupture and necrosis of the myofibers, formation of a hematoma and an important inflammatory reaction. ii) Regeneration phase that presents phagocytosis of damaged tissue followed by myofibers regeneration and leading to satellite cell activation. iii) Remodeling phase: maturation of regenerated myofibers with recovery of muscle functional capacity and also fibrosis and scar tissue formation (Laumonier T and Menetrey J, 2016).

2.3 Myogenesis

The formation of skeletal muscle provides one of the best models for studying the processes of cellular specification and differentiation and of organogenesis. Vertebrate skeletal muscle development originates from the mesoderm primary germ layer (Gros J, et al., 2005). The majority of skeletal muscles in vertebrates, with the exception of certain head muscles, develop from the somites (Bryson-Richardson RJ and Currie PD, 2008; Buckingham M and Vincent SD, 2009). Maturing somites develop the dorsally located epithelial dermomyotome and the ventrally located mesenchymal sclerotome. The sclerotome forms cartilage and bone, tendons arise from the syndetome, while the dermomyotome develops into the dermis and the skeletal muscles of the trunk and limbs (Parker MH, et al., 2003). The first cells in the developmental chain are the progenitor cells. They are undifferentiated multipotent cells that upon the first differentiation signals will give rise to myoblasts. Myoblasts are already committed to the muscle lineage. The next link in the chain are the myotubes which are elongated multinucleated cells formed by the fusion of myoblasts. The myotubes will then continue to elongate and form the muscle basic component, the multinucleated myofiber. The activation of the core myogenic cascade occurs in all tissues destined for the skeletal myogenic lineage; however, the factors that initiate the cascade differ radically in the different locations of the embryo. The myogenic precursor cells (MPCs), which arise in the dermomyotome, are specified by the expression of the paired-box transcription factors Pax3 and Pax7 that activate the quiescent cells (Kassar-Duchossoy L, et al. 2005). It has been observed that Splotch (Sp) mice, lacking a functional Pax3 gene, do not survive to term and fail to form limb muscles due to impaired migration of Pax3-expressing cells originating from the somite (Tremblay et al., 1998). In contrast Pax7 appears to be dispensable for embryonic muscle development although it can compensate for reduced expression of Pax3 and it seems to be essential for myogenesis after birth (Relaix F et al., 2005). It is now well known that the differentiation of the embryonic pluripotent cells would not continue without the governing role of four muscle regulatory factors (MRFs): Myf5, MyoD, Myogenin, and Mrf4 (also known as Myf6 or herculin). All four belong to the basic helix–loop–helix (bHLH) super family of proteins that bind to the E-box sequence, a DNA motif

that contains the sequence CANNTG which is found in the promoters of many muscle-specific genes (Moncaut N et al., 2013). The sequence of MRFs activation throughout the embryonic muscle development is well regulated and it is important a strict order. The first MRFs expressed are Myf5 and MyoD that are considered to be the “commitment” factors (Berkes CA and Tapscott SJ, 2005). After the activation of myogenic linage, the two other MRFs are taking over, Myogenin and Mrf4 are expressed downstream from the first two and are considered as the “differentiation” factors. Under the control of these two factors, myoblasts evolve to myotubes and eventually to myofibers. Mutant mice embryos lacking Myogenin and or Mrf4 genes are dying prenatally because the cells were unable to fuse and form multinucleated myotubes and myofibers. (Parker MH et al, 2003) (Fig. 4). Although the MRFs are indispensable for skeletal muscle formation in the developing embryo, the activation of MRFs is under the control of other extrinsic factors that are secreted and activated upstream such as Wnt and Sonic Hedgehog (Shh). Wnts are expressed in the dorsal regions of the neural tube and induce somitic myogenesis in cooperation with Shh signaling from the notochord, Wnt signaling has also been demonstrated to influence the expression of MRFs (von Maltzahn J et al., 2012).

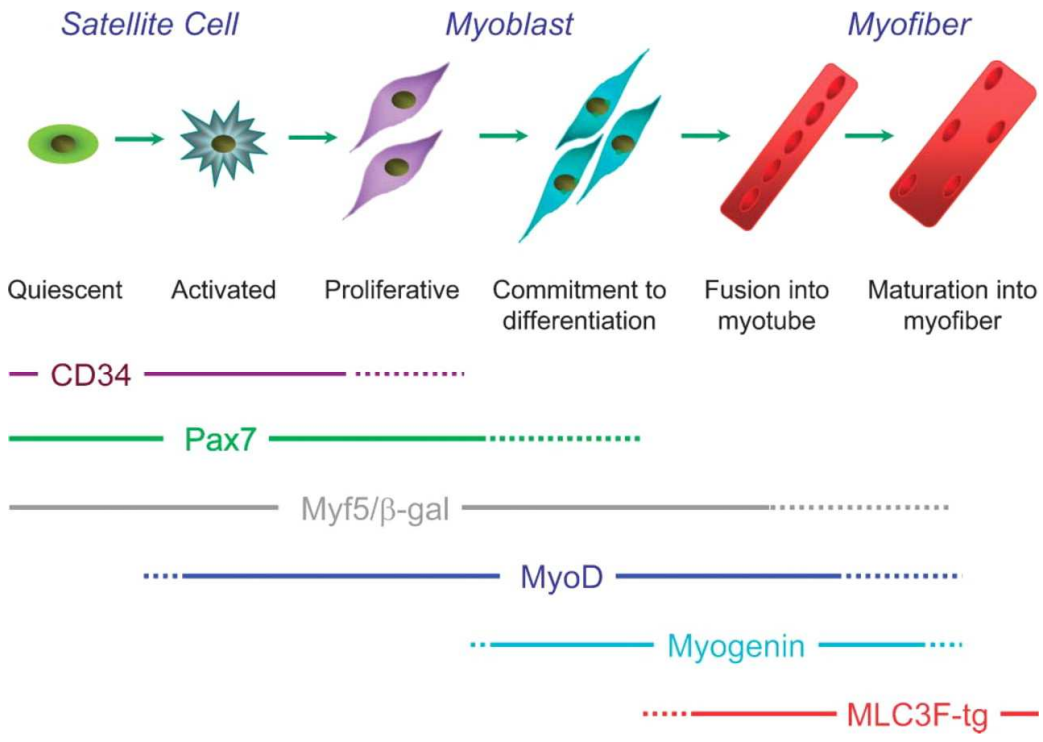

Fig.4. Schematic of satellite cell myogenesis and markers typical of each stage. Satellite cells are quiescent in normal adult muscle and can be activated by muscle damage. Once activated, satellite cells divide to produce satellite cell-derived myoblasts that further proliferate, before committing to differentiation and fusing to form myotubes, which then mature into myofibers. CD34, Pax7, and Myf5/β-gal are expressed in quiescent satellite cells (Zammit PS et al., 2006).

2.4 Tendon anatomy and physiology

Tendons link muscles to bone at the musculo-tendinous junction and osteo-tendinous junction respectively and their essential role is transferring contraction forces. Macroscopically, tendons can take the form of cords or straps of round or oval cross-section. Tendons are generally white and have a smooth surface but in strongest and largest tendons the fasciculi can be enough thick to give a longitudinally striated aspect. Tendon fibers of the same tendon or of adjacent tendons can form cords or bridges. A tendon's shape strictly depends on its function. In general, more the movement is subtle and precise, more the tendon is long and thin (e.g. hand flexors), vice versa if strength and resistance are required, the tendon will be thick and short (e.g. Achille's tendon). When a muscle has a long tendon at one hand, it has a short or aponeurotic tendon at the other one (Williams PL et al., 2003).

Microscopically, tendons are a poorly cellularized tissue and they have a hierarchical structure composed by a cellular unit called tenocytes, a fibroblastic-like cell that produces collagens, lying within a network of ECM (Benjamin M et al., 2008; Magne D and Bougault C, 2015). The fibroblast are termed "tenoblast" when they are still immature spindle shaped and numerous cytoplasmatic organelles reflecting their high metabolic activity. Aged tenoblast become elongated and transform into tenocytes with a lower nucleus-cytoplasm ratio than tenoblast. Together tenoblast and tenocyte account for the 90-95% of the cellular elements of tendons. The remaining 5-10% consist of chondrocytes at the bone attachment and insertion sites, synovial cells of the tendon sheath and vascular cells, including capillary endothelial cells and smooth muscle cells of arterioles (Sharma P and Maffulli N, 2006).

The most important protein is the collagen type 1 that gives tendons their high tensile strength and it is responsible for the hierarchical structure. The tropocollagen molecules consist of triple helix with two alpha-1 chains and one alpha-2 chain that spontaneously self-assemble after secretion and cross-linking into collagen micro fibrils that arrange themselves into larger units, called sub-fibrils, in ligaments or sub-fascicles/primary fiber bundles. The fibrils then gather into collage fibers or fascicles/secondary fiber bundles (Fig. 5)

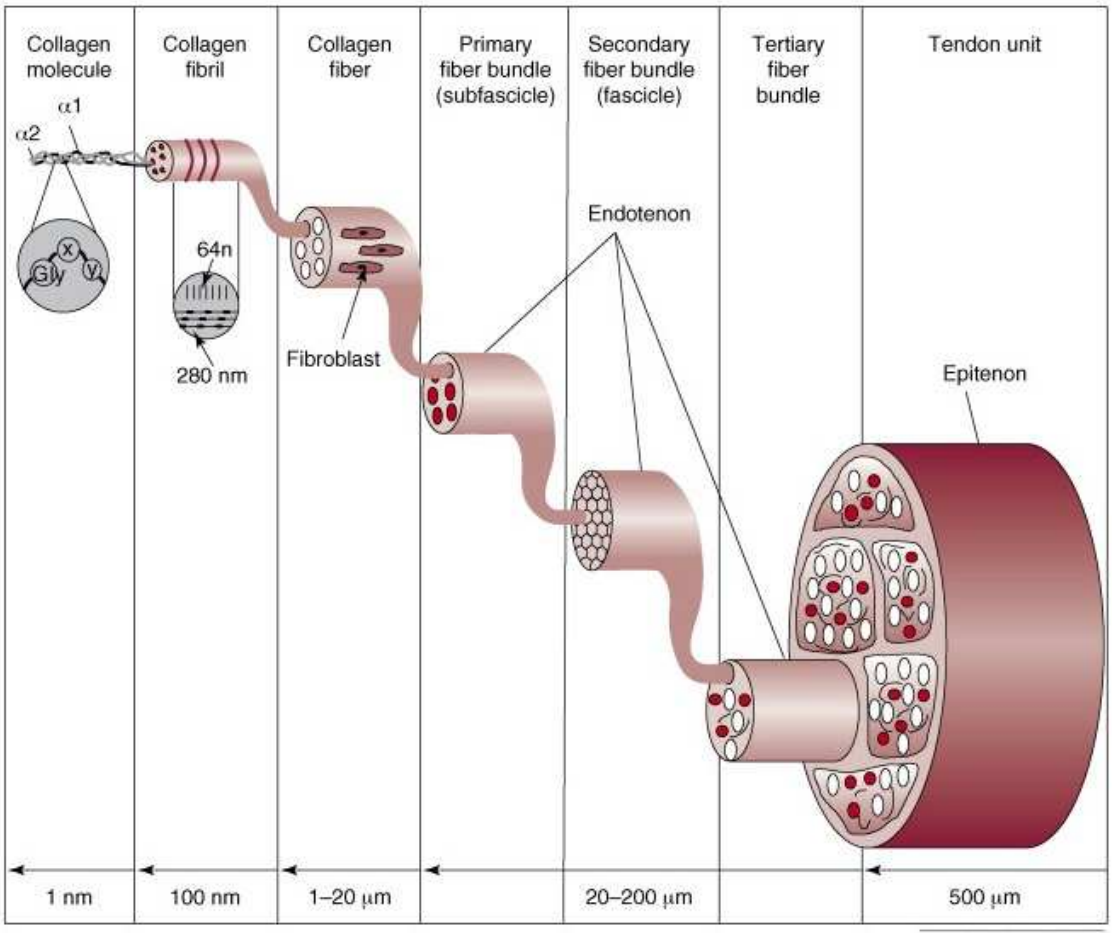

Fig. 5. Schematic of a multi-unit hierarchical structure of the tendon. The tendon has a multi-unit hierarchical structure composed of collagen molecules, fibrils, fibre bundles, fascicles and tendon units that run parallel to the tendon's long axis. This hierarchical structure contributes to the mechanical competence of the tendon (Liu Y et al., 2008)

In addition, tendons are bound together by the endotenon, a loose connective tissue that also includes blood, lymph vessels and nerves and is continuous with the epitenon, which surrounds the whole tendon. Surrounding the epitenon superficially, another thin layer, called paratenon that permits movements within the surrounding tissue. Epi- and paratenon together constitute the peritenon (Liu CF et al., 2011).
Tendons are extraordinarily strong in resisting tensile loads. Given to their low metabolic rate and well-developed anaerobic energy generation capacity, tendons are able to carry loads and maintain tension for long periods. This characteristic reduces the incidence of ischemia and necrosis, but on the other hand, the low metabolic rate entails slow healing after injury. A stress-strain curve helps to demonstrate the behavior of the tendon (Fig. 6).

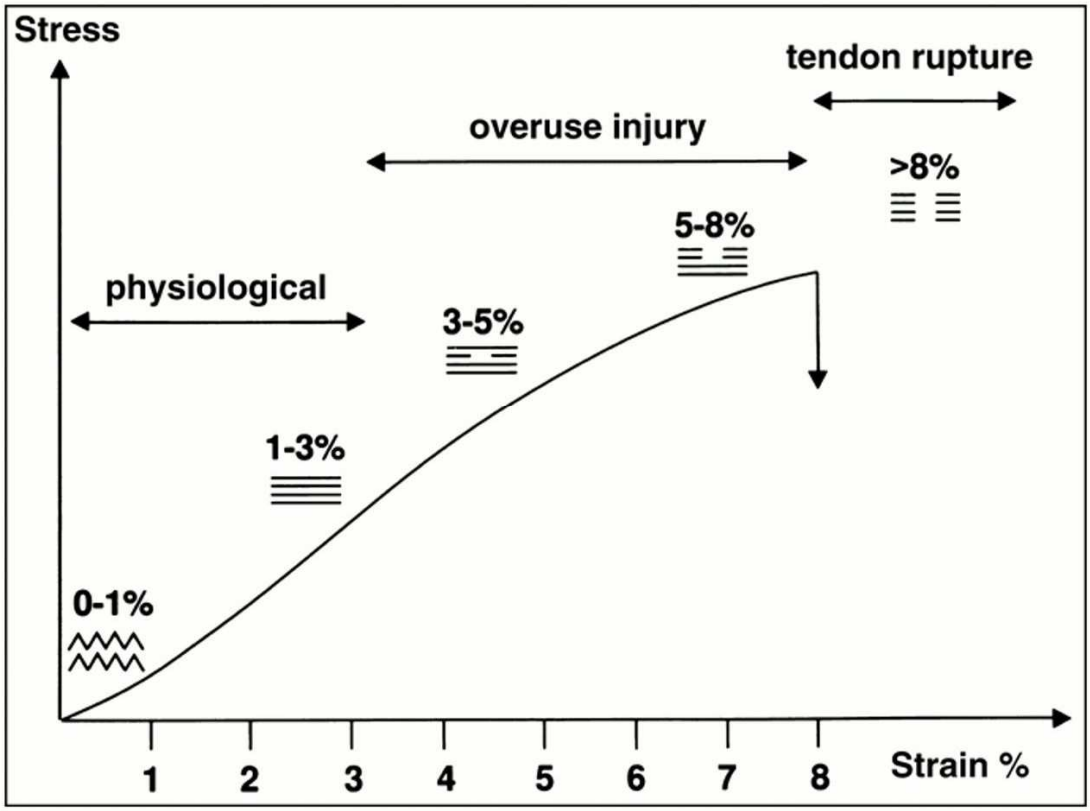

Fig. 6. Stress-strain curve demonstrating the physical properties of the normal tendon (Maffulli N, 1998).

Collagen fibers and fibrils display a crimped configuration. It is possible to distingue three regions: i) physiological response: tendon is lengthened by low tensile loads up to 3-4% of its resting length and quickly resumes its initial length after release (toe region). ii) Overuse injury: at strains of 4-8% pathological irreversible tensile elongation begins to take place. iii) Tendon rupture: tendon breaking point is 9% to 30% strain, depending on type, age and organization on the tendon fiber bundle (Hoffman A and Gross G, 2007).

2.5 Tendon healing process

Tendon injuries, degenerative tendinopathy and overuse tendinitis are very common both in human and veterinary field. It has been estimated that 30 billion dollars are spent on musculoskeletal injuries in the United States each year, and tendon/ligament injuries represent about 45% of these injuries (Praemer A et al., 1999). In general, tendon injury occurs due to acute trauma or inflammation of either the tendon tissue or the surrounding tissues. This includes tendonitis, tendinosis, bursitis, epicondylitis, and complete tendon rupture, depending on the extent of the tissue damage (Biundo JJ Jr et al., 2001; Baring T et al., 2007). In the veterinary field, overstrain injuries to weight-bearing tendons are common in racing animals that can run fast for long distances. The horse is particularly predisposed to overstrain injury of the palmar soft tissue structures of the distal limb due to

hyperextension of the metacarpophalangeal joint during weight-bearing (Kasashima Y. et al., 2004).
Tendon healing starts with hematoma formation, followed by the formation of granulation tissue (Aspenberg P, 2007). Bone morphogenetic proteins (BMPs) and growth and differentiation factors (GDFs) are the main players in the latter process, which is followed by collagen fiber deposition and organization. Basic fibroblast growth factor (bFGF) seems to play a role during cell proliferation and invasion of vascular tissues into the healing tendon (Chang J et al., 1998). Mechanical stimulation seems to be of great significance in tendon healing, contributing to mechanical strength in the regenerating tissue (Aspenberg P, 2007).

2.6 Tenogenesis

In contrast to muscle, tendon morphogenesis is less understood. From most studies of limb tendon development, it seems that tendons arise from the lateral plate mesoderm (Hurle J et al., 1989; Ros M et al., 1995). Tenascin C (TNC) is a protein that can be detected in all tendon blastema and proximal tendons, however, TNC seems not to be a specific marker of tendons, since it is expressed by other cell types, including glial cells and chondrocytes (Kardon G, 1998; Chiquet M and Fambrough DM, 1984). A more specific marker of tendon development is Scleraxis (SCX), a basic helix-loop-helix (bHLH) transcription factor that can bind to DNA sequences containing the E-box consensus sequence through its bHLH motif. In normal condition, SCX is expressed in mature tendons and ligaments of the limbs and trunk, as well as in their progenitors.
SCX gene expression is induced in superficial mesenchyme–derived tendon progenitors by the adjacent ectoderm. Its expression is restricted to these cells by BMPs, which inhibit SCX gene expression, and this inhibition is antagonized by Noggin (Schweitzer, R., et al. 2001). In addition to SCX, the homeobox gene sine oculis–related homeobox 1 homolog (Six1) has been proposed as a potential “player” in the development of tendons. Six1 being expressed in dorsal extensor tendons and Six2 in the ventral flexor tendons of the digits impaired due to the diminution of muscle tissue in these mice (Bonnin, M., et al. 2005).
Other markers of tendon development, such as tenomodulin (TeM), have also been described. Expression of the gene encoding TeM was found in association with the appearance of tenocytes (also called tendon fibroblasts), derived from tendon primordia, during chick development and is upregulated by retrovirus-mediated SCX expression in cultured tenocytes (Shukunami C et al., 2006). These data suggest that TeM as a late marker of tendon morphogenesis and that expression of the gene encoding TeM is positively regulated by SCX in tenocytes (Aslan H et al., 2008). Another marker is Decorin a proteoglycan that regulated tendon structure by stabilizing and aligning collagen fibrils (Zhang G et al., 2006). Decorin-null mice (Dcn–/–) exhibit fragile skin and abnormal fibril morphology (Danielson et al., 1997). Decorin performs an important role in regulating fibril development, growth, fusion, and orientation during tendon development. However, the regulatory role of decorin during advanced aging has not been previously described (Dunkman AA et al., 2013).

Chapter 3: Skin tissue and regeneration

3.1 Skin anatomy

The skin is the largest organ of mammals, accounting for about 15% of the total adult body weight. It performs many vital functions, including protection against external physical, chemical, and biologic assailants, as well as prevention of excess water loss from the body and a role in thermoregulation. The skin is continuous, with the mucous membranes lining the body's surface (Kanitakis J, 2002). The barrier function of the skin is of critical importance, which is evident when this barrier is disrupted following injury, or in atopic dermatitis, ichthyosis, or irritant contact dermatitis (Richmond JM and Harris JE, 2015). The loss of skin integrity may induce important dysfunctions or even death. Once the barrier is disrupted, the rapid but nonspecific innate immune response is recruited in defense, a process that relies on detection of both self and foreign "danger signals" as the initial alarm (Richmond JM and Harris JE, 2015). For superficial wounds, the endogenous healing mechanisms in combination with traditional injuries care are sufficient to achieve functional repair. In contrast, in larger lesions, like third and fourth degree burns, chronic wound or deep ulcers, it is difficult to obtain the restitutio ad integrum and often the result leads to fibrosis and scar tissue formation (Broeckx SY et al 2014, Spaas JH et al., 2016).

3.1.1 General Structure

The integumentary system (Fig. 7) is composed of three layers: the epidermis, the dermis, and subcutaneous tissue (Kanitakis J, 2002).

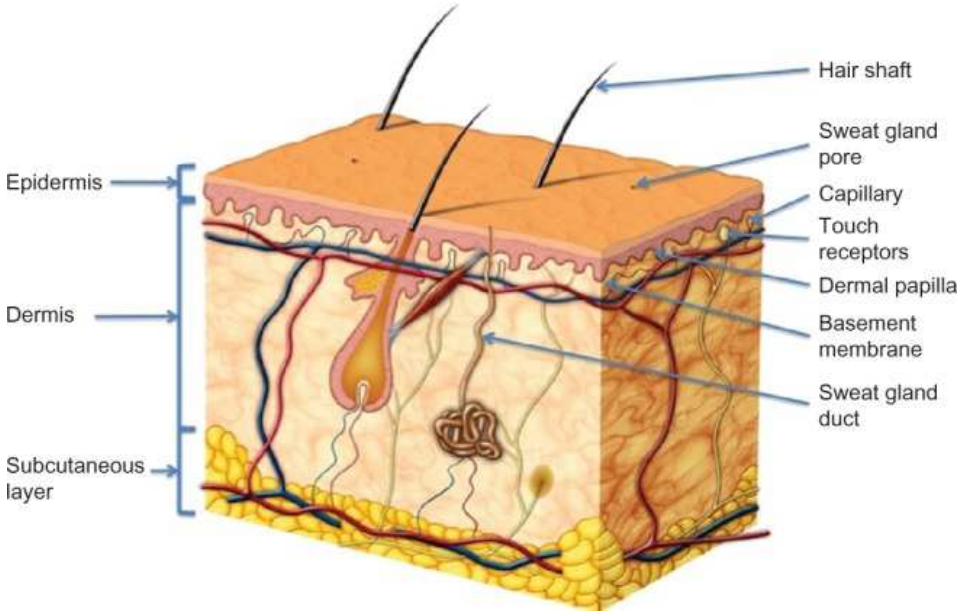

Fig.7. Schematic representation of the cross section of the skin (Poinern GEJ et al., 2011).

The outermost level is the *epidermis* that consists of a specific constellation of cells known as keratinocytes, which function to synthesize keratin, a protein with a protective role. It is originated from the primary germ embryonic layer ectoderm. The middle layer, the *dermis,* derived from the mesoderm layer and is fundamentally made up of the collagen. The subcutaneous tissue or *panniculus* contains small lobes of fat cells known as lipocytes. The thickness of these layers varies considerably, depending on the geographic location on the anatomy of the body and the dermis is thickest on the back, where it is 30–40 times as thick as the overlying epidermis (Kierszenbaum AL, 2006).

3.1.2 Epidermis and its structures

The epidermis is a keratinized squamous epithelium and it is composed of different cell populations such as 1) keratinocytes (85%), 2) melanocytes, 3) Langerhans cells, and 4) Merkel cells, but the keratinocyte cell type comprises the majority of the cells (Reese et al., 2000).

Keratinocytes: At least 80% of cells in the epidermis are keratinocytes. The differentiation process that occurs as the cells migrate from the basal layer to the surface of the skin results in keratinization (Chu DH et al., 2008) a process where, in the synthetic phase, the cell builds up a cytoplasmic supply of keratin, a fibrous intermediate filament arranged in an alpha-helical coil pattern that serves for the cytoskeleton. After bundles of these keratin filaments converge on and terminate at the plasma membrane forming the desmosomes. During the degradative phase of keratinization, cellular organelles are lost, the contents of the cell are consolidated into a mixture of filaments and amorphous cell envelopes, and the cell finally is known as a horny cell or corneocyte (James WD et al., 2006).

Non-keratinocyte cells

Melanocytes are a dendritic, pigment-synthesizing cell derived from the neural crest and confined in the skin predominantly to the basal layer (Chu DH et al., 2008), on the external sheath and hair follicles, in sweat and sebaceous glands. They are responsible for the production of the pigment granules (melanin) (Reese et al., 2000) and its transfer to keratinocytes. Melanin is produced in a rounded, membrane-bound organelle known as the melanosome via a series of receptor mediated, hormone stimulated, enzyme, catalyzed reactions (Haake AR and Hollbrook KA, 1999). The enzyme tyrosinase is involved in the production of melanin. The tyrosinase is absent in albino animals that do not produce melanin even if they present a normal quantity of melanocites (Fig. 8). Melanin protects the skin from ultraviolet radiation.

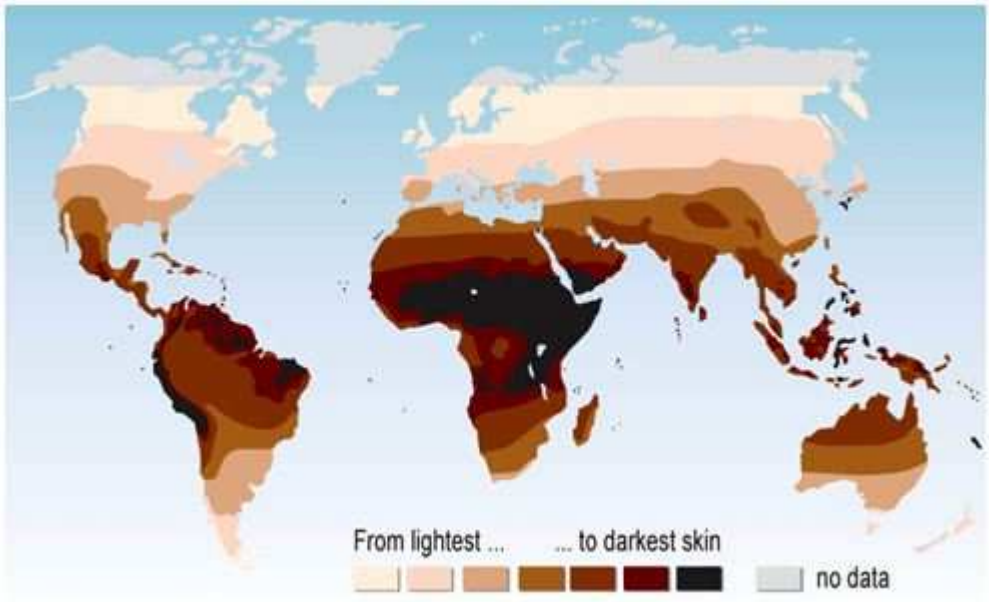

Fig.8. The distribution of skin melanin in the word (www.creofire.wordpress.com)

Langerhans cells are involved in a variety of T-cell responses. Derived from the bone marrow, these cells migrate to a suprabasal position in the epidermis early in embryonic development and continue to circulate and repopulate the epidermis throughout life. The cells are dendritic and do not form cellular junctions with neighboring cells. Langerhans cells constitute 2%–8% of the total epidermal cell population and maintain nearly constant numbers and distributions in a particular area of the body. In the epidermis, the cells mainly are distributed among the squamous and granular layers with fewer cells in the basal layer (Chu DH et al., 2008).

Merkel cells are oval-shaped, slow adapting, type I mechanoreceptors located in sites of high tactile sensitivity that are attached to basal keratinocytes by desmosomal junctions. They are neuroendocrine cells (Reese et al., 2000) and they have a long axis that are parallel to the surface of the skin and are perpendicular to the columnar basal epithelial cells above (Eurell, JA and Frappier BL., 2013). Merkel cells are found in the digits, lips, regions of the oral cavity, and outer root sheath of the hair follicle and are sometimes assembled into specialized structures known as tactile discs or touch domes (Moll I, 1994).

The epidermis commonly is divided into four layers differentiate into 1) the basal cell layer (stratum germinativum), 2) the squamous cell layer (stratum spinosum), 3) the granular cell layer (stratum granulosum), 4) stratum lucidum and 5) the cornified or horny cell layer (stratum corneum) (James et al., 2006; Roosje PJ et al., 1997) (Fig.9).

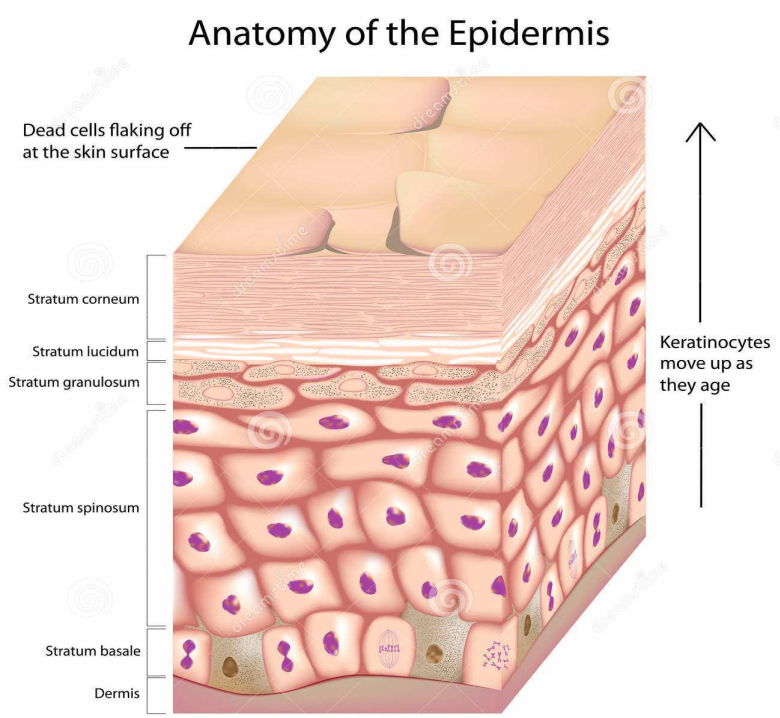

Fig. 9. The anatomy of epidermis: stratum corneum, lucidum, granulosum, spinosum and germinativum (http://www.123rf.com)

The basal layer or stratum germinativum contains column-shaped keratinocytes that attach to the basement membrane zone with their long axis perpendicular to the dermis. These basal cells form a single layer and adhere to one another as well as to more superficial squamous cells through desmosomal junctions. The basal layer is the primary location of mitotically active cells in the epidermis that give rise to cells of the outer epidermal layers (Jones PH et al., 1996). Hyperplasiogenic conditions, such as wounding, can increase the number of cycling cells in the epidermis by stimulating division of stem cells. DNA damage caused by carcinogenic agents may mutate cell proliferation machinery and can also affect the rate of cellular division. Migration of a basal cell from the basal layer to the cornified layer in humans takes at least 14 days, and the transit through the cornified layer to the outermost epidermis requires another 14 days (Chu DH et al., 2008).

The squamous cell layer (stratum spinosum). Overlying the basal cell layer is a layer of the epidermis that is 5–10 cells thick (Roosje PJ et al., 1997). The squamous layer is composed of a variety of cells that differ in shape, structure, and subcellular properties depending on their location. In this layer are present the lamellar granules that bound organelles containing glycoproteins, glycolipids, phospholipids, free sterols, and a number of acid hydrolases, including lipases, proteases, acid phosphatases, and glycosidases. The abundance of hydrolytic enzymes indicates that the lamellar granules are a type of lysosome. Although the lamellar granules primarily are active in cells at the interface between the granular and cornified layers, they also function in cells of the upper spinous layer to deliver precursors of

stratum corneum lipids into the intercellular space (Haake AR and Hollbrook KA, 1999). Keratin filaments in the cytoplasm are bound to desmosomal plaques at one end and remain free at the end closer to the nucleus (Roosje PJ et al., 1997). Gap junctions are another type of connection between epidermal cells forming an intercellular pore, these junctions allow for physiologic communication via chemical signals that is important in the regulation of cell metabolism, growth, and differentiation (Caputo R and Peluchetti D, 1977).

The granular cell layer (stratum granulosum) is composed of flattened cells holding abundant keratohyaline granules in their cytoplasm. These cells are responsible for further synthesis and modification of proteins involved in keratinization (Chu DH et al., 2008). The granular layer varies in thickness: for example, under thin cornified layer areas, the granular layer may be only 1–3 cell layers in thickness, whereas under the palms of the hands and soles of the feet the granular layer may be 10 times this thickness (Roosje PJ et al., 1997). The keratohyaline granules are deeply basophilic and irregular in shape and size, and they are necessary in the formation of the interfibrillary matrix that holds keratin filaments together and the inner lining of the horny cells. Enzymatic action of the keratohyaline granules results in the production of keratin in the epidermis by providing periodic cutting of keratin filaments. In contrast, the hair and nails do not contain keratohyaline granules (Matoltsy AG, 1976; Schwarz R et al., 1979).

Lucidum layer (stratum lucidum) can be found only in a thick skin and in hairless regions (handheld and plantar surfaces and planum nasale). It is compound of several layers of keratinized, closely compact together cells (Eurell, JA and Frappier BL., 2013).

Cornified layer. Horny cells (corneocytes) of the cornified layer offer mechanical protection to the underlying epidermis and a barrier to prevent water loss and invasion by foreign elements (Jackson SM et al., 1993). The corneocytes, rich in protein and low in lipid content (Chu DH et al., 2008), are large, flat, polyhedral-shaped horny cells have without nuclei. The physical and biochemical properties of cells in the cornified layer vary in accordance with position in order to promote desquamation moving outward. Cells in the middle have a much higher capacity for water-binding than the deeper layers because of the high concentration of free amino acids found in the cytoplasm of middle layer cells. The deep cells also are more densely compact and display a greater array of intercellular attachments than the more superficial layers (Haake AR and Hollbrook KA, 1999).

Moreover, the epidermis presents derivative structures, such as eccrine sweat glands, apocrine sweat glands, apoeccrine sweat glands, sebaceous glands, hair follicles, and nails.

Eccrine Sweat Glands are involved in the regulation of heat and are most abundant on the soles of the feet and least plentiful on the back (Roosje PJ et al., 1997); Sato K and Dobson RL, 1970). The sweat glands originate as a band of epithelial cells

growing downward from the epidermal ridge (Mauro T and Goldsmith L, 2008). This tubular structure is modified during development to generate the three composite parts of the eccrine sweat unit that correspond to the intra-epidermal spiral duct, the straight dermal portion, and the coiled secretory duct (James WD et al., 2006; Mauro T and Goldsmith L, 2008). They are found in special skin areas such as the footpads of dogs and cats and the bovine palnum nasolabiale (Eurell, JA and Frappier BL., 2013). The spiral duct opens onto the skin surface and is composed of dermal duct cells that have migrated upward. The secretory coil of the eccrine unit lies deep in the dermis or within the superficial panniculus and is composed of glycogen-rich clear secretory cells, dark mucoidal cells, and myoepithelial cells specialized in contractile properties (James WD et al., 2006; Mauro T and Goldsmith L, 2008). Dark cells have more ribosomes and numerous mucin droplets that occur in the apical part of the cell. Clear cells rest either on the basement membrane or on the myoepithelial cells and form intercellular canaliculi that open directly into the lumen of the gland (Mauro T and Goldsmith L, 2008). Large, glycogen-rich inner epithelial cells initiate the formation of sweat in response to a thermal stimulus. Initially an isotonic solution, the darker mucoidal cells in the secretory coil and in the dermal duct actively reabsorb sodium from sweat in the duct, thereby resulting in the extremely hypotonic solution that is emitted onto skin surface through the intraepidermal spiral duct (James WD et al., 2006).

Apocrine Sweat Glands are involved in scent release (Roosje PJ et al., 1997). They are saccular or tubular glands with a coiled secretory portion and a straight duct. The secretory portion has a large lumen line with flattened cuboidal to low columnar epithelial cells (Kierszenbaum AL, 2006). Apocrine sweat glands in humans are confined mainly to the regions of the axillae and perineum. Instead, the intraepithelial duct opens into pilosebaceous follicles, entering in the infundibulum above the sebaceous duct. The basal secretory coil of apocrine glands, which is normally located entirely in subcutaneous fat, differs from that of eccrine glands in that it is composed exclusively of secretory cells; no ductal cells are present (Roosje PJ et al., 1997). Apocrine sweat glands develop their secretory portions and become active just before puberty, a response induced presumably by hormonal signals. The viscous secretion has distinct odor and can function as a territorial marker, warning signal, and sexual attractant, but its sexual functions may now be vestigial in humans. It is impossible to determine the exact chemical composition of the secretion because is difficult to acquire pure samples of apocrine sweat. (Mauro T and Goldsmith L, 2008).

Apoeccrine sweat glands (AEG) develops during puberty from eccrine-like precursors directly unto the skin. Discovered during the isolation of human axillary sweat from patients with axillary hyperhidrosis, the AEG is found in the adult axillae and the frequency depends from person to person. AEG opens directly to the skin surface (Mauro T and Goldsmith L, 2008).

Sebaceous glands are found in greatest number on the face and scalp but are present on all other locations of the body with the exception of the tarsal plate of the

eye lids, the buccal mucosa and vermilion borders of the lip, the prepuce and mucosa lateral to the penile frenulum, the labia minora, and the female areola (James WD et al., 2006). The secretory portion lies in the dermis and consists of groups of alveoli connected to the excretory duct. Cells of the sebaceous glands contain abundant lipid droplets known as sebum, an oily secretion containing a mixture of lipids and disintegrated cells, in their cytoplasm and are arranged into lobules off the upper segment of the hair follicle. Basaloid germinative cells surrounding the lobule give rise to the lipid-filled cells, which are then expelled into the infundibular segment of the hair follicle via the sebaceous duct. The sebaceous glands are thought to be evolutionarily important in providing a secondary lubrication during the passage through the birth canal. This extra lubrication covers the surfaces that come in direct contact with the birth canal including the vertex, anterior scalp over the forehead and nose to the lower jaw line, and the shoulders, chest, and upper aspect of arms posteriorly (Danby FW, 2005; Thiboutot D, 2004).

Hair and Hair follicles. Hair has many biologic functions including protection from the elements and distribution of sweat-gland products. Hair is a flexible, keratinized structure produced by the hair follicle and it is composed of a cuticle (a single layer of the flat keratinized cells), a cortex with densely packed keratinized cells containing remnants of nuclei and pigment granules and a medulla (the center of the hair with cuboidal or flattened cells). The hair root is the part within the follicle that ends in a hair bulb attached to a dermal papilla (Eurell, JA and Frappier BL., 2013). Sheep have a characteristic hair that is referred to as fibers. There are three types of fibers: wool fibers (tightly crimped of small diameter and without medulla), kemp fibers (coarse with a particular medulla) and coarse fibers with an intermediate size (Reese et al., 2000). Hair follicles vary considerably in size and shape, depending on their location, but they all have the same basic structure. The number and distribution of hair follicles over the body and the future phenotype of each hair is established during fetal development; no extra follicles are added after birth (Kratochwil K et al., 1996, Paus R et al., 1997, Zhou P et al., 1995). The follicle continues to develop until finally widening at the base and forming a bulb around the group of mesenchymal cells from which the dermal Tpapilla is formed (James WD et al. 2006). The differentiation occurs at the lower portion of the hair follicle forming the hair cone and later the hair, the cuticle, and the two inner root sheaths. Along the same side of the follicle but below the sebaceous gland, develops arrector pili muscle (AP) that are a smooth muscle bundle that attaches to the external root sheath of the follicle. The bulge, which is the zone of the AP muscle's follicular attachment, is thought to contain epithelial stem cells responsible for regenerating follicles, a crucial role in the hair growth cycle (Cotsarelis G et al., 1990). The region of the follicle above the sebaceous gland is known as the infundibular segment, and the region between the sebaceous duct and AP attachment is known as the isthmus. The region below the isthmus is known as the inferior portion and contains the bottom of the follicle as well as the hair bulb. The inferior segment undergoes cycles of involution and regeneration throughout life (James WD et al., 2006). The matrix cells are responsible for the production of the hair shaft as well as the inner and

outer root sheaths. The number of cells entering the sheath determines the size of the hair, and the dimensions and curvature of the inner root sheath determine the shape of the hair (Paus R and Cotsarelis G, 1999). The hair bulb contains melanocytes that synthesize melanosomes and transfer them to the keratinocytes of the bulb matrix. Hair color is determined by the distribution of melanosomes in the hair shaft. The hair growth cell cycle present three phases: anagen, catagen, and telogen (Paus R et al., 1996; St-Jacques B et al., 1998).

- Anagen is the active growth stage during which hairs grow at a rate of about 0.33 mm per day. The length of the anagen phase declines with age and decreases dramatically in individuals with alopecia.
- Catagen is a period of involution resulting in hair formation after many cells in the outer root sheath undergo apoptosis.
- Telogen where the hairs in this stage are eventually pushed out by the growing anagen hair shaft (James WD et al., 2006).

Insulin-like growth factor 1 (IGF-1) and fibroblast growth factor 7 (FGF-7) have important roles in hair follicle development and cycling. In addition, estrogens, thyroid hormones, glucocorticoids, retinoid, prolactin, and growth hormone control are able to influence the cell cycle of hair. The most important hormones are the androgens: testosterone and its active metabolite, dihydrotestosterone act through androgen receptors in the dermal papilla. These hormones increase the size of hair follicles in androgen-dependent areas such as the beard area during adolescence. Later in life they can cause miniaturization of follicles in the scalp resulting in androgen alopecia (male pattern baldness) (Kaufman KD, 1996).

Nails. Fingernails provide protection to the fingertips, enhance sensation, and allow small objects to be grasped. The underlying nail bed is part of the nail matrix containing blood vessels, nerves, and melanocytes and has parallel rete ridges. The nail plate is formed from matrix keratinocytes (James WD et al., 2006). Fingernails grow at an average rate of 0.1 mm per day, two to three times faster than the rate of toenail growth. Because of the slow growth rate, toenails can provide information about toxic exposure or disease from many months in the past (James WD et al., 2006).

The interface between the epidermis and dermis is formed by a porous basement membrane zone that allows the exchange of cells and fluid and holds the two layers together (James WD et al., 2006). Basal keratinocytes are the most important components of structures of the dermal-epidermal junction; dermal fibroblasts are also involved but to a lesser extent (Gayraud B et al., 1997). The basal lamina is a layer synthesized by basal cells of the epidermis consisting mainly of type IV collagen as well as anchoring fibrils and dermal microfibrils. This includes the lamina lucida as well as the lamina densa (Aumailley M and Krieg T, 1996). The plasma membranes of basal cells are attached to the basal lamina by rivet-like hemidesmosomes that distribute tensile or shearing forces through the epithelium. The dermal-epidermal junction acts as support for the epidermis, establishes cell

polarity and direction of growth, directs the organization of the cytoskeleton in basal cells, provides developmental signals, and functions as a semipermeable barrier between layers (Stepp MA et al., 1990).

3.1.3 Dermis and Hypodermis

The dermis is an integrated system of fibrous, filamentous, and amorphous connective tissue that accommodates stimulus-induced entry by nerve and vascular networks. The predominant cells are fibrocytes, macrophages, and mast cells. Other blood-borne cells, including lymphocytes, plasma cells, and other leukocytes, enter the dermis in response to various stimuli as well. The dermis comprises the bulk of the skin and provides its pliability, elasticity, and tensile strength. It protects the body from mechanical injury, binds water, aids in thermal regulation, and includes receptors of sensory stimuli. The dermis interacts with the epidermis in maintaining the properties of both tissues. The matrix components, including collagen and elastic connective tissue, also vary in a depth-dependent manner and undergo turnover and remodeling in normal skin, in pathologic processes, and in response to external stimuli (Chu DH, 2008). The constituents of the dermis are mesodermal in origin except for nerves, which, like melanocytes, derive from the neural crest. Until the sixth week of fetal life, the dermis is merely a pool of dendritic-shaped cells full of acid-muco-polysaccharides, which are the precursors of fibroblasts. By the 12^{th} week, fibroblasts are actively synthesizing reticulum fibers, elastic fibers, and collagen. The principal component of the dermis is collagen that is found in tendons, ligaments, the lining of bones, and the skin (James WD et al., 2006). The major constituent of the dermis is type I collagen. Type IV collagen is found in the basement membrane zone, and the major structural component of anchoring fibrils is collagen type VII, which is produced primarily by keratinocyte. Hyaluronic acid is a minor component of the normal dermis but is the major mucopolysaccharide that accumulates in pathologic states (James WD et al., 2006).

In the dermis are present many structures:

Vasculature is made up of three intercommunicating plexuses. The subpapillary or superficial plexus composed of postcapillary venules found at the junction of the papillary and reticular dermis and the lower plexus at the dermal-subcutaneous interface. The dermal papillae are supplied by capillaries, end arterioles, and venules of the superficial plexus. The middle or cutaneous plexures are located at the papillary and reticular layers of the dermis give branches to the subpapillary plexus. The deeper plexus is supplied by larger blood vessels and is more complex surrounding adnexal structures. Vasodilation and increased skin blood flow, along with sweating, are crucial to heat dissipation during heat exposure and exercise. During exposure to cold, vasoconstriction in the skin decreases heat loss from the body to prevent hypothermia. Altered control of skin blood flow can considerably impair the ability to maintain normal body temperature (James WD et al., 2006).

Muscles involuntary or smooth muscle of the skin occurs as AP, tunica dartos of the external genitals, and the areolas around the nipples. The location of the nucleus in the center of the muscle cell and the absence of striation distinguishes smooth muscle from striated muscle. The muscle fibers of the arrectores pilorum are located in the connective tissue of the upper dermis and are attached to the hair follicle below the sebaceous glands (James WD et al., 2006).

Nerves bundles, together with arterioles and venules, are found in great quantity in neurovascular bundles of the dermis (James WD et al., 2006). Meissner corpuscles, found in the dermal papillae, help to mediate touch and are found predominantly on the ventral sides of the hands and feet. Meissner corpuscles occur in greater abundance on the hands, with greatest concentration in the fingertips. Vater-Pacini corpuscles are large nerve-end organs that generate a sense of pressure and are located in the deeper portion of the dermis of weight-bearing surfaces and genitalia. Pain, temperature, and itch sensation are transmitted by unmyelinated nerve fibers that end around hair follicles and the papillary dermis (James WD et al. 2006).

Mast cells are specialized secretory cells derived from bone marrow and distributed in connective tissues throughout the body. Although present in greatest numbers in the papillary dermis, they also are present in the subcutaneous fat (Chu DH, 2008). In the normal dermis, mast cells appear as oval to spindle-shaped cells with a centrally located round to oval nucleus. Numerous mast cells are located around blood vessels, especially postcapillary venules. Mast cell granules are round, oval, or angular membrane-bound structures containing histamine, heparin, serine proteinases, and certain cytokines. The cell's surface contains hundreds of thousands of glycoprotein receptor sites for IgE. Type I or connective tissue mast cells are located in the dermis and submucosa. Type II or mucosal mast cells are located in the respiratory tract mucosa and in the bowel (James WD et al., 2006). Traditionally associated with the allergic response, more recent studies suggest that these cells also may be capable of regulating inflammation, host defense, and innate immunity. After activation, mast cells express histamine, leukotrienes, prostanoids, proteases, and many cytokines and chemokines (Krishnaswamy G et al., 2006).

Subcutaneous fat toward the end of the fifth month fat cells begin to develop in the subcutaneous tissue. These lobules of fat cells or lipocytes are separated by fibrous septa made up of large blood vessels and collagen. The panniculus varies in thickness depending on the skin site. Considered an endocrine organ, the subcutaneous tissue provides the body with buoyancy and functions as a storehouse of energy. Hormone conversion takes place in the panniculus, converting androstenedione into estrone by aromatase. Lipocytes produce leptin, a hormone that regulates body weight by way of the hypothalamus (James WD et al., 2006)

3.2 Skin pathology

The skin is the largest organ of vertebrates and is crucial for defense as well as survival. Injury induces loss of the integrity of the skin resulting in functional imbalance, eventually accompanied by disability or even death (Theoret C, 2009).

3.2.1 Epidermis

The epidermis can suffer of different pathologies.

Disorders of cornification. Seborrhea or inflammation, trauma, metabolic or nutritional disorders.

Hyperkeratosis is an increase in the thickness of the stratum corneum in presence of chronic stimuli.

Epidermal hyperplasia is an alteration in the epidermal growth or differentiation. It is characterized by an increase in the number of cells. In early stages the epidermal-dermal interface can appear undulating and with the progression of the condition the ridges can extend into the dermis and interdigitate with the dermal papillae receiving an elongation that can appear regular or irregular. In this group is possible to find the Pseudocarcinomatous hyperplasia that is a chronic late stage due to chronic injury such as long term actinic radiation or persisting and nonhealing ulcers. It can developed after the regular or irregular forms (McGavin D, 2013).

Dyskeratosis is an alteration in the proliferation and maturation of the epidermis that is characterized by premature keratinization of cells. The keratinocytes appear shrunk and separated from adjacent cells with pyknotic nucleus and brightly eosinophilic cytoplasm because of the accumulation of keratin filaments (McGavin 2013).

Apoptosis is the programed cell death of the keratinocytes. The apoptotic cells are phagocytosed by adjacent keratinocytes before the cellular disintegration to prevent the development of an acute inflammatory response. Apoptosis is tipical of diseases such as lupus erythematosus and erythema multiforme (McGavin D, 2013).

Necrosis is the death of cells characterized by nuclear pyknosis, nuclear karyorrhexis (a rupture of the nuclear membrane with fragmentation and the release of contents) or nuclear karyolysis (a complete dissolution of the nucleus with a loss of chromatin material). This process is accompanied by an acute inflammatory response typical of thermal burns, lacerations, irritant contact dermatitis, and injury as a result of ischemia and infarction (McGavin D, 2013).

Dysplasia is an abnormal development of the keratinocytes, characterized by alterations in size, shape and organization. In this condition, the dysplasia can precede to a formation of noninvasive (in situ) carcinoma (McGavin D, 2013).

Alterations in epidermal fluid balance

Edema is a fluid accumulation between the cells and spongiosis is the term to indicate the intracellular edema of the epidermis. It can result in the formation of spongiotic vesicles that are common in epidermal inflammation by Staphylococci or Malassezia. The histological appearance is a swelling of the keratinocytes because of the fluid accumulation in the cytoplasm.

Ballooning degeneration is an intracellular accumulation of keratinocytes in the superficial layers such as stratum spinosum. It is characterized by swollen cells that have lost their intracellular attachments. This type can be seen in a virus infection such as pox and parapox virus (McGavin D, 2013).

Acantolysis is the disruption of intracellular junctions in particular the desmosomes between keratinocytes of the epidermis. It occurs with immune-mediated injury like in “pemphigus foliaceus” or with a neutrophilic enzymatic destruction as seen in superficial pyoderma. The aspect can be the presence of subcorneal vescicles and pustules or a separation of the upper epidermis from the basal cells attached to the basal lamina (McGavin D, 2013)

Inflammatory lesions. Acute inflammation begins in the dermis layer with hyperemia, an edema fluid that arises from dilated veins causing spongiosis and the leukocytes migrate in the site of injury (exocytosis) (McGavin D, 2013). If the inflammation progresses, the migrating leukocytes form pustules within the epidermis or in the stratum corneum. In thermal burns, there is the formation of vesicles due to fluid accumulation within or below the epidermis. When the fluid reaches the surface, it dries and forms a crust.
The type of leukocyte recruit depends on the pathogenesis of the disease: a population of eosinophils can be seen in case of ectoparasitic bites while lymphocytes are often seen with immune-mediated diseases (Lupus erythematosus) (McGavin D, 2013).

Pustules are accumulations of inflammatory cells within the epidermis. The presence of degenerated neutrophils and coccoid bacteria is usually due to a superficial bacterial infections and will be localized beneath the stratum corneum (McGavin D, 2013).

Crusts is dried fluid and cellular debris exudate located on the epidermal surface.

Alteration of epidermal pigmentation

Hyperpigmentation: is a result of an increased production of melanin from melanocytes or an increased amount of melanocytes (McGavin D, 2013). The increased production of melanin occur during chronic inflammatory diseases such as hyperadrenocorticism (McGavin D, 2013).

Hypopigmentation: can be congenital/hereditary and develops because of a lack of melanocytes, or a failure to produce melanin or to transfer it to the epidermis.

The acquired form is due to a loss of melanocytes or of their pigment such as in the case of copper deficiency (McGavin D, 2013).

3.2.2 Dermis

Alterations of growth, development and maintenance

Dermal atrophy is a decrease of collagen fibrils and fibroblasts with a reduction in the thickness of the layer. Macroscopically the skin appears thin, translucent and the vessels are more (McGavin D, 2013).

Fibrosis (fibroplasia) consists in the proliferation of fibroblasts and newly formed collagen fibrils as a response to various injuries. It is a gradual deposition and maturation of collagen production, the fibroblast and capillary decreases, resulting in the formation of a scar.

Granulation tissue: is a term for early stage fibroplasia. The long axis of the fibroblasts and collagen fibrils are parallel to the surface of the skin and are perpendicular to vertically align the vessels (McGavin D, 2013)

Collagen dysplasia usually is an inherited condition of decreased tensile strength but with an increased ability to stretch. This results in a very fragile skin. Collagen fibers can vary in size and shape and consist of tangled fibers with abnormal organizational patterns (McGavin D, 2013).

Collagen degeneration disorders is a brightly eosinophilic granular to amorphous material boarding the fibers and somewhat obscuring them. The fibers can have a “flame figures” due to irregular radiating, edges and brightly eosinophilic staining intensity such as in the case of insect bites, must cell tumors and eosinophilic granulomas. Eosinophilic granuloma, ulcerated skin of a cat with fragmented collagen, bordered by de-granulated eosinophils (McGavin D, 2013).

Inflammation of the dermis

Acute dermatitis: i) Complete resolution in case of a little tissue damage that can be completely repaired. ii) Formation of an abscess due to bacterial infections with the formation of pus. iii) Formation of a scar after a replacement of the injured area by fibrous connective tissue in case of a significant tissue destruction, for example a deep burn. iv) Progression to chronic dermatitis (McGavin D, 2013).

Chronic dermatitis is an inflammation that lasts weeks or months. The histological features are accumulation of macrophages, lymphocytes and plasma cells (McGavin D, 2013).

Perivascular dermatitis in presence of eosinophils. There is a situation of hypersensitivity due to parasites or other antigens.

Interface dermatitis is a mild inflammation affecting the basilar epidermis and superficial dermis. With the presence of lymphocytes is suggestive of an immune response such as Lupus erithematosus.

Nodular to diffused dermatitis with infectious agents. There are macrophages (granulomatous dermatitis) that can indicate a persistent stimulus by a bacteria or fungi (McGavin D, 2013).

Adnexa: represents alterations in hair follicles and glands.

Atrophy can be physiologic or pathologic; it can be associated with hormonal or nutritional abnormalities, alterations of the blood supply, inflammation or stressful events. Damage to germinal epithelium can result in destruction or a total loss of the adnexa with replacement by a scar.

Hypertrophy is an increase in the unit size of a structure. Follicles are longer and wider; this pathology is common to find in the case of chronic allergic dermatitis (McGavin D, 2013).

Abnormalities of hair cycle stages is characterized by a disruption in the normal progression of the hair cycle: anagen→ catagen→ telogen → exogen. Those abnormalities vary from animal to animal, in fact, some can have a failure of hair to regrow after clipping (alopecia or hypothyroidism), others can have a sudden shedding like in case of “telogen effluvium” or it can be associated with endocrine diseases such as hyperadrenocorticism (McGavin D, 2013).

Follicular dysplasia is an incomplete or abnormal development of follicles and hair shafts. Microscopically appears as the presence of abnormal keratinocytes in the hair matrix and the lesions appear with a color mutant alopecia (McGavin D, 2013).

Skin vessels

Vasculitis appear as an inflammation of the vessels by microbes, toxins, immunologic injury or disseminated intravascular coagulation. Histologic lesions include damage to the vessel wall, necrotic cells or foci of fibrinoid necrosis, mural infiltrates of leukocytes. Intramural or perivascular edema or fibrin exudation (McGavin D, 2013)

Panniculus

Panniculitis is an inflammation of the subcutaneous adipose tissue. It can be caused by infectious agents (bacteria, funghi), immune-mediated disorders (lupus erithematosus), physical injury (trauma, foreign bodies), nutritional disorders (vitamin E deficiency) or pancreatic diseases (pancreatitis or tumor of the pancreas) (McGavin D, 2013). There are two types of this pathology. Primary panniculitis is typical of feline panosteatitis due to diets that are high in polyunsaturated fats and a lack of vitamin E that causes oxidation of the lipids and pyogranulomatous inflammation. Secondary panniculitis where the subcutis is affected by inflammation primarily involving the dermis (McGavin D, 2013).

Physical Injury

Acral lick dermatitis is a common psychogenic dermatitis mostly localized on the extremities. It is caused by a persistent licking or chewing and, usually, a single

lesion is found. They are circumscribed, hairless and sometimes ulcerated. Microscopically, the dermis is thickened by fibrosis. Capillaries and collagen fibers are oriented parallel to hair follicles. Sebaceous glands and hair are hypertrophic, and there is a perivascular and a periadnexal plasmacytic dermatitis. There can be some secondary complications of the lesions due to bacterial infections (McGavin D, 2013).

Pyotraumatic dermatitis is common in dogs. Longhaired and dense undercoat dogs are more predisposed. Lesions are more commonly to develop in humid weather. It is secondary to irritation due to scratching and biting because of pain or itching by allergies, parasites (fleabite dermatitis) or irritant chemicals. The lesions are hairless, red, with exudate fluid and circumscribed edges (McGavin D, 2013).

Feline ulcerative dermatitis syndrome is an uncommon disorder with an unknown pathogenesis although a self-trauma appears to contribute to the lesions. Lesions are mostly located in the dorsal neck or intercapsular regions and consist of a non-healing ulcer with serocellular exudate. Microscopically the lesions consist of ulcers covered by fibrinecrotic crust. The dermis below the ulcer contains necrotic epidermis and adnexa intermixed with degenerate neutrophils (McGavin D, 2013).

Temperature extremes

Direct freezing: causes disruption of cells and vascular damage leading to tissue anoxia. With the formation of extracellular ice crystals that damage the cellular membranes leading to the death of the cells. As freezing continues, a shift in intracellular water to the extracellular space leads to cellular dehydration and increased intracellular sodium concentration that leads to intracellular ice crystal formation. Lesions are located in the extremities (McGavin D, 2013).

Slow chilling produces vasoconstriction with endothelial and parenchymal cell damage. Secondary vasodilatation causes increased vascular permeability, which leads to edema and neutrophilic inflammation.

Thermal burns are categorized as partial (first or second degree) or full-thickness (third degree). Dry heat causes desiccation and carbonization, whereas moist heat causes coagulation of tissue. First-degree burn is present only the epidermis is harmed, there will be erythema and edema. Second degree hits the epidermis and a part of the dermis are injured. Formation of “burn blister” (vesicle formation as a result of fluid accumulation at the dermal-epidermal junction). Third degree is a coagulation of the epidermis and all dermal components. Desiccation of the epidermis with underlying amorphous accretion of connective tissue representing the coagulated dermis and adnexa. Forth degree burns: the damage extends to the subcutaneous fascia and underlying tissue (McGavin D, 2013).

3.3 Skin healing

Acute wounds normally heal in a very orderly and efficient manner characterized by four distinct, but overlapping phases: hemostasis, inflammation, proliferation and remodeling (Diegelmann RF and Evans MC, 2004). The stages of wound healing based on microscopic characteristics and events that are initiated, mediated and sustained by biochemical mediators known as cytokines and growth factors (Hosgood G, 2006)

3.3.1 Normal and pathological response to injury

The term wound has been defined as a disruption of normal anatomical structure and, more importantly, function. Therefore, healing is the complex and dynamic process that results in the restoration of anatomical continuity and function (Lazarus GS et al., 1994).
Four basic responses can occur following an injury.
Normal repair is the response where there is a re-established equilibrium between scar formation and scar remodeling.

The pathological responses to tissue injury stand in sharp contrast to the normal repair response. In excessive healing, there is too much deposition of connective tissue that results in altered structure and, thus, loss of function (van Zuijlen PP et al., 2002). Fibrosis, strictures, adhesions and contractures are examples of excessive healing. Keloids and hypertrophic scars in the skin are examples of fibrosis (Rahban SR and Garner WL, 2003).

Deficient healing is the opposite of fibrosis; it exists when there is insufficient deposition of connective tissue matrix and the tissue is weakened to the point where it can fall apart.

Chronic non-healing ulcers are examples of deficient healing.
Regeneration is the elegant process that occurs when there is loss of structure and function but the organism has the sophisticated capacity to replace that structure by replacing exactly what was there before the injury. All dermal wounds heal by three basic mechanisms: connective tissue matrix deposition, contraction and epithelization. Wounds that are simple and can be closed by sutures, tape or staples heal by Primary Intention. The main mechanism of healing during primary intention is connective tissue matrix deposition, where collagen, proteoglycans and attachment proteins are deposited to form a new extracellular matrix. In contrast, wounds that remain open heal mainly by contraction do not present interaction between cells and matrix. The underlying mechanisms responsible for contraction are not fully understood but there appears to be a complex interaction between contractile fibroblasts sometimes referred to as "myofibroblasts" and the matrix components (Tomasek JJ et al., 2002).

3.3.2. The healing cascade

Homeostasis

Immediately after injury, hemorrhage from damaged vessels and lymphatics fills the wound and cleans the surface. Catecholamines and other vasoactive compounds such as serotonin, bradykinin and histamine cause vasoconstriction, which lasts for only 5 to 10 minutes. After that, there is a vasodilatation and through diapedesis, intravascular cells and fluid pass through the vessel walls into the extravascular space.

The healing cascade begins immediately following injury when the platelets come into contact with exposed collagen. As platelet aggregation proceeds, clotting factors are released resulting in the deposition of a fibrin clot at the site of injury. The fibrin clot serves as a provisional matrix and sets the stage for the subsequent events of healing (Clark RA, 2001). The most important component is fibronectin. With the presence of factor XIII, fibronectin becomes covalently cross-linked to fibrin providing a limited wound strength and forms a provisional extracellular matrix. This provisional extracellular matrix has multiple binding sites for adhesive molecules such as neutrophils, macrophages and connective tissue cells. The blood clot has the ability to provide protection in the form of a barrier against infection and it prevents fluid loss and provides a substrate for the early reorganization of the wound (Slatter DH, 2003).

The two most important signals involved in this phase are platelet-derived growth factor (PDGF) and transforming growth factor-beta (TGF-β). The PDGF initiates the chemotaxis of neutrophils, macrophages, smooth muscle cells and fibroblasts. In addition, it also stimulates the mitogenesis of the fibroblasts and smooth muscle cells. TGF- β adds another important signal for the initiation of the healing cascade by attracting macrophages and stimulates them to secrete additional cytokines including FGF (fibroblast growth factor), PDGF, TNFα (tumor necrosis alpha) and IL-1 (interleukin-1). In addition, TGF-β further enhances fibroblast and smooth muscle cell chemotaxis and modulates collagen and collagenase expression. The net result of these redundant signals is a vigorous response of the matrix producing cells to ensure a rapid deposition of new connective tissue at the injury site during the proliferative phase that follows the inflammatory phase.

Inflammation

Chemoattractants encourage neutrophils and monocytes to appear in wounds approximately 6 hours and then 12 hours after injury.

Neutrophils are the next predominant cell marker in the wound within 24 hours after injury. Neutrophils increase in number for 2-3 days, they prevent infection and phagocytize organisms and debris (Fossum T et al., 2007). The major function of the neutrophil is to remove foreign material, bacteria and non-functional host cells and damaged matrix components that may be present in the wound site. Bacteria give off chemical signals, attracting neutrophils, which ingest them by the process of phagocytosis. During bacterial protein synthesis a waste product represented by a tri-peptide called f-Met-Leu-Phe is released which in turn attracts inflammatory

cells. Neutrophils will engorge themselves until they are filled with bacteria and constitute what is called "laudable pus" in the wound (Thurston AJ, 2000). The mast cell is another marker cell of interest in wound healing that release granules filled with enzymes, histamine and other active amines. These mediators are responsible for the characteristic signs of inflammation around the wound site. The active amines released from the mast cell, causes surrounding vessels to become leaky and thus allow the speedy passage of the mononuclear cells into the injury area. In addition, fluid accumulates at the wound site and the characteristic signs of inflammation begin. The signs of inflammation have been well recognized since ancient times: rubor (redness), calor (heat), tumor (swelling) and dolor (pain). Monocytes become wound macrophages in 24-48 hours and they secrete collagenases removing necrotic tissue, bacteria and foreign material (Fossum T et al., 2007). These highly phagocytic macrophages are also responsible for removing nonfunctional host cells, bacteria- filled neutrophils, damaged matrix, foreign debris and any remaining bacteria from the wound site. The presence of wound macrophages is a marker that the inflammatory phase is nearing an end and that the proliferative phase is beginning. Macrophages also recruit mesenchymal cells, stimulate angiogenesis and modulate matrix production in wounds.
Lymphocytes appear later and secrete soluble factors that may stimulate or inhibit migration and protein synthesis by other cells (Fossum T et al., 2007) and they are not considered to be major inflammatory cells involved in the healing response (Diegelmann RF and Evans MC, 2004).

Proliferation

The transition from inflammation to proliferation is marked by the invasion of fibroblasts and an increased accumulation of collagen in the wound. In addition, there is a new endothelial structures formation. Those mechanisms are angiogenesis, fibroplasia and epithelialization.
Angiogenesis is the growth of new capillaries from preexisting vessels at wound edges into areas previously unoccupied by vascular tissue. In the earliest phase, local factors in the wound microenvironment such as low pH, reduced oxygen tension and increased lactate actually initiate the release of factors needed to bring in a new blood supply. Intact or recently broken capillary blood vessels are stimulated and this allows the migration of capillary endothelial cells toward the site of injury and simultaneously endothelial proliferation initiates (Hosgood G, 2006).
The early granulation tissue appears with deep red color because of the multiple new capillaries that have recently formed. As the healing process progresses, the new blood vessels disintegrate because of apoptosis and the wound color becomes paler (Hosgood G, 2006).
Fibroplasia. Wound fibroblast have a characteristic myofibroblastic appearance, with abundant contractile filaments, intracellular tight junctions, and distorted nuclear envelope. They contain actin, a smooth muscle protein, desmin and vimentin. The fibroblastic cells are responsible for the synthesis of the true extracellular matrix by gradually replacing the provisional one. It starts with the presence of type 3 collagen. A collagen molecule that is relatively abundant in blood

vessels and is associated with the capillary content of the granulation tissue. With the formation of collagen type 1 by the fibroblasts the production spreads quickly till it overtakes the type 3 collagen (Slatter DH, 2003). The elaboration, orientation and contraction of the extracellular matrix components by the myofibroblasts leads to the progression of re-organization of the fibrin filled wound into a durable connective tissue. The accumulation of connective tissue reaches its peak from 7 to 14 days after injury. After reaching that peak, the fibroblasts stop producing collagen and there is a regression of the capillary content of the granulation tissue. The granulation tissue then results as an acellular scar as the cells undergo apoptosis (Slatter DH, 2003).

Epithelialization starts with the mobilization of epithelial cells at the margin of the wound and follows by proliferation of epithelial cells from the origin site 1-2 days after injury (Slatter DH, 2003).

The process of epithelization is stimulated by the presence of EGF, TGFα, KGF (Keratinocyte growth factor) produced by epithelial cells, wound fibroblasts and wound macrophages (Zanaboni G 2000). As the Proliferative phase progresses the predominant cell in the wound site is the fibroblast. This cell of mesenchymal origin is responsible for producing the new matrix needed to restore structure and function to the injured tissue. Fibroblasts attach to the cables of the provisional fibrin matrix and begin to produce collagen (Clark RA, 2001). At least 23 individual types of collagen have been identified to date but type I is predominant in the scar tissue of skin. Dermal collagen on a per weight basis approaches the tensile strength of steel; in normal tissue it is a strong and highly organized molecule. In contrast, collagen fibers formed in scar tissue are much smaller and have a random appearance; scar tissue is always weaker and will break apart before the surrounding normal tissue. The regained tensile strength in a wound will never approach normal. In fact, the maximum tensile strength that a wound can ever achieve is approximately 80% of normal skin. Finally, in the process of collagen remodeling, collagen degradation also occurs (Parks WC, 1999).

Contraction is the reduction of the wound size that corresponds to changes in the tension of the wound and the surrounding tissue. During the second week of repair, fibroblasts assume a myofibroblast phenotype characterized by actin-containing microfilaments disposed along the cytoplasmic face of the plasma cell membrane and by cell-to-cell and cell-to-matrix linkages.

The stimulation of the contraction occurs with the TGF-β1 or TGF-β2 and platelet derived growth factor, attachment of fibroblasts to the collagen matrix through integrin receptors, and cross-links between individual bundles of collagen. During the contraction, the surrounding skin stretches and the wound takes on a stellate appearance. Once the contraction begins it continues until the wound edges meet and the contraction ends (Slatter DH, 2003).

Remodeling

This phase begins once collagen has been adequately deposited in wounds (17-20 days) and may continue for years. The cellularity of granulation tissue is reduced as cell die. There is a reduction in collagen content of the extracellular matrix. Collagen

fibers remodel with alteration of their orientation and increased cross-linking which improves wound strength. (Fossum et al. 2007)

Fibers orient along lines of stress with the decrease of type 3 collagen and increase in type 1. The degradation occurs due to proteolytic enzymes (matrix metalloproteinases) secreted by macrophages, epithelial cells, endothelial cells and fibroblasts within the extracellular matrix. The most rapid gain in wound strength occurs between 7 to 14 days after injury. Slower increase in wound strength then occurs, but normal tissue strength is never regained. As the number of capillaries also decrease the scar becomes paler, less cellular, flattened and soften (Fossum T et al., 2007). Many clinical problems are associated with excessive scar formation. Fibrosis can be defined as the replacement of the normal structural elements of the tissue by distorted, non-functional and excessive accumulation of scar tissue. Keloids can be used as a clinical example of fibrosis to define some of the biochemical and cellular markers characteristic of fibrosis (Rahbam SR and Garner WL, 2003). Fibroblasts isolated from keloids produce about 2 to 3 times more collagen compared to fibroblasts isolated from normal skin in the same patients (Diegelmann RF and Evans MC, 2004). It appears that keloids have increased expression of TGFβ and also an up-regulation of receptors for TGFβ. Hypertrophic scars are also characterized by excessive accumulation of scar collagen and are frequently misdiagnosed as keloids. There is one very significant biological marker that distinguishes keloids from hypertrophic scars and that is the absence of myofibroblasts in keloids and an abundance of these contractile cells in hypertrophic scars (Ehrlich HP et al., 1994).

3.4 Treatments of skin injuries

3.4.1 Conventional treatments

Treatment	Specie	Deliver Methods	Results
Hyaluronic Acid (Fidia Farmaceutici, Italy)	Non experimental open wound in dog	Application in defect side	Wound area reduction (Ferrari R. et al., 2015)
CMHA-S	2x2 cm wound in dog	Application in defect side	Scar minimization (Hadley H. et al., 2013)
Aloe Vera or Aloe Barbadensis	Diabetic rat wounds	Gel applications in defect side	Accelerated healing process (Pereira R. et al., 2014)
Acemannan	Full-thickness skin excisional wound (1x1 cm)	Injected subcutaneously in four sites surrounding the wound.	Accelerated skin wound closure and proliferation (Xing W. et al., 2014)
Manuka Honey	Second degree burns created in rabbit model	Cream application in defect side	Increase in the healing process and wound contraction (Pereira R. et al., 2014)
Mono Flower Honey of Thymus plant + Propolis	Open wound healing in Wistar rat	Cream application in defect side	Accelerated wound healing process, shortened inflammatory phase, increased tissue granulation and angiogenesis. (Takzaree N. et al., 2016)
PRP and growth factors: PDGF, EGF, FGF, IGF1, IGF2, VEGF, TGF-β and KGF)	Diabetic mouse model, human chronic wounds	Application in defect side	Production of inflammatory cells, vascular endothelial cells, fibroblasts and keratinocytes and contribute to re-epithelialization, angiogenesis and granulation tissue formation

CMSA-S: thiolated carboxymethylhyaluronic acid

3.4.2 Innovative treatments

Treatment	Specie	Deliver Methods	Results
PLGA-collagen-ASC	Full-thickness lesion (3x3 cm) on skin of large pig	Implanted of PLGA-collagen-ASC	Granulation tissue formation, inhibition of inflammation and of scarring (Domingues JA et al., 2016)
BM-MSC seeded on Integra matrix enriched with PRP (Ematrix)	Lesion (2x4 cm) on skin of Lewis rat.	Integra matrix and Ematrix fixed to the skin	Regenerated skin, reduced collagen deposition, reepithelization, neo-angiogenesis, hair follicles and sebaceous glands (Formigli L et al., 2015)
ASC + scaffold CMC	Circular lesion (7mm) on skin of Wistar rat	Implanted of scaffold CMC with ASC	Improved epithelization, granulation tissue and cell proliferation (Rodrigues C et al., 2014)
Allogeneic putative EpSC in combination with autologous PRP	Full-thickness skin wound (4x4 cm) in horse	Intradermal injection and topical application in defect side	Accelerated wound healing process (Broeckx SY et al., 2014)
Autologous and allogeneic EpSC	Full-thickness lesion on skin of horse	Intradermal injection and topical application in defect side	Highest expression of IL-6, VEGF and IGF-1 mRNA (Spaas J et al., 2016)
Plasma (Ionized gas)	Animal and human living tissue sterilization and medical application	Application in defect side	Regulation of blood vessel tone and blood coagulation, immune system and early apoptosis (Fridman G et al., 2008)

PLGA: Poly(Lactic-co-Glycolic Acid), ASC: adipose stem cells, BM-MSC: bone marrow MSC, PRP: platelet rich plasma, CMC: sodium carboxymethylcellulose, EpSC: epithelial stem cells.

GENERAL AIM

Adult MSCs gained general attention, both in human and veterinary research fields, due to the great promise they offer as a resolute alternative for the treatment of disease that currently not guarantee the *restitution ad integrum*. Moreover adult MSCs possess considerable advantages with respect to embryonic stem cells (ES), since the latter receives attention because of the ethical controversies associated with the destruction of human embryos, and the possibility to give rise to tumors, when clinically applied. Improved understanding of MSCs function holds great promise for the application of cell therapy and also for the development of powerful cell-derived therapeutics for human and veterinary regenerative medicine (Brehm W et al., 2012; Spees JL et al., 2016).
The present work has been focused on two main topics correlated between each other: the reprogramming of adult mesenchymal stromal cells isolated from peripheral blood of horses and sheep towards tenogenic and myogenic fate *in vitro*, and the application of autologous and allogeneic MSCs in skin injuries *in vivo*.

The major aim of the first part of this work has been to reprogram PB-MSCs into new cell lines.
Tendons are structures that present low cellularity and low vascularity and they are constituted of dense connective tissue that reduce the self-healing and regenerative potential of tendons (Jiang D et al. 2014; Veronesi F et al. 2015). Autologous tenocytes can be used to repair injured tendons (Cao Y et al., 2002), nevertheless tenocytes may not be an ideal source for tendon repair (Bi Y et al., 2007) because these cells have a limited proliferative potential and ethic committees from many countries prohibit invasive collection of tendon tissue to obtain tenocytes (Tan Q et al., 2012). The same problems can be found in the regeneration of muscle tissue: it is very important to have differentiated cells to regenerate muscle disease such as injuries, muscle degeneration (Duchenne dystrophy) and inflammation.
With the increasing perspective to use MSCs for clinical purpose, growth factors (GFs) TGFβ3 (transforming growth factor), EGF2 (Epidermal growth factor), bFGF2 (Fibroblast growth factor) and IGF-1 (insulin-like growth factor) in presence or without Low Level Laser Technology (LLLT) were tested to differentiate equine PB-MSCs towards tenogenic fate.
To obtain myoblasts to regenerate muscle diseases, PB-MSCs were induced to differentiate towards myogenic fate using the complex TAT-MyoD in presence of a conditioned medium obtained from co-culturing PB-MSCs with C2C12 without a direct contact.
Lastly, to reduce the time and to increase efficiency of cell transfection, novel surface-active maghemite nanoparticles (SAMNs) were tested as vectors for eukaryotic cell transfection of coding gene in PB-MSCs without the application of external magnetic fields. Molecular and immunostaining analysis were performed to assess the cellular differentiation.

The goal of this section was to obtain reprogrammed MSCs to implant in the recellularized scaffold to use for tendon, muscle, skin and other type of tissue lesions in human and veterinary medicine.
The second major topic of this work was to study the regenerative capacity of MSCs derived from peripheral blood in the skin healing process.
Wound healing is a complex multi-stage process that organizes the reconstitution of the dermal and epidermal layers of the skin. In many pathological circumstances such as diabetes or severe burns, the normal wound healing process fails to adequately restore function to the skin, leading to potentially severe complications from ulcers or resulting infections (Beckles GL, Chou CF, 2006). In human medicine, the treatment of open wounds has been already described thousands of years ago and currently, there are many choices for the topical treatment of open wounds in veterinary medicine.
The aim of this second section was the evaluation of the PB-MSCs regenerative potential in wounds performed on animal skin. The first study shows wounds induced in the gluteus region of six horses and treated with autologous epithelial stem cells (EpSCs), allogeneic EpSCs, vehicle treatment or untreated control. The second project evaluates the use of sheep allogeneic PB-MSCs to treat experimental lesions on the back of six sheep. This project is part of a large scheme where conventional treatments (Manuka Honey, Connettivina and Acemannane) were compared to innovative cures (MSCs and gas-ionized plasma). In this thesis, only the data about skin regeneration with PB-MSCs was reported.
In particular, in the first work, the aim was achieved by molecular analysis performed from biopsies collected in the areas of the lacerations after 1 and 5 weeks of treatments with equine allogeneic and autologous PB-MSCs. In the second project, the effect of sheep allogeneic PB-MSC on skin lesions was analyzed from biopsies obtained after 15 and 42 days of trial. Clinical and molecular analysis and histological and immunohistochemical staining were performed to evaluate time of healing, inflammation, neovascularization and cell proliferation.
Overall, the major aim of this second part of the present work was the evaluation of MSCs ability to suppress excessive inflammation and decrease scarring while stimulating de novo angiogenesis in the wound bed, all leading to promising outcomes in chronic wound repair *in vivo*.

FIRST PAPERWORK SECTION

Studies of MSCs reprogramming *in vitro* towards tenogenic and myogenic fate

Tendon injuries may result from an acute trauma (e.g. tendon laceration or rupture) or, more commonly, from overuse (chronic tendon injury) (Nirchls RP, 1990, Riley G, 2004). Abuse tendon injuries are a major cause of musculoskeletal morbidity and often compromise the return to the same level of activity. In humans, an estimated 30–50% of all sports lesions that affect professional and recreational athletes are tendon injuries (Kannus P and Natri A, 1997). In racing thoroughbreds, tendon and ligament injuries are the most common orthopedic injuries (Pinchbeck GL et al, 2004, Kasashima Y et al, 1999). In addition, a large number of event, dressage and show jumping horses (Singer ER et al, 2008) suffer from tendon injuries. Although it has been reported in early chronic tendinopathies that tendon structure may normalize in some cases (Cook JL and Purdam CR, 2009) in other cases, scar tissue may have important consequences for the individual in terms of reduced performance and a substantial risk of reinjure (Dowling BA et al, 2000). The primary need to restore tendon functionality has therefore encouraged the development of regenerative therapies. Hereby, several growth factor (GF) and cell-based therapies have been introduced for the treatment of tendon injuries, with the aim to accelerate the healing and to improve the quality of the repaired tissue (Richardson LE et al, 2007; Waselau M et al, 2008). Frequently, tendon injuries are associated with skeletal muscle lasions that can generate from a variety of events, including direct trauma such as muscle lacerations and contusions, indirect insults and also from degenerative diseases such as muscular dystrophies (Huard J et al. 2002; Jarvinen TA et al. 2005; Cossu G and Sampaolesi M, 2007). Currently, there is no cure for any of the muscular dystrophies, although improved understanding of the genetics of muscular dystrophies has led to important insights into the basic pathophysiological mechanisms (Townsend D et al, 2011).
Skeletal muscle can regenerate completely and spontaneously in response to minor injuries, such as strain. In contrast, after severe injuries, muscle healing is incomplete, often resulting in the formation of fibrotic tissue that impairs muscle function. Various strategies, including growth factors injections, transplantation of muscle stem cells in combination or not with biological scaffolds, anti-fibrotic therapies and mechanical stimulation, may become therapeutic alternatives to improve functional muscle recovery. Current therapeutic approaches have limited effectiveness and there is still no gold standard treatment (Laumonier T and Menetrey J, 2016). For this reason, researchers are aware of having to find new cures to obtain the *restitutio ad integrum* of tendon and muscle injuries, the common goal for humans and animals patients. In this section, new techniques are described to ameliorate the tendon and muscle regenerative capabilities.

Tenocytes are adult and specialized cells that demonstrated biomechanical and histologic regenerative properties in a full-size tendon defect when compared to

undifferentiated MSCs. Unfortunately, autologous tenocytes present a limited proliferative potential and ethic committees prohibit invasive collection of tendon tissue to obtain tenocytes (Pietschmann MF et al, 2013, Tan Q et al. 2012). Vice versa, scarce data are present in literature about the differentiation of MSCs into myoblasts (Dugan JM et al, 2014). As it is known that stem cells can be reprogrammed (Takahashi K et al., 2007; Yu J et al., 2007) to differentiate toward the tenogenic and myogenic fate, new techniques are tested during the three years of PhD project to obtain this goal.

In the first paper, it was demonstrated that equine MSCs isolated from peripheral blood can develop the tenogenic pathway using four specific growth factors such as TGFβ3 (transforming growth factor), EGF2 (epidermal growth factor), bFGF2 (fibroblast growth factor) and IGF-1 (insulin-like growth factor) in presence or without Low Level Laser Technology (LLLT). Expression levels of genes Early Growth Response Protein-1 (EGR1), Tenascin (TNC) and Decorin (DCN) were quantified with real time-PCR (rt-PCR) to demonstrate the tenogenic induction.
In the second paper, equine PB-MSCs may differentiate into skeletal muscle cells with conditioned medium as well as in co-culture with C2C12 without direct contact in presence of the construct TAT-MyoD.
The Tat proteins of human immunodeficiency viruses types 1 and 2 (HIV-1 and HIV-2) are powerful transcriptional activators of viral gene expression. HIV-1 Tat is a small polypeptide of 101 amino acids (Fittipaldi A and Giacca M, 2005) and it is essential for the transcription of viral genes and for viral replication. After infection of susceptible cells, the HIV-1 genome integrates into the host cell DNA (Greene WC and Peterlin BM, 2002). For these properties, TAT can be used as a cargo to introduce new cDNA or protein in the cells. In our study, Myf5, Myogenin and exogenous MyoD expression were evaluated by rt-PCR analysis to quantify the myogenic differentiation of MSCs. Therefore, the effective process was evaluated observing the localization of Myf5 and Myogenin by immunofluorescence.
To increase in the future the effectiveness and the speed of transfection of MSCs with cDNA, SAMNs (novel surface active maghemite nanoparticles) are proposed as vectors for eukaryotic cell transfection. These nanoparticles present peculiar colloidal properties, surface characteristics and, for their ability to covalently and reversibly bind biomolecules are perfect to join exogenous cDNA and to penetrate the lipid membrane cells (Venerando R, 2013).
In the third paper, equine PB-MSCs were transfected with nude SAMNs to study the delivery of nanoparticles into the cells. In a second time, MSCs were incubated with SAMNs-pDNA coding of GFP to test the new transfection method. Immunofluorescence reveled a consistent cytoplasmic green fluorescence light originated by GFP protein in ePB-MSCs treated with SAMN@pDNA, demonstrating the correct cell transfection.
Further studies will be necessary to develop this methodology for clinical purposes such as the induction of MSCs toward tenogenic and myogenic fate without the use of viral vector.

The following publications are focused on tenogenic and myogenic differentiation and SAMNs transfection:

1) **Tenogenic induction of equine mesenchymal stem cells by means of growth factors and low-level laser technology.**
   Gomiero C, Bertolutti G, Martinello T, Van Bruaene N, Broeckx SY, Patruno M, Spaas JH.
   Veterinary Research Communication 2016 Mar;40(1):39-48.

2) **TAT-MyoD fused proteins, together with C2C12 conditioned medium, are able to induce equine adult mesenchymal stem cells towards the myogenic fate.** Patruno M, **Gomiero C**, Sacchetto R, Topel O, Negro A, Martinello T. Journal of Veterinary Science.
   Submitted for pubblication.

3) **Intelligent colloidal nano-vector for mesenchymal stem cells transfection.**
   Draft paper in preparation (nor submitted yet).

Tenogenic induction of equine mesenchymal stem cells by means of growth factors and low-level laser technology

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ORIGINAL ARTICLE

Tenogenic induction of equine mesenchymal stem cells by means of growth factors and low-level laser technology

Chiara Gomiero[1] · Giulia Bertolutti[1] · Tiziana Martinello[1] · Nathalie Van Bruaene[2] · Sarah Y. Broeckx[2] · Marco Patruno[1] · Jan H. Spaas[2]

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Abstract Tendons regenerate poorly due to a dense extracellular matrix and low cellularity. Cellular therapies aim to improve tendon repair using mesenchymal stem cells and tenocytes; however, a current limitation is the low proliferative potential of tenocytes in cases of severe trauma. The purpose of this study was to develop a method useful in veterinary medicine to improve the differentiation of Peripheral Blood equine mesenchymal stem cells (PB-MSCs) into tenocytes. PB-MSCs were used to study the effects of the addition of some growth factors (GFs) as TGFβ3 (transforming growth factor), EGF2 (Epidermal growth factor), bFGF2 (Fibroblast growth factor) and IGF-1 (insulin-like growth factor) in presence or without Low Level Laser Technology (LLLT) on the mRNA expression levels of genes important in the tenogenic induction as Early Growth Response Protein-1 (EGR1), Tenascin (TNC) and Decorin (DCN). The singular addition of GFs did not show any influence on the mRNA expression of tenogenic genes whereas the specific combinations that arrested cell proliferation in favour of differentiation were the following: bFGF2 + TGFβ3 and bFGF2 + TGFβ3 + LLLT. Indeed, the supplement of bFGF2 and TGFβ3 significantly upregulated the expression of Early Growth Response Protein-1 and Decorin, while the use of LLLT induced a significant increase of Tenascin C levels. In conclusion, the present study might furnish significant suggestions for developing an efficient approach for tenocyte induction since the external administration of bFGF2 and TGFβ3, along with LLLT, influences the differentiation of PB-MSCs towards the tenogenic fate.

Keywords Growth factors · Low level laser technology · Mesenchymal stromal cells · Tenocytes · Tenogenic induction

Introduction

Tendons are made of dense connective tissue and present low cellularity and low vascularity. These properties confer characteristics such as stiffness and elasticity (Brehm et al. 2012; Smith et al. 2013; Patruno and Martinello 2014), which are essential in transmitting forces between muscles and bones (Freedman et al. 2014). However, the not-vascularized nature and low number of tenocytes, reduce the self-healing and regenerative potential of tendons (Jiang et al. 2014; Veronesi et al. 2015). In veterinary medicine, several therapies are used to improve tendon regeneration although long rehabilitation periods are needed and relapses are frequent (Riley 2008; Spaas et al. 2012). In order to augment tendon healing, innovative techniques are being used to treat tendon lesions, including tissue engineering and cell therapies (Delincé and Ghafil 2012) such as platelet-rich plasma (PRP), growth factors (GFs) (Maia et al. 2009), mesenchymal stem cells (MSCs) (Brehm et al. 2012) and low level laser irradiation (LLLI) (Sperandio et al. 2014; Iacopetti et al. 2015). Since it should exists a synergy action between GFs present in PRP and

Marco Patruno and Jan Spaas Contributed equally.

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✉ Marco Patruno
marco.pat@unipd.it

[1] Department of Comparative Biomedicine and Food Science, University of Padova, Viale dell'Università 16, Legnaro, 35020 Padova, Italy

[2] Global Stem Cell Technology-ANACURA Group, Noorwegenstraat 4, 9940 Evergem, Belgium

low-level laser technology (LLLT), the aim of this study was to verify their influence in reprogramming MSCs into tenocytes fate.

PRP is an important source of autologous GFs (Abate et al. 2012) such as PDGF (Platelet-Derived Growth Factor), VEGF (Vascular endothelial growth factor), thromboxane, fibronectin/vitronectin, TGFβ (transforming growth factor), EGF (Epidermal growth factor), FGF (Fibroblast growth factor) (Anitua et al. 2015), IGF (insulin-like growth factor) (Schär et al. 2015) and cell fractions that can accelerate wound healing, decrease the inflammatory reaction and promote regeneration of affected tissues (Dimauro et al. 2014). In horses autologous platelet concentrates (APCs) are recently intensively studied in order to find the most appropriate protocols for clinical purposes (Hessel et al. 2015). Moreover, it has been demonstrated that several GFs, such as EGF, bFGF, PDGF-BB and TGF-β1, influence tenogenic differentiation and extracellular matrix production (Goncalves et al. 2013).

MSCs can be isolated from several sources (Martinello et al. 2010; Martinello et al. 2011; Zhu et al. 2013, Toupadakis et al. 2010), are able to generate several types of mature and differentiated cells and to improve tendinopathy treatment (Smith et al. 2013). In equine medicine, bone marrow and peripheral blood are commonly used (Crovace et al. 2010; Martinello et al. 2013; Broeckx et al. 2014a, 2014b). MSCs isolated from horse peripheral blood (PB-MSCs) are able to differentiate into diverse mesenchymal lineages and could be used in cell-based therapies for the treatment of tendon, ligament, and bone pathologies as well as cartilage defects (Koerner et al. 2006; Giovannini et al. 2008; Martinello et al. 2010; Martinello et al. 2011; Spaas et al. 2013; Barberini et al. 2014).

LLLT is a therapy being used by veterinarians to reduce inflammation and pain and accelerate tissue healing (Hawkins and Abrahamse 2006). In vivo and in vitro studies have demonstrated the stimulatory capacity of low-level laser energy on cell populations obtained from different tissues (Posten et al. 2005). Chen et al. (2015) showed that LLLT is able to stimulate proliferation and collagen synthesis of tenocytes as well as keratinocyte proliferation. Pyo et al. (2013) demonstrated that LLLI increase cell proliferation and promote the expression of BMP-2, osteocalcin, and TGFβ1 hypothesizing an influence in bone regeneration through a stimulatory effect on osteoblasts favouring their growth and maturation.

Although these new treatments have shown some positive effects on tendon regeneration, researchers are starting to combine different strategies to improve tendon regenerative capabilities. Another recent approach for enhancing tendon regeneration is the tenogenic induction of MSCs before clinical use. Using this methodology, Pietschmann et al. (2013) showed that tenocytes demonstrated increased biomechanical and histologic regenerative properties in a full-size tendon defect when compared to non-induced MSCs. Unfortunately, autologous tenocytes have a limited proliferative potential and ethic committees from many countries prohibit invasive collection of tendon tissue to obtain tenocytes (Tan et al. 2012). Our hypothesis was to evaluate the synergic action of GFs, usually present in PRP preparations, and LLLT for inducing the differentiation of low immunogenic MSCs (Broeckx et al. 2014a, 2014b) towards the tenogenic fate; therefore, we have used different combinations of GFs, with and without LLLT, in order to check the gene expression of EGR1 (Early growth response protein 1), Tenascin C (TNC) and Decorin (DCN), all crucial genes involved in tenogenesis (Pajala et al. 2009; Tao et al. 2015; Dunkman et al. 2013).

Materials and methods

Isolation and culture of PB-MSCs

Twenty millilitres of blood were collected into sterile EDTA (Ethylenediaminetetraacetic acid) tubes from the external jugular vein of four adult mares between five and seven years of age. Animal Ethics Committee approval numbers and date are: EC_2012_001 (21–11-2012) and EC_2014_001, (17–02-2014) by Global Stem Cell Technology, GST, Belgium; EC_2014_020 (07–04-2014) by Faculty of Veterinary Medicine, Ghent University, Belgium.

Blood samples were transported to the GST-ANACURA laboratory at room temperature within 4 h of sampling. Blood samples were centrifuged at 1000 *g* for 20 min at room temperature (RT) and the buffy coat collected and diluted 1:2 with Phosphate Buffered Saline (PBS). The cell suspension was layered on Percoll gradient (density 1.080 g/mL; GE Healthcare) and centrifuged at 600 *g* for 15 min at RT. The interphase was collected, washed three times with PBS and centrifuged at 200 *g* for 10 min at RT (Spaas et al. 2013). The putative MSCs were seeded in a T75 flask (BD Falcon) with an expansion medium (Exp) made as follows: Dulbecco's Modified Eagle Medium (DMEM, Euroclone ECM0728L), 20 % fetal calf serum (FCS, (Euroclone ECS0180L), 1 % antibiotics/antimycotics (Euroclone ECB3001D) and 10^{-11} M dexamethasone (Sigma Aldrich). The medium was refreshed twice a week and cells cultured at 37 °C and 5 % CO_2. At 70 % of confluence, the cells were trypsinized with 0.25 % trypsin- ethylenediaminetetraacetic acid (Euroclone ECB3051D) passage 0 (P0) and further cultured for 10 additional passages (P1 → P10) with the same medium, but without dexamethasone (Sigma Aldrich). Cells were used at specific passages (P1-P10 for PDT experiment, P5 and P10 for Flow cytometry and P5 for GF and laser experiments) as described below.

Population doubling time (PDT)

PDT was calculated from P1 to P10 using the following formula: PDT = cell culture time (T)/cell doubling time (CDT). T was expressed as days from Ni to Nf, where Ni is the initial and Nf the final number of cells for each passage and CDT was obtained with the following formula: CDT = ln(Nf/Ni)/ln(2) (Hoynowski et al. 2007).

Flow cytometry

For quality control purposes, immunophenotypic characterization (Spaas et al. 2013) of all donor PB-MSCs was performed at P5 and P10. Briefly, PB-MSCs were tested for the stem cell Cluster of Differentiation (CD) markers CD29, CD44 and CD90 and for the adult blood cell (negative) markers CD45, major histocompatibility complex (MHC) type II and monocyte/macrophage marker by flow cytometry. The cells were prepared as described in Spaas et al. (2013). The following combinations of markers and their respective clones were assessed: CD29-APC (TS2/16, Biolegend), CD44-FITC (CVS18, AbD Serotec), CD90 (DH24A, VMRD) + IgG1-PE-Cy7 (Biolegend)/as positive subset; CD45-PeCy5.5 (F10-89-4, AbD Serotec), mono- & macrocyte marker-Alexa 488 (MAC387, AbD Serotec), + MHCII-PE (CVS20, AbD Serotec) as negative subset. Control isotypes were tested for each marker to define the threshold (the range for isotype controls was defined at 0–1 % of positive signals). The samples were analyzed on a FACSCanto II (BD Biosciences) instrument, equipped with two lasers (488 nm solid state and a 633 nm HeNe laser). The optimal settings for the MSC were determined by Compbeads Plus beads. Flow cytometer performance was monitored on a daily basis by Cytometer Setup & Tracking beads (BD Biosciences). The data were analyzed with FacsDiva software.

Addition of growth factors (GFs)

PB-MSCs obtained from four donor horses were seeded, at passage 5, each in T25 flasks (1500 cells/cm^2) to test the effects of the addition of some growth factors (GFs) on the mRNA expression levels of genes important in the tenogenic induction such as EGR1, TNC and DCN (see below the Real Time PCR method). The GFs added were the following: TGFβ3 (transforming growth factor), EGF2 (Epidermal growth factor), bFGF2 (Fibroblast growth factor) and IGF-1 (insulin-like growth factor). The latter GFs were tested singularly with and without LLLT (data not shown) in order to confirm previous observations (Schneider et al. 2011) that described no changes when GFs were tested alone. However, our preliminary results indicated that bFGF2 increased, although not significantly, the expression of the tested genes; consequently, and also because bFGF2 is essential in the tenogenic differentiation and wound repair, it has been decided to always use it in the following GFs combinations (see the panel below).

Combination	Growth factors					
1	Exp					
2	Exp					LLLT
3	Exp	bFGF2				
4	Exp	bFGF2				LLLT
5	Exp	bFGF2	TGFβ3			
6	Exp	bFGF2	TGFβ3			LLLT
7	Exp	bFGF2		IGF1		
8	Exp	bFGF2		IGF1		LLLT
9	Exp	bFGF2			EGF2	
10	Exp	bFGF2			EGF2	LLLT

The expansion medium (Exp) was therefore used alone (combination N.1) or with LLLT (combination N.2) or supplemented with: bFGF2 (combination N.3), bFGF2 and LLLT (combination N.4), bFGF2 and TGFβ3 (combination N.5), bFGF2, TGFβ3 and LLLT (combination N.6), bFGF2 and IGF1 (combination N.7), bFGF2, IGF1 and LLLT (combination N.8), bFGF2 and EGF2 (combination N.9), bFGF2, EGF2 and LLLT (combination N.10). All GFs are by Sigma-Aldrich and were added to the medium at 10 ng/ml. PB-MSCs were cultured in duplicate to evaluate the effect of the GFs treatment alone and in addition to LLLT. Cells were cultured in supplemented media for five days, with the medium refreshed at day three. Five days after seeding cells reached 70 % confluency and were trypsinized, counted and frozen at −80 °C with 90 % expansion medium with 10 % DMSO (Dimethyl sulfoxide) for subsequent Real Time polymerase chain reaction (Real time-PCR) analyses. These experiments were repeated four times.

Low level laser technology (LLLT)

PB-MSCs seeded at 1500 cells/cm2 in T25 flasks were conditioned with GFs, as previously described in the paragraph above, and submitted to LLLT (ASAlaser, M6 handpiece) equipped with combined, synchronized and overlapping continuous and pulsed emissions emitted by a single handpiece. The emission was produced by an InGa(Al)As diode laser with the following parameters: for continuous wave the wavelength was of 660 nm while for pulsed laser diode the wavelength was of 905 nm (peak optical power 25 W). A daily dose of 5 J/cm^2 for 2 min performed by the same operator. The first irradiation was performed 24 h after seeding to allow cell attachment and conditioning of GFs. Subsequently, LLLT was performed once a day up to the fifth day after seeding;

when cells reached 70 % of confluence they were trypsinized, counted and frozen.

Cell count

Cells were trypsinized and collected after each treatment with GFs and LLLT. The number were determined by Burker chamber counting and the cells were marked with Trypan Blue (Sigma B-7021) in order to calculate the average number of dead and live cells. This evaluation allowed discerning the effect of treatments between the arrest of proliferation and cell death.

RNA isolation and gene expression analysis

Total RNA extraction was performed using TRIzol (Life Technologies) reagent following the manufacturer's instructions. RNA was quantified on a Nanodrop (Thermo Scientific) spectrophotometer and a complementary single strand DNA (cDNA) was synthesized from 2 μg of purified RNA to perform real time-PCR using the ABI 7500 Real Time PCR system (Applied Biosystem). The relative expression of the following genes was used to evaluate tenogenic induction after five days of treatments: EGR1, DCN and TNC. Each sample was tested in triplicate and untreated PB-MSCs were used as a calibrator sample. The $2^{-\Delta\Delta ct}$ method was used to analyze and normalize the RNA expression of the target genes with respect to the endogenous housekeeping gene Glyceraldehyde- 3- phosphate dehydrogenase (GAPDH). PCR primers were designed using Primer Express 3.0 software (Applied Biosystems).

Statistical analysis

Normally distributed data were expressed as the mean ± standard deviation. Normality of the data was confirmed using the Kolmogorov–Smirnov test (α = 5 %). Statistical analyses were performed using the paired Student *t* test (SPSS software, version 11.0, SPSS, IBM). The level of statistical significance was set at $P \leq 0.05$ for all analyses.

Results

Flow cytometric analysis of isolated PB-MSCs

PB-MSCs were successfully isolated from peripheral blood of four healthy horses. The expression profile of cell-surface antigen in adherent PB-MSCs was evaluated by flow cytometry in cell samples at P5 (Fig. 1a) and P10 (Fig. 1b). Cells from the four horses were uniformly positive for adhesion markers CD29 (100 % ± 0 at P5 and P10), CD44 (92 % ± 4 at P5, 99.3 % ± 0.5 at P10) and CD90 (100 % ± 0 at P5 and P10). In addition, the analysis demonstrated less than 5 % positivity for the hematopoietic lineage marker as CD45 (1.7 % ± 0.6 at P5, 1.2 % ± 0.3 at P10), for the leukocytes markers Mo/Ma (0.8 % ± 0.3 at P5, 1.4 % ± 2 at P10) and for the MHCII (0 % ± 0 at P5 and P10). In Fig. 1c, the CD percentages of different markers used in the study are summarized.

Fig. 1 Detection of Cluster of Differentiation (CD) markers of peripheral blood cells using a flow cytometry assay. Peripheral Blood-MSCs (PB-MSCs) tested at P5 (**a**) and P10 (**b**) showed a positivity for antibodies against CD29, CD44 and CD90 (*in blue*) and a negativity for Mo/Ma, MHCII and CD45 (*in red*). The table shows the average expression ± standard deviation of CD positivity and negativity of PB-MSCs at P5 and P10. Control isotypes were tested for each marker to define the threshold

Population doubling time of isolated PB-MSCs

To examine PDT of PB-MSC samples (n = 4), the proliferation potential was measured according to the passage number (from P1 to P10). The PDT showed an initial lag time (supplemental figure), but PDT was relatively constant from P3 until P10.

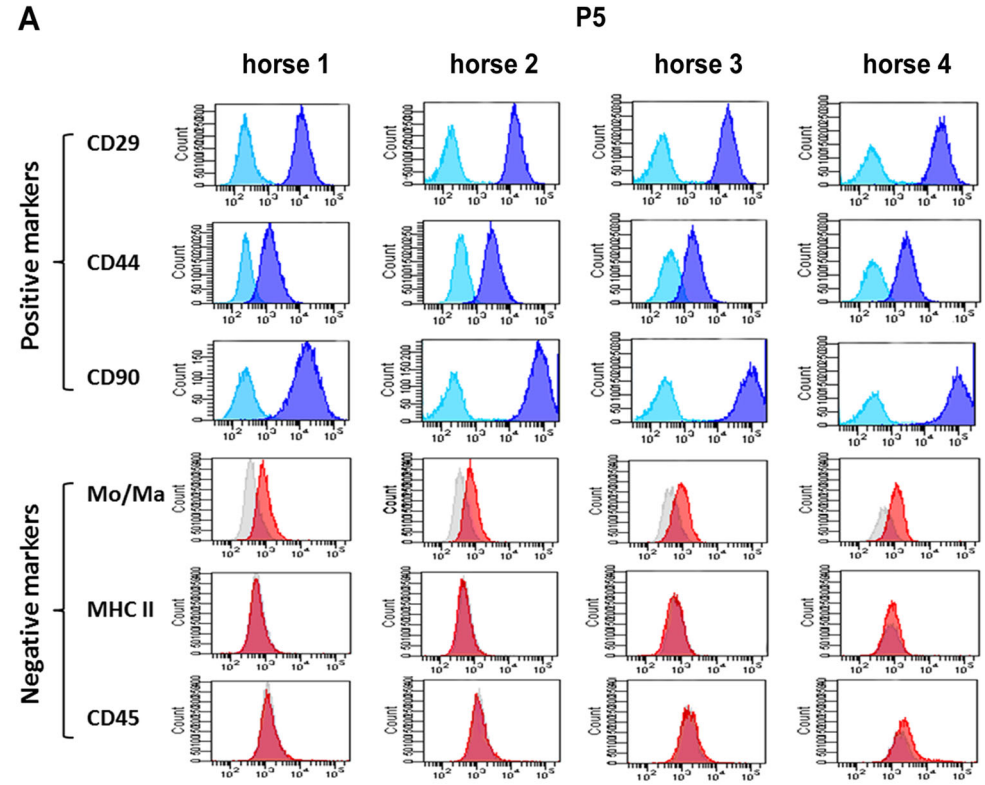
Cell proliferation of PB-MSCs treated with GFs and LLLT

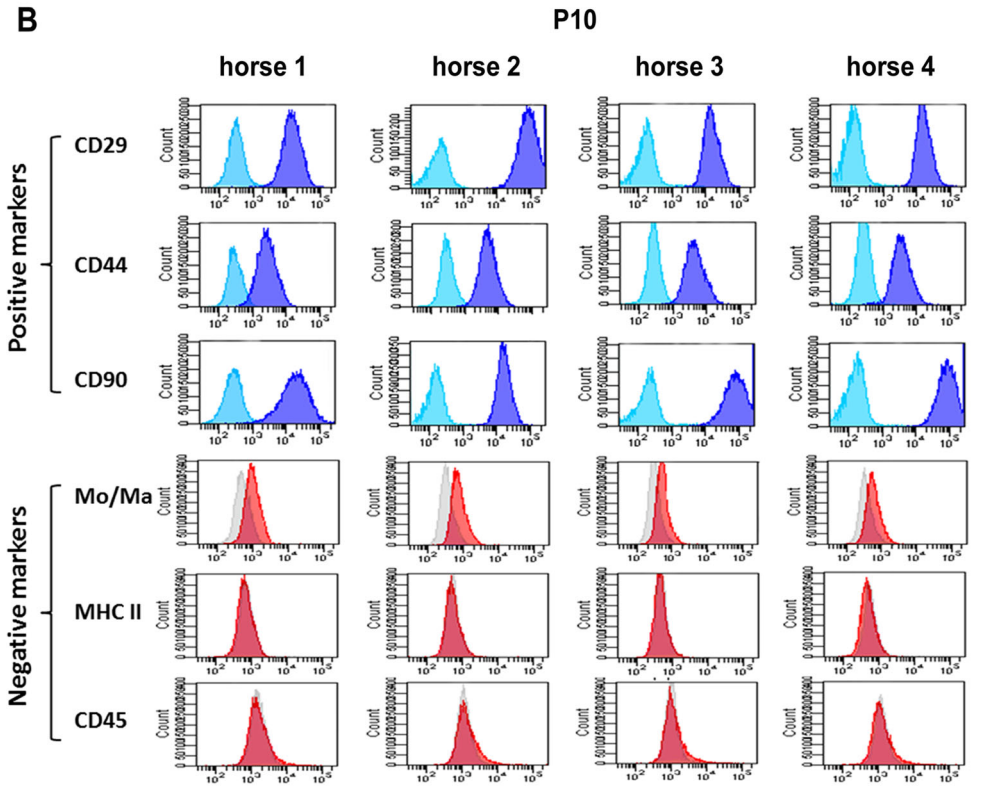
Figure 2 shows the concentration of dead cells (dark grey bars) and live cells (light grey bars) detected in cell culture after each treatment. All combination of GFs caused the same cytotoxicity (dead cells 31.5 ± 14.2, Fig. 2) and it was slightly greater than control medium and laser treatment. The combinations of bFGF2 + TGFβ3 and bFGF2 + TGFβ3 + LLLT, arrested cell proliferation compared to control cells and other GFs treatments (Fig. 2 light grey bars) without increasing the cell death. This data indicate that TGFβ3 influences the proliferation of MSCs, while EGF2 and IGF1 do not modify cell growth.

MRNA expression of EGR1, TNC and DCN

PB-MSCs were used to test the effects of the addition of some GFs (TGFβ3, IGF1, bFGF2 and EGF2) on the mRNA expression levels of genes involved in the tenogenic differentiation such as EGR1, TNC and DCN. TGFβ3, IGF1, bFGF2 and EGF2 were previously tested individually with and without LLLT (data not shown) without obtaining any significant results; therefore, we started to use combinations of GFs with and without LLLT (see panel in the material and methods).

Figure 3a and 3c indicate that EGR1 and DCN expression, respectively, increased significantly in the presence of bFGF2 and TGFβ3 with and without LLLT treatment (combination n. 5). The level of the TNC gene (Fig. 3b) appears to be influenced by treatment with bFGF2, TGFβ3 and LLLT (combination n. 6) since its expression increased significantly,





Average ± SD	CD44	CD90	CD29	Mo/Ma	MHCII	CD45
P5	92.0 ± 4.0	100.0 ± 0.0	100.0 ± 0.0	0.8 ± 0.3	0.0 ± 0.0	1.7 ± 0.6
P10	99.3 ± 0.5	100.0 ± 0.0	100.0 ± 0.0	1.4 ± 2.0	0.0 ± 0.0	1.2 ± 0.3

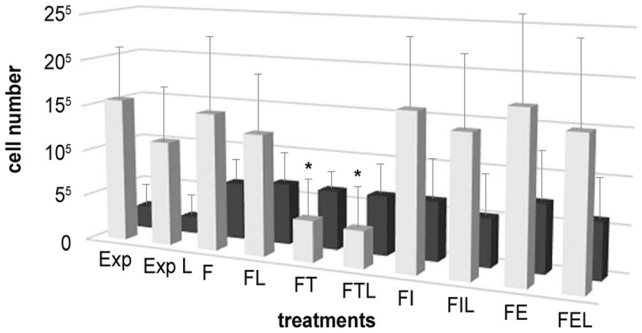


Fig. 2 The histogram shows the average ± SD of the cell number counted after different treatments. The number of alive cells is rapresented by light grey bars while the number of dead cells is indicated by dark grey bars. The treatments did not modify the cell mortality while influenced the proliferation activity. Asterisk indicates significant differences (*$P < 0.05$)

whereas, treatment with bFGF2 and TGFβ3 without LLLT showed a similar, but not-significant increasing trend. The factors bFGF2-EGF2 and bFGF2-IGF1 did not cause variations in EGR1, DCN and TNC mRNA levels in comparison to control PB-MSCs (Table 1).

Discussion

Novel treatments used for tendon regeneration include PRP, MSCs and LLLT. Unfortunately, these methods do not typically result in complete healing of injured tendons and a return to total function is very difficult to achieve (Liu et al. 2011). Obtaining in vitro functional tenocytes from MSCs is important for tissue healing and engineering applications (Bi et al. 2007; Tan et al. 2012). We have hypothesized that a specific mixture of GFs in combination with LLLT induced the differentiation of MSCs towards a tenogenic fate.

The MSCs used in this study were obtained from peripheral blood (PB) of healthy horses ($n = 4$) and the cells were subjected to flow cytometric analysis for characterization. In the present study it was chosen to use the allogeneic PB-MSCs between P5 and P10 due to the insufficient cell yield at earlier passages and in order to generate multiple doses and perform all necessary quality assessments (Broeckx et al. 2014c; Vandenbergh et al. 2016).

PB-derived MSCs showed a positive expression for adhesion and the MSC markers CD29, CD44 and CD90, both at passage P5 and P10. In contrast, PB-MSCs were not positive for the hematopoietic lineage marker CD45, the monocyte/macrophage (Mo/Ma) leukocyte markers and MHC II. The present results showed that PB-MSCs did not lose their stem markers and the cells maintained their proliferative capacity through P10 after an initial lag phase.

In experimental studies conducted in vivo, LLLT produced an anti-inflammatory effect by decreasing the number of fibroblasts and neovascularization in tendon lesions of sheep

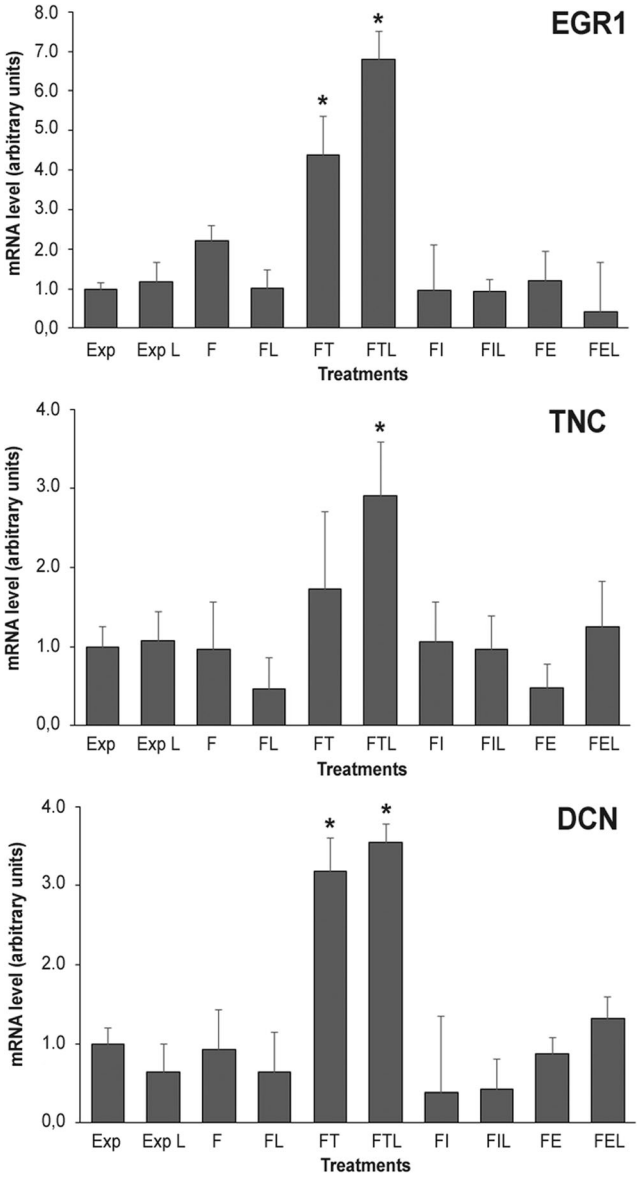


Fig. 3 mRNA expression of EGR1 (early growth response protein 1), TNC (tenascin c) and DCN (decorin) in PB-MSCs before treatment (Exp) and after different treatments (Exp L: laser treated cells; F: bFGF2; FL: bFGF2 + laser; FT: bFGF2 + TGFβ3; FTL: bFGF2 + TGFβ3 + laser; FI: bFGF2 + IGF1; FIL: bFGF2 + IGF + laser; FE: bFGF2 + EGF2; FEL: bFGF2 + EGF2 + laser) detected by Real-Time PCR. Each graph rappresents the average ± SD of cells isolated from four hoses and Exp sample was used as calibrator. Asterisk indicates significant differences (*$P < 0.05$)

Table 1 Primers used for the quantitative Real time- PCR analysis

Gene	5′ Forward primer 3′	5′ Reverse primer 3′
GAPDH	gcatcgtggagggactca	gccacatcttcccagagg
EGR1	cggacatgacaacaacctttc	cctttgccctttcctttagca
DCN	gagagctgcgtgtccatgag	agtgggttggtgccaagttc
TNC	catccaccatcatccaggagtt	tggcaaacacacggatgaa

GAPDH (glyceraldehyde- 3- phosphate dehydrogenase); EGR1 (early growth response protein 1); DCN (decorin); TNC (tenascin C)

included in the study (Iacopetti et al. 2015). In addition, LLLT used on tendinopathies in rats reduced the mRNA expression of pro-inflammatory mediators such as TNFa (tumor necrosis factors), IL1b and IL6 (interleukin), and TGFβ (Pires et al. 2011). It is important to precise here that in vivo studies include many variables and, therefore, the contribution of LLLT is very difficult to understand. In vitro Huertas et al. (2014) observed a correlation between the energy density applied and cell growth rate while Pyo et al. (2013) attributed an increase in osteoblastic BMP2 (bone morphogenetic protein) and TGFβ1 expression to LLLT. In the latter study, it is possible to comment that LLLT induces a general increase of the superfamily TGF-β (BMPs are a subfamily of TGF-β) because it probably stimulates cell proliferation in regenerative processes. Surely, diverse energy densities differently stimulate cellular metabolic processes (i.e. increasing the oxidation, as discussed by Hamblin and Demidova 2006) and in our in vitro experiments a density that should influence cellular proliferation/differentiation was used, as previously observed in myoblasts (Monici et al. 2013). Several studies have demonstrated the involvement of bFGF2 in regulating cell growth and differentiation (Hoffmann and Gross 2007). In this regard, Cai et al. (2013) indicated that bFGF2 is able to activate the MAPK pathway promoting the differentiation of MSCs into tendons. In fact, the expression of bFGF2 induced tendon matrix protein collagen type I, collagen type III and scleraxis expression. Furthermore, it was demonstrated that bFGF2, introduced with a viral vector, modulated the expression of genes for multiple GFs, increased the gene level of TGF-β1, CTGF and VEGF and down-regulated IGF1 during the tendon repair process (Tang et al. 2014). Overall, bFGF2 has been reported as the most potent growth stimulator among the various GFs (Takehara 2000) and it also plays a pivotal role in wound repair (Molloy et al. 2003). However, in the present study, bFGF2 alone was not sufficient to induce a significant increase of expression of EGR1, TNC and DNC. Therefore, we evaluated the synergic action of bFGF2 in combination with TGFβ3, IGF1 or EGF2 to promote an increase of those tenogenic genes.

TGFβ is a well-known cytokine that regulates various processes in tendon healing. Increased TGFβ levels are associated with tendon adhesion (Khan et al. 2000) as well as tendinosis (Fu et al. 2002). TGFβ is a major anti-proliferative and pro-differentiation signal for hematopoietic stem/progenitor cells (Zhao and Chen 2014). Decreased TGFβ expression reduces migration and proliferation of keratinocytes (Hameedaldeen et al. 2014) and the addition of TGFβ decreases the number of tendon sheath, epitenon and endotenon cells in culture (Klein et al. 2002). Our results confirm the anti-proliferative activity of TGFβ3 in PB-MSCs. In fact, this GF decreased significantly the cellular proliferation but it did not modify cell death respect other GF treatments. The activation of a mechanism of cellular differentiation of PB-MSCs towards the tenogenic fate is hypothesized. Moreover, our results are supported by Rider et al. (2008), which demonstrated that TGFβ3 inhibits endogenous bFGF activity for human adipose stem cells (hASCs) proliferation. In 3D culture this GF increased the expression of TNC, COL1 (collagen 1), COMP (cartilage oligomeric matrix protein) and Tenomodulin (Barsby et al. 2014).

For the first time in equine MSCs, the genes EGR1, TNC and DCN were evaluated with the exogenous addition of GFs.

TGFβ3, IGF1 and EGF2 were tested individually (data not shown) but, as described by Schneider et al. (2011), were not able to increase genes involved in the tenogenic pathway.

On the contrary, the combination of bFGF2 and TGFβ3 significantly upregulated the expression of EGR1 and DCN while the use of LLLT induced a significant increase in TNC levels. The EGR1 is one of the first transcription factors involved in the tenogenic cascade; it is associated with increased collagen formation during embryonic tenogenesis and induces Scleraxis (SCX) and Col1a1 genes (Lejard et al. 2011). TNC although rarely present in most adult tissues is upregulated in embryonic and developing tissues or in tissues experiencing a fast rate of growth and influences cell adhesion and migration (Goncalves et al. 2013). DCN is a proteoglycan that regulates tendon structure by stabilizing and aligning collagen fibrils (Zhang et al. 2006).

The combination of bFGF2-IGF1 and bFGF2-EGF2 did not alter the expression of EGR1, TNC and DCN in treated PB-MSC versus control cells. In this case, the addition of LLLT to GF treatments did not promote the stimulation of EGR1, DCN and TNC tendon markers. Moreover, the combination bFGF2-IGF1 and bFGF2-EGF2 induced a level of proliferation activity similar to untreated MSCs, although IGF1 and EGF2 are known for their proliferative and differentiating effects. EGF2 is a single-chain polypeptide that has the ability to enhance migration and cell proliferation of bone marrow derived MSCs (Tamama et al. 2006), and has been well documented for its mitogenic and chemotactic effects on fibroblasts (Brown et al. 1991). Regarding IGF1, it is known that it has a stimulating effect on collagen synthesis in tendon-ligament tissue since it stimulates fibroblast proliferation and synthesis of extracellular matrix protein (Durgam et al. 2012; Hansen et al. 2013). Most likely, in our study, the effect of IGF1 on tenogenic induction was not seen because the use of bFGF2 treatment might have down-regulated its expression (Tsubone et al. 2004).

Conclusions

The effects of GFs on cell cultures are conditioned by numerous variables, such as concentration and timing of incubation, combination of GFs, evaluated markers and cell type. However, we consider that this study might furnish significant

suggestions for developing an efficient approach for tenogenic induction since from our results, the external administration of bFGF2 and TGFβ3, along with LLLT, surely optimize and accelerate the differentiation of PB-MSCs.

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Compliance with ethical standards

Conflict of interest statement JS and SB are employers of GST and inventors of several patents owned by the company.

None of the other authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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Veterinary Research Communications

Tat-MyoD FUSED PROTEINS, TOGETHER WITH C2C12 CONDITIONED MEDIUM, ARE ABLE TO INDUCE EQUINE ADULT MESENCHIMAL STEM CELLS TOWARDS THE MYOGENIC FATE.

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Corresponding Author:	Marco Patruno, PH.D Faculty of Medicine Veterinary Legnaro, Padova ITALY	
Corresponding Author Secondary Information:		
Corresponding Author's Institution:	Faculty of Medicine Veterinary	
Corresponding Author's Secondary Institution:		
First Author:	Marco Patruno, PH.D	
First Author Secondary Information:		
Order of Authors:	Marco Patruno, PH.D	
	Chiara Gomiero	
	Roberta Sacchetto	
	Ohad Topel	
	Alessandro Negro	
	Tiziana Martinello	
Order of Authors Secondary Information:		
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Abstract:

The Tat protein is able to translocate through the plasma membrane and when it is fused with other peptides may act as a protein transduction system. This ability appears particularly interesting to induce tissue-specific differentiation when the Tat protein is associated to transcription factors. In the present work, the potential of the complex Tat-MyoD in inducing equine peripheral blood mesenchymal stem cells (PB-MSCs) towards the myogenic fate, was evaluated. Results showed that the internalization process of Tat-MyoD happens only in serum free conditions and that the nuclear localization of the fused complex is observed after 15 hours of incubation. However, the supplement of Tat-MyoD only was not sufficient to induce myogenesis and, therefore, in order to achieve the myogenic differentiation of PB-MSCs, conditioned medium was added. The latter was obtained coculturing PB-MSCs with C2C12 without direct contact. These results suggest that TAT- transduction of Tat-MyoD, when supported by conditioned medium, represents a useful methodology to induce myoblasts differentiation.

Tat-MyoD FUSED PROTEINS, TOGETHER WITH C2C12 CONDITIONED MEDIUM, ARE ABLE TO INDUCE EQUINE ADULT MESENCHIMAL STEM CELLS TOWARDS THE MYOGENIC FATE.

Marco Patruno [a*], Chiara Gomiero [a], Roberta Sacchetto [a], Ohad Topel [a], Alessandro Negro [b], Tiziana Martinello [a*].

[a] *Department of Comparative Biomedicine and Food Science, University of Padova, Italy*
[b] *VTH - Koret School of Veterinary Medicine, The Robert H. Smith Faculty of Agriculture, Food and Environment, The Hebrew University of Jerusalem, Israel*
[c] *Department of Biomedical Sciences University of Padova,, Italy*

*= Corresponding authors

Abstract

The Tat protein is able to translocate through the plasma membrane and when it is fused with other peptides may act as a protein transduction system. This ability appears particularly interesting to induce tissue-specific differentiation when the Tat protein is associated to transcription factors. In the present work, the potential of the complex Tat-MyoD in inducing equine peripheral blood mesenchymal stem cells (PB-MSCs) towards the myogenic fate, was evaluated. Results showed that the internalization process of Tat-MyoD happens only in serum free conditions and that the nuclear localization of the fused complex is observed after 15 hours of incubation. However, the supplement of Tat-MyoD only was not sufficient to induce myogenesis and, therefore, in order to achieve the myogenic differentiation of PB-MSCs, conditioned medium was added. The latter was obtained coculturing PB-MSCs with C2C12 without direct contact. These results suggest that TAT-transduction of Tat-MyoD, when supported by conditioned medium, represents a useful methodology to induce myoblasts differentiation.

KEYWORDS: Tat-MyoD, equine PB-MSCs, C2C12, coculture, myogenic induction.

1. Introduction

Adult skeletal muscle presents a low cellular turnover in the absence of disease or damages (Cheung et l 2013). On the contrary, during regenerative mechanisms the muscle tissue becomes very dynamic thanks to the involvement of satellite cells. The use of these cells for therapeutic purpose appears promising for treatment of diseases and injuries affecting skeletal muscle, including muscular dystrophy (Partridge 2003). Both skeletal muscle injuries and disorders are actually quite common among athletic animals such as horses (Freestone and Carlson, 1991; Lee et al., 2016). However, the self-renewal potential of adult satellite cells is per se limited, decreases with age, sarcopenia (Chen and Goldhamer 2003) and is depleted by wasting muscular dystrophies (Yusuf and Brand-Saberi 2012). Given the need to use an unlimited cell population, mesenchymal stem cell (MSCs) deserves a particular attention to offer an alternative therapeutic solution for muscle diseases (Mizuno 2010). MSCs can be isolated from various anatomical districts such as bone marrow, adipose tissue, amniotic fluid, peripheral blood (Kuznetsov et al. 2001; Kern et al. 2006; Koerner et al. 2006; Martinello et al. 2010; Martinello et al. 2011) and they share the ability to differentiate along several pathways (Chamberlain et al. 2007; Giovannini et al. 2008). Up to now, scarce data are present in literature about the differentiation of MSCs into myoblasts. *In vitro*, it has been shown that MSCs may differentiate into skeletal muscle cells with conditioned medium as well as in coculture with a fusion between MSCs and myoblasts (Dezawa et al. 2005; Sung et al. 2013; Dugan et al. 2014). Specific signaling molecules, such as dexamethasone together with insulin and EGF (epidermal growth factor) (Tehrani et al. 2014), are able to induce the differentiation into skeletal muscle. Furthermore, MSCs isolated from bone marrow and treated with FGF (Fibroblast Growth Factors), forskolin, PDGF (Platelet-Derived Growth Factor) and transfected with an NICD plasmid were able to express MyoD (Dezawa et al. 2005), although the frequency of spontaneous cell fusion was very low. Recently, Rabiee et al. demonstrated that the overexpression of FND5, using an inducible lentivirus system, increased the transcription level for cardiac progenitors in embryonic stem cells (Rabiee et al. 2014) and Sung et al. induced equine MyoD expression in equine adipose-derived mesenchymal stem cell using a MyoD lentiviral vector

(Sung et al. 2016). Moreover, embryonic stem cells were induced to differentiate also into smooth muscle cells if Olfm2 (olfactomedin 2) overexpression was promoted (Shi et al. 2014). In a coculture of stem cells from amniotic fluid and cardiac cells, the physical contact between the two types of cells seems to be necessary but not sufficient to induce the cardiogenic potential (Gao et al. 2014); this fact means that a specific microenvironment is required to induce the maturation of myogenic cells. Therefore, the innovative approach of protein transduction with Tat domain fused with various transcription factors (Lin and Kao 2015; Woo et al. 2015), including MyoD (Sung et al. 2013; Hidema et al. 2014), appears to be a valid technical approach. Even though some data indicate that Tat-MyoD induces myogenic differentiation in naturally predisposed cells only, like the C2C12 cell line (Noda et al. 2009) or the mouse muscle primary cells (Hidema et al. 2014) Sung et al. demonstrated that myogenic differentiation of human adipose-derived stem cells was reached usingTat-MyoD transduction when the cells were fused with C2C12 myoblasts (Sung et al. 2013). In the present study, we described that myogenic differentiation of equine peripheral blood mesenchymal stem cells (PB-MSCs) using the Tat-MyoD transduction can be achieved simply with a coculture C2C12 myoblasts. .

2. Materials and methods

2.1.Generation of Tat-MyoD fused proteins

The nucleotide sequence encoding human MyoD was amplified from a human cDNA library with the following oligonucleotides (CAGCTAGCATGTCCTTCGCCATGCTGCGTTCAG - TGCAAGCTTCTAACTTCGAATCGCCGTCTTTTC) and cloned in plasmid Tat-Prp (Vicario et al. 2014) between *NheI* and *HindIII* restriction site, in order to obtain plasmid pTat-MyoD. The plasmid pTAT-MyoD is able to coding for MyoD sequence fused to peptide containing the translocation of HIV-1 protein TAT with 6x Histidine tag at N-terminus.

(NH_2-MRGSHHHHHHGMARGYGRKKGRQRRR-).

The plasmid pTat-MyoD was trasformed in Escherichia Coli BL21 bacteria cells. The bacteria were grown at 37°C in Luria Broth (LB) medium containing ampicillin (100 µg/ml) to an OD600 of 600 nm. Protein expression was induced by adding IPTG (Isopropil-β-D-1-Thiogalactopyranoside) about 4 hours at 25°C. To collect the Tat-MyoD protein, bacteria were harvested and cell membrane was lysed by sonication under denaturing condition using 6 M guanidinium. The proteins were bound to the resin IMAC and then were eluted with 8 M urea and 300 mM imidazole (pH 6.3). The fractions containing the larger quantity of protein were purified using a gel filtration PD10 column (GE Healthcare) to eliminate urea and imidazole. The purified protein was quantified using a spectrophotometer and then an SDS-PAGE was made to verify the purity of Tat-MyoD (44 KDa). The final protein concentration obtained was 0,5 mg/ml.

2.2.Transduction of Tat-MyoD into peripheral blood derived-mesenchymal stem cells (PB-MSCs)

MSCs were isolated from equine peripheral blood (Martinello et al. 2010) and were cultured in GM (growth medium, DMEM Dulbecco's Modified Eagle's Medium, 10% fetal bovine serum FBS, and antibiotics 100 mg/ml streptomycin, 100 U/ml penicillin, Euroclone) at 37°C. In order to evaluate the internalization of Tat-MyoD, PB-MSCs (when reaching confluence) were incubated in the presence of 0,1µg/ml Tat-MyoD for 2, 6, 15, 24 and 48 hours in medium without serum. The time course analysis was repeated in quadruplicate.

2.3.Coculture of PB-MSCs and C2C12

PB-MSCs and C2C12 cells were cocultered independently by using transwell insert (BD Falcon) with a 1µm pore size of membrane to separate each cell type. PB-MSCs were plated at the bottom

of 6-well plates at concentration of 1,5x10^5 cells/well in GM and the day after the cells were treated with Tat-MyoD for 15h in medium without serum. Concurrently, C2C12 were seeded at density of 3x10^5 cells per insert in GM, when the cells reached 80% of confluence the medium was changed to DM (differentiation medium, DMEM, horse serum 2%, antibiotics 1%, Euroclone). After 3 days the inserts with C2C12 were transferred into the wells with PB-MSCs in DM. The coculture was maintained for 7 days in DM and the experiment was repeated in triplicate.

2.4.Immunostaining

To perform immunostaining experiments cells were washed with PBS and fixed in 4% paraformaldehyde for 10 min; after further washing they were permeabilized with 0,3% Triton X-100 for 5 min and blocked for 1h using 1% FBS. Anti-His tag antibody (1:100, Sigma) was employed to evaluate the internalization of Tat-MyoD. To evaluate the differentiation of cells, anti-MyoD (1:100, Santa Cruz), anti-Myf5 (1:100, Santa Cruz) and anti-Myogenin antibodies (1:500, Chemicon) were used. All antibodies were maintained overnight at 4°C. Fixed cells were washed with PBS followed by addition of anti-mouse or anti-rabbit Alexa 568 conjugated antibody (Molecular Probes) at a 1:500 (v/v) dilution. Finally, staining of nuclei was obtained with DAPI (Sigma). As controls, PB-MSCs treated with Tat-MyoD without coculture and PB-MSCs in coculture, but without Tat-MyoD treatment, were used.

3. Results

3.1.Purification of Tat-MyoD protein

Tat-MyoD was expressed in *E. Coli* Bl21 and purification was performed using a Ni-NTA column. Tat-MyoD purified to homogeneity shows and apparent molecular weight of 44 KDa on SDS-

PAGE and migrate on gel slower respect its theoretical molecular weight of 37905.1 Da (Fig. 1). This common behavior may be explained due to the high number of basic amino acids (17.2% respect to total amino acids).

3.2.Localization of Tat-MyoD into PB-MSCs

In order to evaluate the cellular pathway of Tat-MyoD protein construct, an immunofluorescence assay was chosen (Fig. 2). Using confocal microscopy, it was found that after 2 and 6 hours of PB-MSCs treatment with MyoD-Tat, the protein permeated cell membrane and was present in the cytoplasm; only after 15 hours of incubation, the construct was confined in the nucleus and this localization was persistent after 24 and 48 hours of treatment (Fig. 2). Experiments were performed in serum free medium since the latter inhibits this process (data not shown).

3.3.Myogenic differentiation of PB-MSCs

Myogenic differentiation was achieved using Tat-MyoD transduction and the inductive medium of the cellular line C2C12. To study the effect of our set up on myogenic marker expression in PB-MSCs, we performed an indirect coculture using transwell insert (Fig. 3B). The scheme of experiment is illustrated in Figure 3A. The effective differentiation was evaluated observing the localization of Myf5 and Myogenin by immunofluorescence (Fig. 4). Results indicated that to activate the myogenic pathway in mesenchymal stem cells it was necessary the co-action of MyoD transduction and the molecular signals present in the medium of C2C12. Figure 4 (A, B) shows Myf5 and Myogenin expression in PB-MSCs treated for 15 hours with Tat-MyoD in serum free medium and, subsequently grown for 7 days in coculture with C2C12 myotubes in differentiative medium. The myogenic differentiation of PB-MSCs was not achieved using, separately, Tat-MyoD

(Fig. 4 D, E) or the C2C12 conditioned medium (Fig. 4 G, H). Fig 4C and 4F show the internal localization of Tat-MyoD complex by means of His-Tag antibody and fig. 4I confirms the absence of myogenic differentiation with only C2C12 conditioned medium with the use of MyoD antibody.

4. Discussion

The equine model offers a unique opportunity to explore treatment strategies for musculoskeletal disorders under conditions similar to the pathophysiology of human patients. Current treatments are often restricted to the management of symptoms or replacement with inert materials; therefore, there is a need for alternative biological approaches. MSCs may differentiate into cell types relevant to amend musculoskeletal diseases (Gupta et al. 2007; Lee et al. 2011; Galli et al. 2014) and are able to secrete growth factors to promote a repairing environment. However, for cell therapy purposes is necessary that MSCs are able to participate in the formation of new muscle fibers, a critical process that has not been fully elucidated so far. In vitro, hASCs (Human adipose-derived stem cells) treated with 5-azacytidine and fibroblast growth factor-2 (FGF-2) stimulates the early muscle differentiation steps (Eom et al. 2011); more, the expression of MyoD using high efficient lentiviral transduction induces myogenic differentiation while adipogenic differentiation is inhibited (Goudenege et al. 2009). Moreover, using MyoD lentiviral vector Sung et al. induced the expression of MyoD but not of Myogenin, (Sung et al. 2016). However, these methods are not appropriated for clinical use due to their mutagenic potential. In the last decade, several groups have demonstrated that the Tat protein transduction domain (PTD) is a great transactivator of gene expression (Dietz and Bähr 2004; Fittipaldi and Giacca 2005); its short amino acid motif, highly enriched in basic amino acids, binds to the cell surface and internalize in a variety of different cell types. In the recent past, various cellular proteins were described to interact with Tat and mediate or control its transcriptional activity (Kashanchi et al. 1996; Benkirane et al. 1998; Marzio et al. 1998; Col et al. 2001). In the present study, the human MyoD protein was engineered with the Tat sequence in order

to evaluate a safe method for the induction of mesenchymal stem cells towards the myogenic differentiation. This approach was already proposed in cells that naturally follow the myogenic fate, as mouse myogenic primary cells (Noda et al. 2009) and C2C12 cell line (Hidema et al. 2012) but to our knowledge was never tried on PB-MSCs. Additionally, experiments from Sung et al. (2013) underlines the importance of the extracellular environment, as they were able to differentiated human adipose-derived stem cells into myogenic cells using a fusion with C2C12 cells.

We were successful in inducing myoblasts differentiation in PB-MSCs. Our experiment indicates that the development of myogenic phenotypes of mesenchymal stem cells by Tat-MyoD construct depends on time and culture conditions, highlighting the role of *in vitro* microenvironment in terms of secreted factors and cell contacts.

Indeed, an important observation raised from our experiments was the necessity to add Tat-MyoD in a cell culture with serum free medium. It has been demonstrated that short peptides (Green and Loewenstein 1988) rich in arginine (Suzuki et al. 2002) are rapidly internalized by cells, in a receptor-independent manner and without energy consumption. This does not happen for Tat basic domain when fused to protein cargos (Fittipaldi and Giacca 2005). It was suggested that the process of Tat internalization occurs through adsorptive endocytosis. Several investigators (Hakansson et al. 2001; Mann and Frankel 1991) state that Tat sequence binds homologue of heparin sulfate (HS) glycosaminoglycan (GAG), a major constituent of extracellular matrix, suggesting that the bound HS/Tat might be involved in the internalization process. In accordance with this hypothesis, our study suggests that the presence of heparin in serum competes with the bound of HS/Tat, decreasing the uptake progression. To stimulate myogenic differentiation, Tat-MyoD has to be localized in the nucleus. Our results demonstrated that after 2 and 6 hours the construct remained in the cytoplasm, probably in vesicle as hypothesized by (Noda et al. 2009). Only after 15 hrs of incubation, Tat-MyoD was localized in the nucleus where it persisted after 24 and 48 hrs. However, the activation of myogenic pathway by nuclear MyoD was not sufficient to induce cellular differentiation..

Likewise PB-MSCs cocultured with C2C12 grown in cell insert (prevent the cell direct contact but permits the interaction of culture medium) was not enough to induce the myogenic commitment.

To our knowledge, this is the first study that shows a myogenic differentiation in equine adult stem cells using the TAT-mediated protein transduction system; the advantage of our method consists in obtaining committed myogenic cells derived from an abundant cell source, as PB-MSCs, without the need of fusion with other cells. It is important to state that our model might easily be reproduced also in human mesenchymal stem cells too (Martinello et al, unpublished results) although further studies will be necessary to develop this methodology for clinical purposes.

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Conflict of Interest: None

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Figure Legends

Fig. 1. Purification of Tat-MyoD by Ni-NTA column. Lane 1, BL21 cell and Sumo-hyrudin. Lane 2, BL21 and pTat-MyoD before induction. Lane 3, BL21 and pTat-MyoD after induction with 0.5 mM IPTG. Lane 4, Purified Tat-MyoD after Ni-NTA column.

Fig. 2. Immunofluorescence analysis of PB-MSCs treated with Tat-MyoD for 2, 6, 15, 24 and 48 hours using the anti-His Tag antibody (red) and DAPI (blue). From 15 hours of incubation anti-His Tag and DAPI colocalized. Bottom right image shows PB-MSCs after 48 hours of Tat-MyoD incubation (PC = Phase contrast). Scale bars: 58µm

Fig. 3. (A) Scheme of coculture between PB-MSCs treated with Tat-MyoD and C2C12, GM indicates growth medium and DM differentiation medium. (B) Scheme of transwell insert used for the coculture.

Fig. 4. Myogenic differentiation of PB-MSCs. Immunofluorescence of PB-MSCs after the Tat-MyoD treatment and the contemporary coculture with differentiated C2C12 (A, B, C). Immunofluorescence of PB-MSCs after 7 days of Tat-MyoD treatment (D, E, F) and after 7 days of coculture with differentiated C2C12 (G, H, I). The images show the merge between nuclear DAPI

staining (blue) and anti-Myf5 (A, D, G), anti-Myogenin (B, E, H), anti-His Tag (C, F), and anti MyoD (I) antibodies (red staining). Scale bars: 58 µm.

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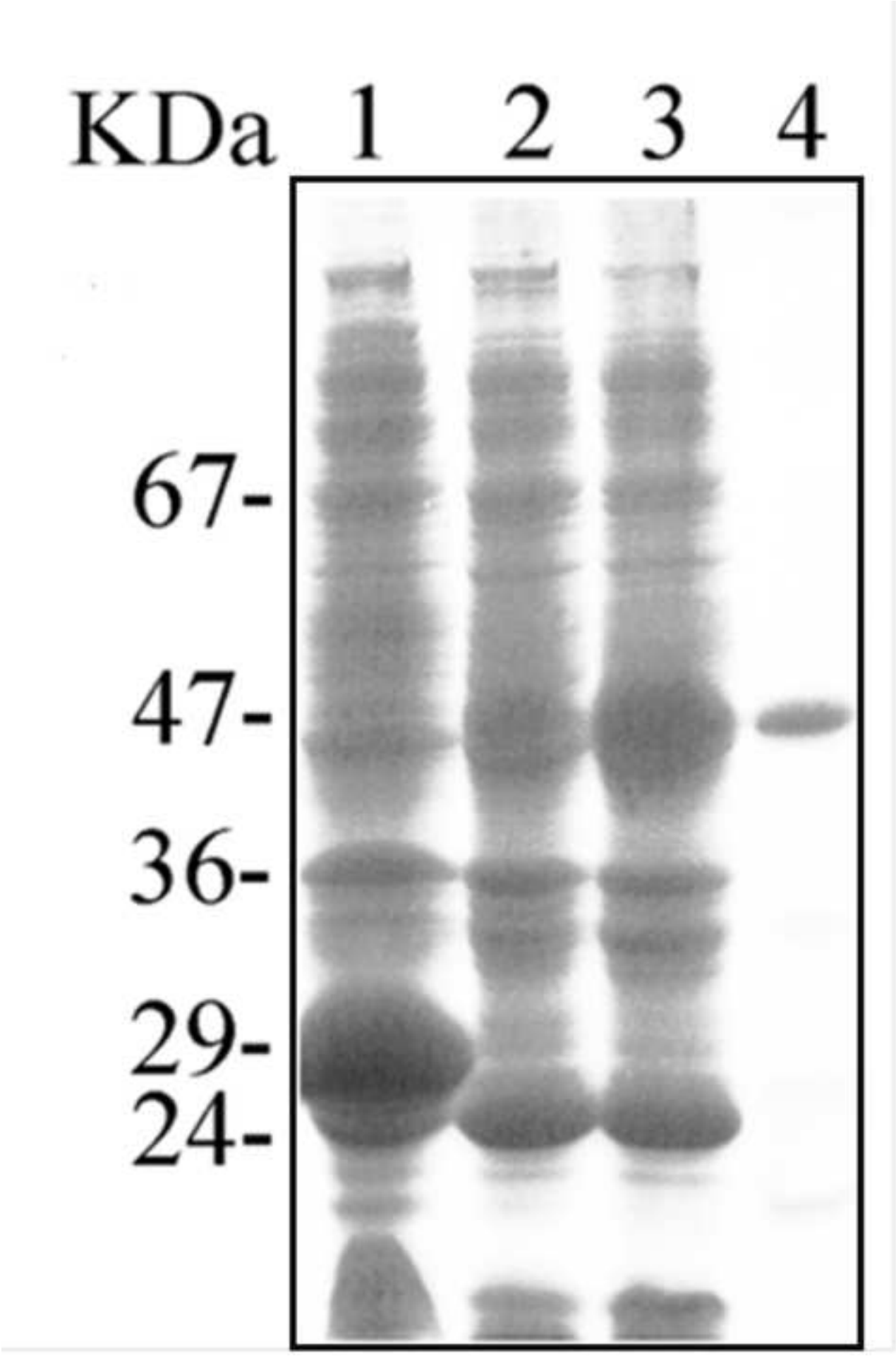

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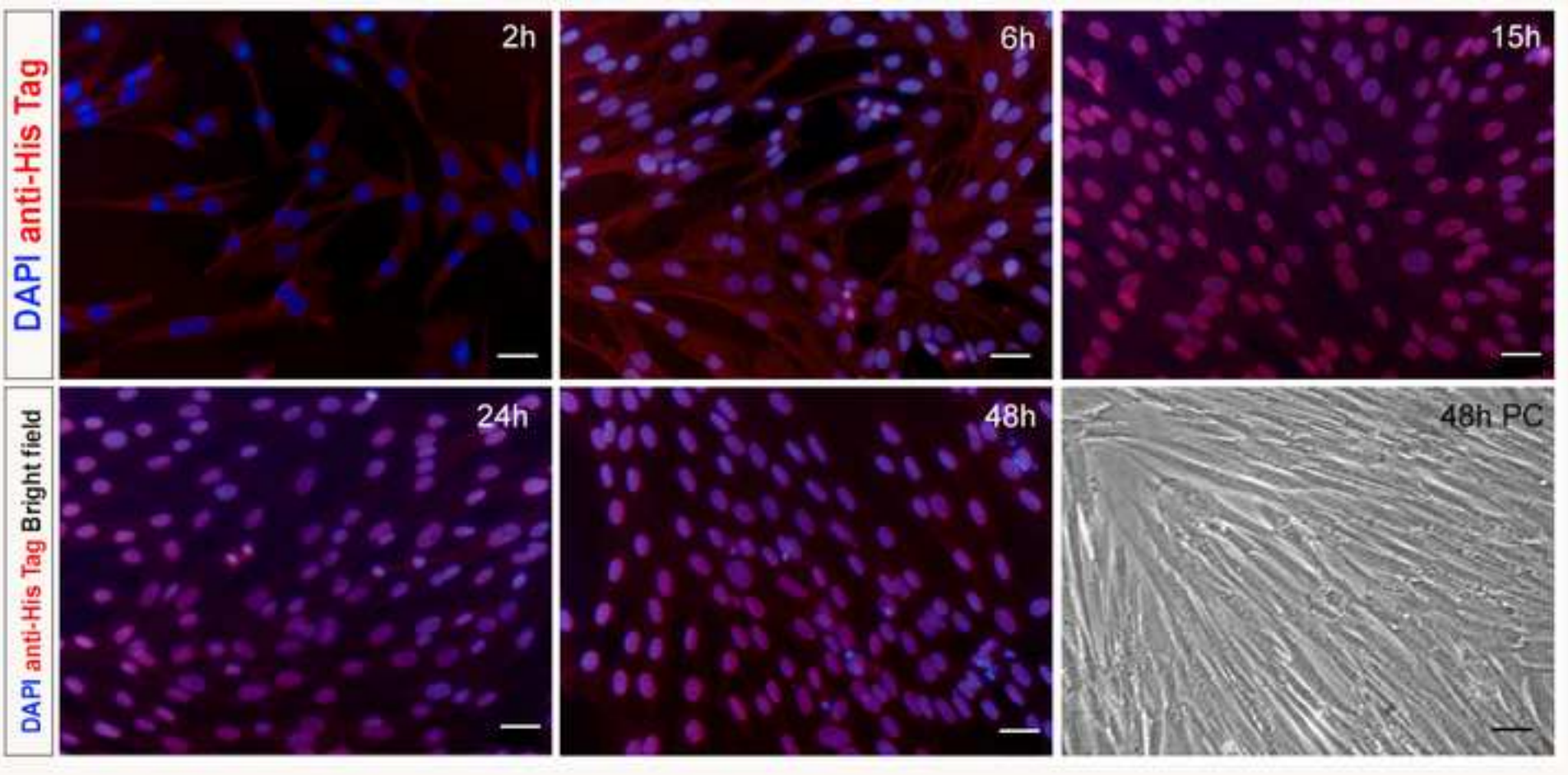

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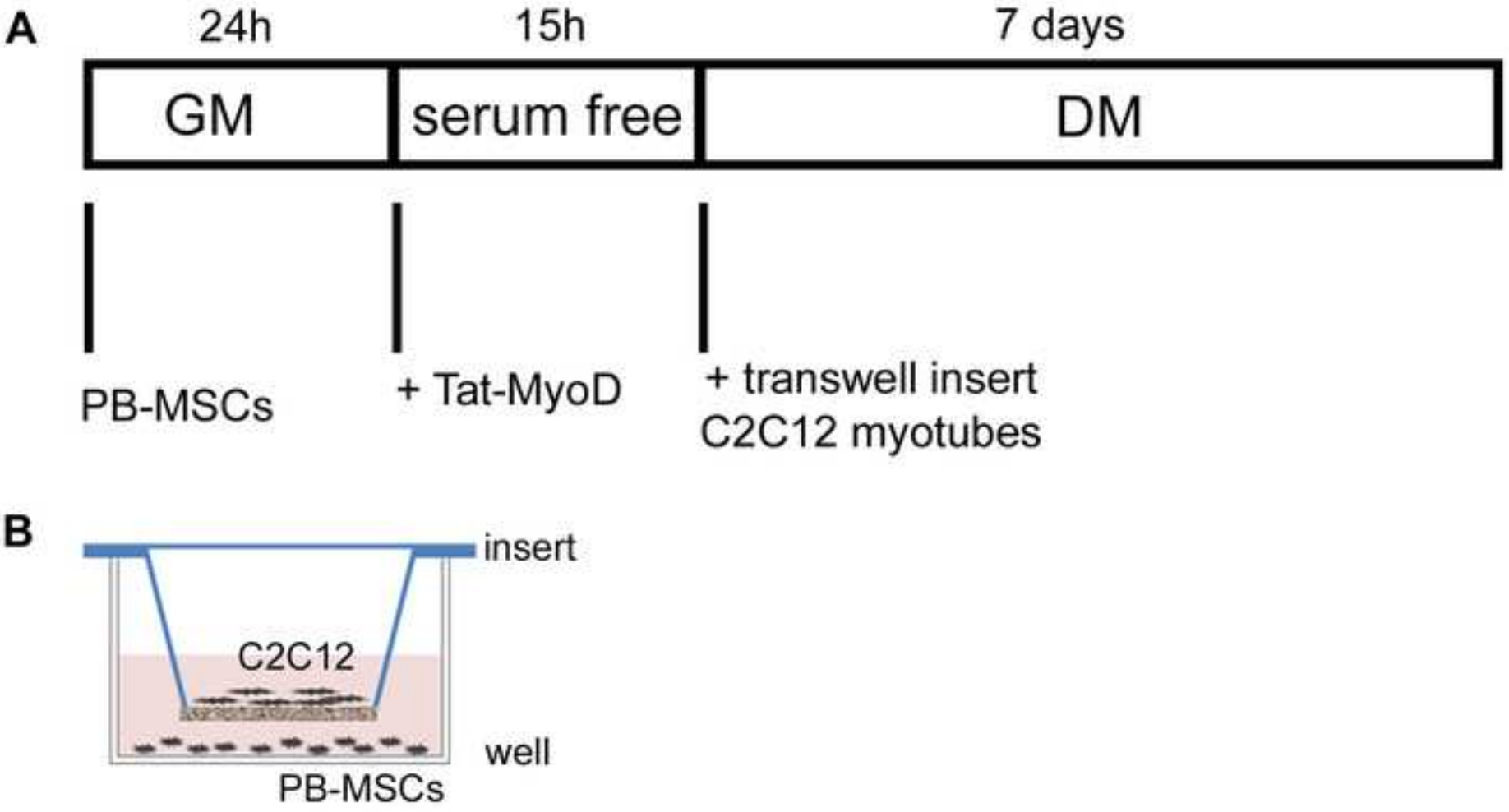

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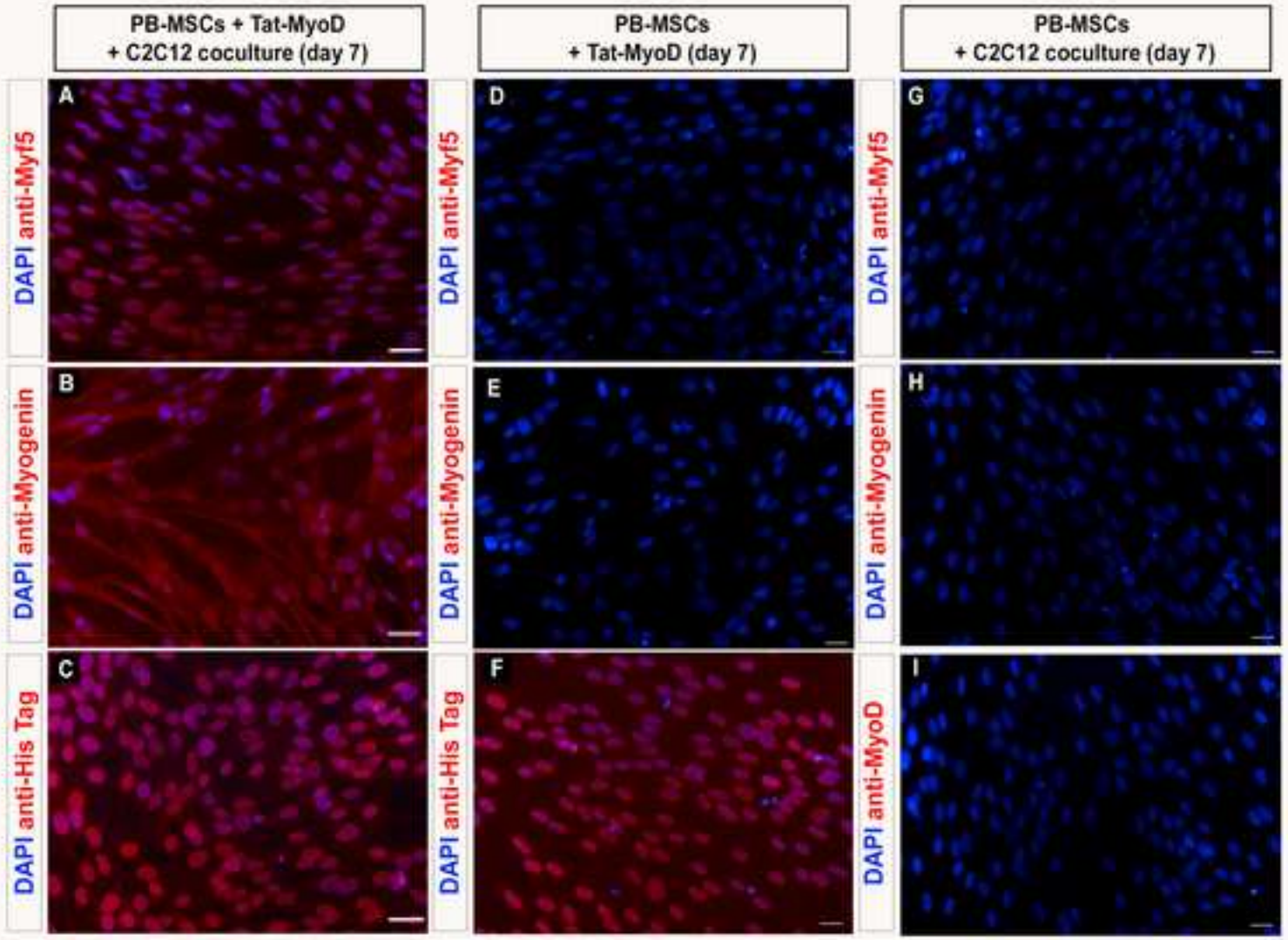

ABSTRACT

Intelligent colloidal nano-vector for mesenchymal stem cells transfection (Draft paper in preparation, nor submitted yet).

DNA does not possess the ability to independently translocate through the cellular and nuclear membranes; therefore, certain agents are employed to carry out this task. Novel surface active maghemite nanoparticles (SAMNs), standing out for their peculiar colloidal properties, surface characteristics and for their ability to covalently and reversibly bind biomolecules, are proposed as vectors for eukaryotic cell transfection. Due to their unique size-related properties and peculiar behavior, nanoparticles are said to bear great potential for scientific innovation. A magnetic drivable DNA nano-vector (SAMN@pDNA) was synthetized by self-assembly of SAMNs and plasmidic DNA (pDNA). SAMN@pDNA was characterized by TEM, light scattering, zeta-potential, Electron Paramagnetic Resonance, UV-Vis and x-ray photoelectron spectroscopy. Conversely, to coated iron oxide nanoparticles, complexing nucleic acids by electrostatic interactions, naked SAMNs, due to the covalent nature of the binding with DNA, lead to an extremely robust gene delivery tool. On the other hand, SAMN@pDNA showed a higher efficiency with respect to lipofectamine in the transfection of GFP coding gene in equine peripheral blood-derived mesenchymal stem cells, without the necessity of application of an external magnetic field. Thus, SAMNs are intelligent DNA nano vectors, able to match DNA protection, due to the unusual covalent nature of the interaction, with a pronounced transfection proclivity. This, along with the extensively demonstrated colloidal stability, excellent cell uptake, stability in being maintained in the host cells, low toxicity and great MRI contrast agent properties make of SAMN elective vector for a novel strategy in gene therapy. This work could provide a new, more effective, transfection method with numerous potential biomedical applications such as targeted drug delivery. SAMN based nano-bio-composites can be employed for the preparation of self-assembled opsonized nanoparticles as future candidates for biomedical applications.

Keywords: magnetic nanoparticles, transfection, mesenchymal stem cells, green fluorescent protein (GFP), rhodamine isothiocyanate (TRITC).

SECOND PAPERWORK SECTION

Studies of skin regeneration *in vivo*

Skin is a soft tissue that forms about 8% of the total body mass and covers the entire surface area. It is a self-repairing, self-renewing organ in the body that forms an important barrier from the outer to the inner environment (William PL et al., 1995). Therefore, damage to the skin leads to debilitating effects forming wounds that is an impairment of the anatomical structure and function of the skin (Atiyeh BS et al., 2002). Cutaneous wound healing is comprised of a network of biological processes, collectively restoring the integrity of the skin after injury. Unfortunately, the ideal outcome of cutaneous wound healing, which encompasses complete tissue regeneration, is often sacrificed in favor of quickly closing a wound with formation of fibrotic scar tissue (Van den Broek LJ et al., 2014). Fibrotic scar formation is an undesirable result of cutaneous wound healing, not only for cosmetic reasons but because scar tissue has compromised mechanical strength and is more sensitive to pain than healthy skin (Clark JA et al., 1996). Treating cutaneous skin wounds and reducing scar tissue cause a financial burden worldwide, and annual expenditures on products designed to minimize scarring exceed $5 billion (Jackson WM et al., 2012). For these reasons, different strategies are being used to generate skin component. Implantation of biodegradable scaffolds with or without cells (Sun G et al., 2011; Kawai K et al., 2000; Cornwell KG et al., 2009) such as Integra™ (Johnson & Johnson, New Brunswick, NJ) can regenerate dermal components. This scaffold presents a dermal component derived from bovine collagen, and chondroitin-6-sulphate that forms the dermal component and degrades slowly. Some disadvantages associated with the clinical use of dermal substitutes include slow vascularization, poor integration, and rejection. Furthermore, the dermal substitute Integra™ can be uneconomical and other disadvantages include poor handling properties, short shelf life, high manufacturing and distribution costs, and restriction to wounds of relatively low severity (Branski LK et al., 2007). These problems have been partially solved with the use of MSCs. The interest in the use of stem cells for potential wound healing applications is increasing for the treatment of deep burn wounds (Gohari S et al., 2002; Lattari V et al., 1997). MSCs actively contribute to regenerative processes, as they are involved in the inflammatory (Beckrich K and Aronovitch SA, 1999), proliferative (Boyce ST and Warden GD, 2002), and remodeling (Falanga V, 2004) phases of tissue regeneration and they are good therapeutic alternatives for regeneration and repair of damaged organs and tissues in various diseases (Mimeault M et al., 2007; Parker AM and Katz AJ, 2006). Although embryonic stem cells (Levenberg S et al., 2002) and induce pluripotent stem cells (iPSCs) (Park IH et al., 2008) are able to generate tissue cells of whole organism, these cells present various limitations: genetic manipulation of iPSCs, ethical consideration, and control of cellular differentiation of embryonic stem cells make them less attractive for translational approaches. Adult stem cells on the other hand do not pose any ethical issues and are also available in abundant supply.

To date, lot of research has focused on bone marrow-derived stem cells (BMSCs). However, the procurement of these cells is a painful procedure and usually leads to low yield and may lead to donor site morbidity. On the other hand, stem cells derived from adipose tissue are easy to obtain and provide a much higher yield (Musina R et al., 2005; Liu TM et al., 2007; Fraser JK et al., 2006). Due to their ease of collection and similar properties, ASCs (Hassan WU et al., 2014) are an attractive source and worthy of attention for clinical translation. Recently, the focus has shifted to regenerate the dermal component of the skin as well to overcome the lack of dermis in skin grafts (Compton CC et al., 1993; Burke JF et al., 1981). Actually, peripheral blood seems to be a good alternative source compared to bone marrow and adipose tissue. Frequent isolation success in rabbits, mice and guinea pigs (Kuznetsov et al. 2001), some difficulty was found in isolation in humans, dogs and horses (Roufosse et al. 2004; Koerner et al. 2006; da Silva et al. 2006; Giovannini et al. 2008). Equine peripheral blood represents an interesting source of MSCs because of the low invasivity, ease of harvesting, and low pain levels involved in the harvesting procedure. It is evident that MSCs can be obtained from peripheral blood, albeit in low numbers (Dhar M et al., 2012) and the differentiation of equine peripheral blood-derived MSCs into adipocytes, chondrocytes and osteocytes could be observed but only following induction with modified differentiation protocols or prolonged incubation time (Koerner et al. 2006; Giovannini et al. 2008).

In the works that follow, MSCs derived from peripheral blood have been used in attempt to regenerate sheep and equine skin lesions. In the first paper, wounds of 6 cm^2 were induced in the gluteus region of 6 horses and treated with (i) autologous epithelial stem cells (EpSCs), (ii) allogeneic EpSCs, (iii) vehicle treatment or (iv) untreated control. RT-PCR was performed on tissue biopsies collected after 1 and 5 weeks of treatment and IFN-y, IL-6, VEGF, EGF, IGF-1 and eKER were analyzed. Equine wounds treated with allogeneic EpSCs demonstrate a significant increase in mRNA expression of IL-6, VEGF and IGF-1 in the acute phase. In the longer term, an increase in IFN-y, VEGF and eKER mRNA was detected in the wounds treated with allogenic EpSCs, autologous EpSCs or their vehicle.
This study showed that there are no differences in cellular immune response between autologous and allogeneic EpSC-treated wounds and the wounds heal faster in presence of the two different types of cells.
In the second study, sheep allogeneic PB-MSCs were utilized to treat experimental lesions on the back of 6 sheep. Two biopsies were collected after 15 and 42 days of treatments and used to performed clinical and molecular analysis and histological and immunohistochemically staining.
Clinical analysis allowed us to analyze different parameters such as the healing time, the presence, the color and the nature of exudate, the aspect of gauze, the hydration of the wound, the percentage of re-epithelization and contraction of the lesions. Clinical evaluation showed that the healing time of the cell PB-MSCs treated group (30,05 ± 1,7 days) was faster than placebo (31,80 ± 1,9 days) and that the closure of the wound is better than the group control. Molecular analysis was performed to study the expression level of genes Collagen 1α1 (Col1α1) and Keratin

of hair (hKER). Sheep PB-MSCs are able to stimulate the Col1α1 gene expression after 15 days of treatment raising further after 42 days. The expression level of gene hKER increases significantly only in PB-MSCs-treated lesions after 42 days of trail. Dermal and subcutaneous inflammation, immature and undifferentiated mesenchymal tissue (mature granulation tissue) and skin adnexa were evaluated using histological analysis: PB-MSCs-treated wounds after 42 days have better healing than control group, in fact, dermal and subcutaneous inflammation were absent, undifferentiated mesenchymal tissue was present and skin adnexa were perfectly regenerated. Lastly, MHCII, vWF and KI67 were evaluated with immunohistochemical staining: PB-MSCs-treated wounds showed a protein expression raising of the major histocompatibility complex II (MHCII), neovascularization and cellular proliferation respect PBS-treated lesions.
This project is part of a large scheme where conventional treatments (Manuka Honey, Connettivina and Acemannane) were compared to innovative cures (MSCs and gas-ionized plasma). In this thesis, only the article about skin regeneration with PB-MSCs was reported.

It follows the paperwork focused on skin regeneration using equine and ovine PB-MSCs.

1) **Wound-healing markers after autologous and allogeneic epithelial-like stem cell treatment.**
   Spaas JH, **Gomiero C**, Broeckx SY, Van Hecke L, Maccatrozzo L, Martens A, Martinello T, Patruno M.
   Cytotherapy. 2016 Apr;18(4):562-9.

2) **Mesenchymal stem cells improve the wound healing process of mammalian skin.**
   Draft paper in preparation (nor submitted yet).

Cytotherapy, 2016; 18: 562–569

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Wound-healing markers after autologous and allogeneic epithelial-like stem cell treatment

JAN H. SPAAS[1,2], CHIARA GOMIERO[3], SARAH Y. BROECKX[1,2], LORE VAN HECKE[4], LISA MACCATROZZO[3], ANN MARTENS[4], TIZIANA MARTINELLO[3] & MARCO PATRUNO[3]

[1]Global Stem cell Technology, ANACURA group, Evergem, Belgium, [2]Pell Cell Medicals, ANACURA group, Evergem, Belgium, [3]Department of Comparative Biomedicine and Food Science, University of Padova, Italy, and [4]Department of Surgery and Anaesthesia of Domestic Animals, Faculty of Veterinary Medicine, Ghent University, Merelbeke, Belgium

Abstract

Background aims. Several cytokines and growth factors play an essential role in skin regeneration and epithelial-like stem cells (EpSCs) have beneficial effects on wound healing in horses. However, there are no reports available on the expression of these growth factors and cytokines after EpSC therapy. *Methods.* Wounds of 6 cm^2 were induced in the gluteus region of 6 horses and treated with (i) autologous EpSCs, (ii) allogeneic EpSCs, (iii) vehicle treatment or (iv) untreated control. Real time polymerase chain reaction was performed on tissue biopsies taken 1 and 5 weeks after these treatments to evaluate mRNA expression of *interferon (IFN)-γ*, *interleukin (IL)-6*, *vascular endothelial growth factor* (*VEGF*), *epidermal growth factor* (*EGF*), *insulin-like growth factor (IGF)-1* and *epidermal keratin* (*eKER*). *Results.* One week after treatments, mRNA levels of *IL-6* ($P = 0.012$) and *VEGF* ($P = 0.008$) were higher in allogeneic EpSC-treated wounds compared with controls. Also, mRNA levels of *IGF-1* were higher at 1 week in both autologous ($P = 0.027$) and allogeneic ($P = 0.035$) EpSC-treated wounds. At week 5, all EpSC- and vehicle-treated wounds demonstrated significantly higher *IFN-γ*, *VEGF* and *eKER* mRNA expression compared with controls and compared with their respective levels at week 1. *Conclusions.* Equine wounds treated with allogeneic EpSCs demonstrate a significant increase in mRNA expression of *IL-6*, *VEGF* and *IGF-1* in the acute phase. In the longer term, an increase in *IFN-γ*, *VEGF* and *eKER* mRNA was detected in the wounds treated with allogenic EpSCs, autologous EpSCs or their vehicle.

Key Words: *allogeneic, autologous, cytokines, growth factors, horse, skin, stem cells*

Introduction

The skin is the largest organ of the mammalian body, and its healing and regeneration has been extensively studied [1]. Currently, scientists mainly focus on enhancing skin wound repair because wounds might result in severe dysfunction and can be life threatening when they are chronic or involve an extended skin surface [2]. In general, wound healing consists of a dynamic process driven by cell proliferation and differentiation and is mediated by different types of growth factors, cytokines and chemokines [3,4].

It has been reported in horses that autologous as well as allogeneic epithelial-like stem cells (EpSCs) improve different wound-healing parameters, resulting in significantly enhanced wound repair [5,6]. The authors described a significant increase in early cellular immune response and vascularization to result in reduced tissue granulation and earlier wound closure. This is reinforced by other studies, which have reported that an earlier inflammatory peak in wounds of ponies leads to enhanced wound contraction and epithelialization compared with wounds in horses [7–9]. Additionally, it has been described that keratinocytes would also be able to induce a cellular immune response after exposure to pro-inflammatory cytokines, such as interferon (IFN)-γ [10,11] which results in enhanced wound healing as well [12].

Besides inflammatory parameters, growth factors also play a pivotal role during the wound-healing process. Vascular endothelial growth factor (VEGF), for example, promotes skin wound angiogenesis by exerting a paracrine effect on endothelial cells [13], and epidermal growth factor (EGF) influences epithelial

Correspondence: **Jan H. Spaas**, PhD, DVM, Global Stem cell Technology, ANACURA group, Evergem, Belgium. E-mail: Jan.spaas@anacura.com; **Marco Patruno**, PhD, Department of Comparative Biomedicine and Food Science, University of Padova, Italy. E-mail: marco.pat@unipd.it

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cell proliferation and migration and enhances angiogenesis [14,15]. Another important growth factor is insulin-like growth factor (IGF), which promotes keratinocyte migration [16], and research has shown that wounds with lower IGF levels display less healing capacity [17]. Additionally, epidermal keratin (eKER) is an interesting structural parameter to investigate because this fibrous protein can be found in wool, hair, nails, mammalian claws, equine and bovine hooves and horns [18]. Indeed, these intermediate filaments are abundantly present in stratified epithelia, particularly in the suprabasal layers of the epidermis, and may therefore be considered an epidermal reconstitution marker [19]. The present study is the first to investigate the aforementioned growth factors, cytokines and keratin mRNA expression levels at two time points in an *in vivo* experimental wound-healing study where the following treatments are evaluated: (i) autologous EpSCs, (ii) allogeneic EpSCs, (iii) vehicle treatment and (iv) untreated control wounds.

Methods

Skin sampling for EpSC isolation and wound induction

Six French trotter mares between 5 and 7 years of age were included in this study. A 1-cm^2 skin sample was retrieved from the neck region of these horses for EpSC isolation and characterization as previously described [20]. Twelve weeks after EpSC harvesting, 12×6 cm^2 wounds were created in the gluteus region of all horses to allow the evaluation of diverse treatments (three wounds per treatment group) at different time points, as reported by our group [5]. Before skin harvesting, horses were sedated with detomidine (0.04 mg/kg intravenous [IV]; Medesedan), and analgesia was achieved using butorphanol (0.1 mg/kg IV; Dolorex). Procaine 4% plus adrenalin was used for local subcutaneous anesthesia. Samples for real-time polymerase chain reaction (rt-PCR) analyses were taken at 1 and 5 weeks after treatment by means of a 3-mm punch biopsy after aforementioned sedation and analgesic drug administration and with a subcutaneous anesthesia consisting of procaine 4% without adrenaline. The experimental procedure was approved by the ethics committee of Global Stem Cell Technology (EC_2012_002, EC_2013_003 and EC_2014_001) and the Faculty of Veterinary Medicine, Ghent University (EC_2014_020).

Different treatment groups

Four treatment groups were considered: (i) autologous EpSCs, (ii) allogeneic EpSCs from two randomly chosen donors within the same group of horses, (iii) Dulbecco's Modified Eagle's Medium (DMEM) as a vehicle control and (iv) untreated controls. Twenty minutes after wound induction, 4×10^6 cells in 2 mL DMEM were injected subcutaneously in the wound margins and 4×10^6 cells in 1 mL DMEM were applied topically for the autologous and allogeneic treatment. Two of the six horses received half the doses in both the autologous and allogeneic treated group because the obtained number of cells for autologous treatment in these horses was insufficient. In the vehicle control wounds, 2 mL DMEM was injected subcutaneously, and 1 mL DMEM was applied topically. The remaining group of control wounds was left untreated to monitor the normal healing process. The horses did not receive any other medication.

RNA isolation and gene expression analysis

Total RNA extraction was performed using Trizol (Life Technologies) reagent following the manufacturer's instructions. RNA was quantified on a Nanodrop (Thermo Scientific) spectrophotometer and a complementary single strand DNA (cDNA) was synthesized from 2 µg of purified RNA to perform rt-PCR using the ABI 7500 Real Time PCR system (Applied Biosystems). The relative expression of the following genes was used to evaluate pro-inflammatory cytokines *IFN-γ* and *IL-6*; growth factors *VEGF*, *EGF* and *IGF-1*; and epidermis reconstitution marker *eKER*.

Each sample was tested in triplicate, and untreated skin was used as a calibrator sample. Real-time conditions were 2 min at 50°C, 10 min at 95°C, 40 cycles of denaturation at 95°C for 15 sec and annealing/extension at 60°C for 1 min. Wells contained 30 µL of PCR mixture (SYBR Green PCR Master Mix, Applied Biosystems), including 3 µL of cDNA at a dilution of 1:10. The 2-ΔΔct method was used to analyze and normalize the RNA expression of the target genes with respect to the endogenous housekeeping genes *GAPDH* (glyceraldehyde-3- phosphate dehydrogenase) and *18S*. The value Δct was calculated as the difference between the average Ct for each target gene and the *GAPDH*/*18S* genes. The value ΔΔct was obtained as the difference between the average ΔCT for each treatment and the calibrator sample. PCR primers were designed using Primer Express 3.0 software (Applied Biosystems). All primer sequences used in the present study are listed in Table I. Wherever possible, primers were designed to span introns in the genomic DNA to minimize non-specific fluorescence signals due to contaminating genomic DNA.

Statistical analysis

Normally distributed data were expressed as the mean ± SEM. Normality of the data was confirmed using the Kolmogorov-Smirnov test (a = 5%). Statistical analyses were performed using the paired

Table I. Sequences of primers related to genes INF-γ, IL-6, VEGF, EGF, IGF-1, eKER, *GAPDH* and *S18* used for the rt-PCR analysis.

Gene	Forward Sequence	Reverse Sequence
INF-γ	AGGCCTAACTCTCTCCGAAACA	CGCGGCCTGGCAGTAATA
IL-6	CCCCTGACCCAACTGCAA	GGCTGAACTGCAGGAAATCC
VEGF	ACCCCGATGAGATCGAGTACA	GCAGTGGGCACGCACTCTA
EGF	GGATGCATTGTCTAGACTCGACTGT	CCGTCATAGGACTGGGAACATT
IGF-1	GCACATCATGTCCTCCTCACA	CTCAGCCCCGCAGAGTGT
eKER	GGGCGTGGACCCAGAGAT	GAACCGCACCTTGTCAATGA
GAPDH	GCATCGTGGAGGGACTCA	GCCACATCTTCCCAGAGG
S18	AAACGGCTACCACATCCAAG	TCCTGTATTGTTATTTTTCGTCAC

Student's *t*-test comparing data within each time point with the untreated controls at week 1 or week 5 (Figures 1A,B and 2); when the expression levels were compared between week 1 and week 5, the untreated sample at week 5 was used as calibrator (Figures 1C,D and 3) (SPSS software, version 11.0, SPSS Inc.). The level of statistical significance was set at $P < 0.05$ for all analyses.

Results

Isolation and characterization of equine skin-derived EpSCs

Isolated and purified cells were characterized as EpSCs based on sphere-forming assays, multilineage differentiation capacities and immunophenotypic properties as previously described [6,20]. For safety reasons, one

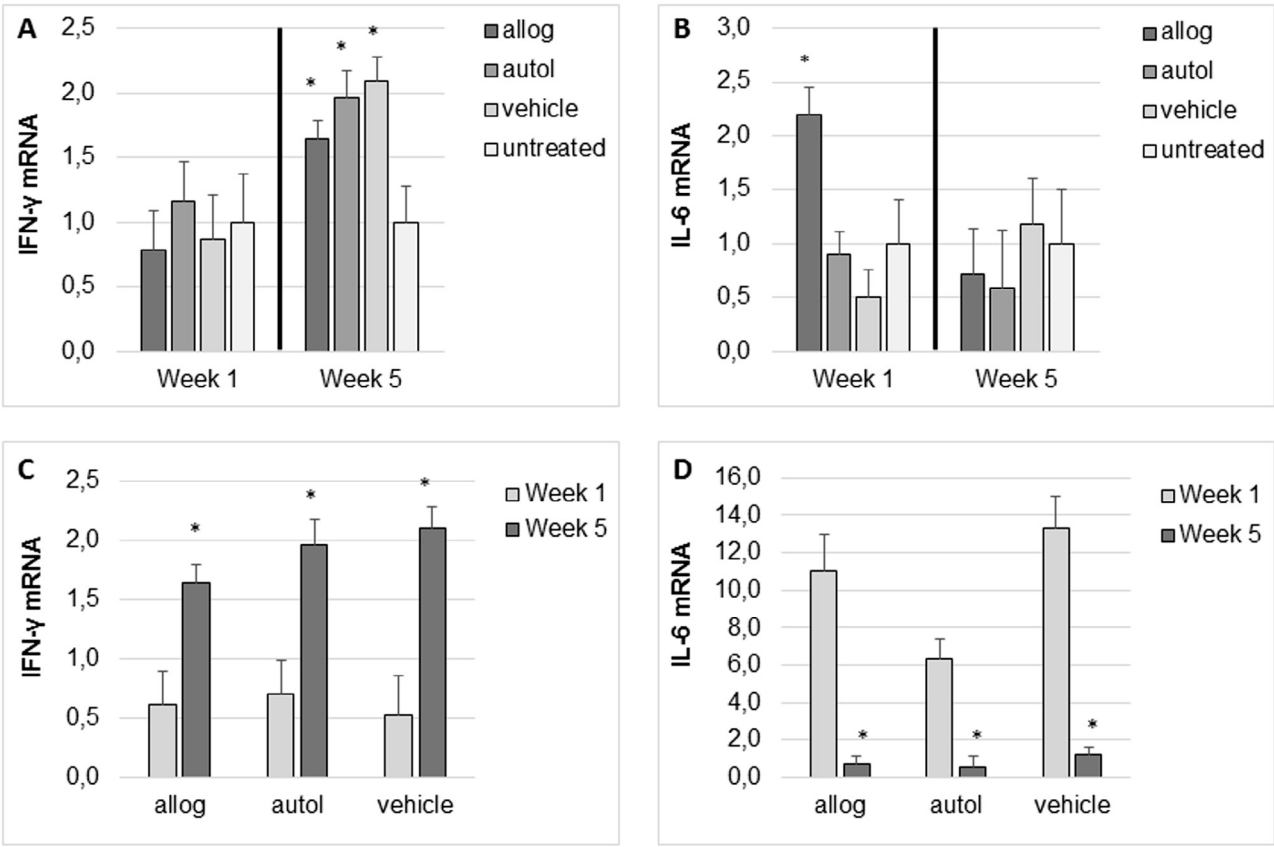

Figure 1. Tissue mRNA expression levels of *IFN-γ* and *IL-6* at weeks 1 and 5 after treatment with autologous (autol) or allogeneic (allog) epithelial-like stem cells (EpSCs) or vehicle treatment. Untreated wounds were used as sample calibrator at each time point: 1 week for the left side of the panel (A, B) and 5 weeks for the right side of the same panel; the black bar indicates that the two sides of the panel were produced with the two different sample calibrators (A, B). *IFN-γ* and *IL-6* expression were compared between the two time points (the untreated wounds at week 5 were used as calibrator) (C, D). Histograms indicate averages ± SEM. *Statistically significant difference between treatment groups (A, B) or over time (C, D), $P < 0.05$.

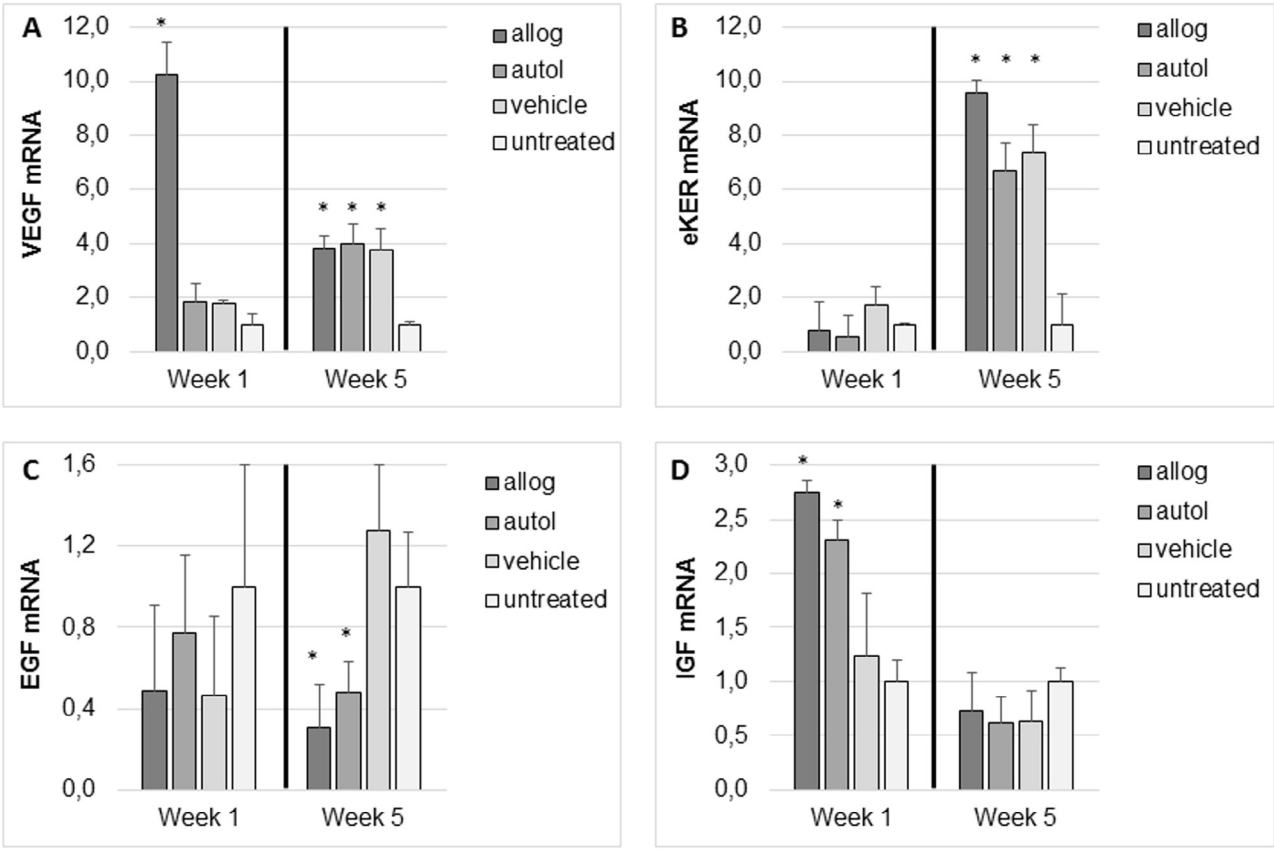

Figure 2. Tissue mRNA expression levels of *VEGF* (A), *eKER* (B), *EGF* (C) and *IGF* (D) at weeks 1 and 5 after treatment with autologous (autol) or allogeneic (allog) epithelial-like stem cells (EpSCs) or vehicle treatment. Untreated wounds were used as sample calibrator at each time point: 1 week for the left side of the panels and 5 weeks for the right side of the same panels. The black bar indicates that the two sides of the panels were produced with two different sample calibrators. Histograms indicate averages ± SEM. *Statistically significant difference between treatment groups, $P < 0.05$.

of the six horses had to be positioned in lateral decubitus, and the topical treatments were compromised. Therefore, the biopsies of this horse were excluded from the study, and only the data from the other five horses were used. Because there were no considerable differences in any of the evaluated wound-healing parameters after administering a total (subcutaneously and topically) of 8×10^6 EpSCs (n = 3) versus 4×10^6 EpSCs (n = 2) per wound, the data of all five horses were taken together for further analyses.

Pro-inflammatory cytokine expression

The rt-PCR analyses revealed no differences in *IFN-γ* mRNA expression at 1 week after different treatments (Figure 1A, left). All injected wounds (also vehicle control) demonstrated a significantly higher *IFN-γ* mRNA expression after 5 weeks in comparison to the untreated wounds (Figure 1A, right side of the panel). When the mRNA expression levels of *IFN-γ* were compared between the two time points (using the untreated at week 5 as calibrator) a significant increase was observed at 5 weeks after treatments (Figure 1C).

The *IL-6* mRNA expression showed the highest level in allogeneic EpSC treated wounds (P = 0.012) at 1 week (Figure 1B, left side of the panel) while at 5 weeks after treatment no significant differences were observed. When the expression levels of *IL-6* mRNA were compared between the two time points (using the untreated at week 5 as calibrator) a significant decrease was observed at 5 weeks after treatment (Figure 1D).

Growth factor expression

Compared with all other treatments, allogeneic EpSC-treated wounds showed the highest *VEGF* mRNA expression ($P = 0.008$) at week 1 (Figure 2A). At week 5, by contrast, *VEGF* mRNA expression was significantly higher in all injected wounds (also vehicle) compared with untreated controls (Figure 2A) and compared with the respective levels of all different treatments at week 1 (Figure 3A). The *eKER* mRNA expression levels were similar in all wounds at 1 week

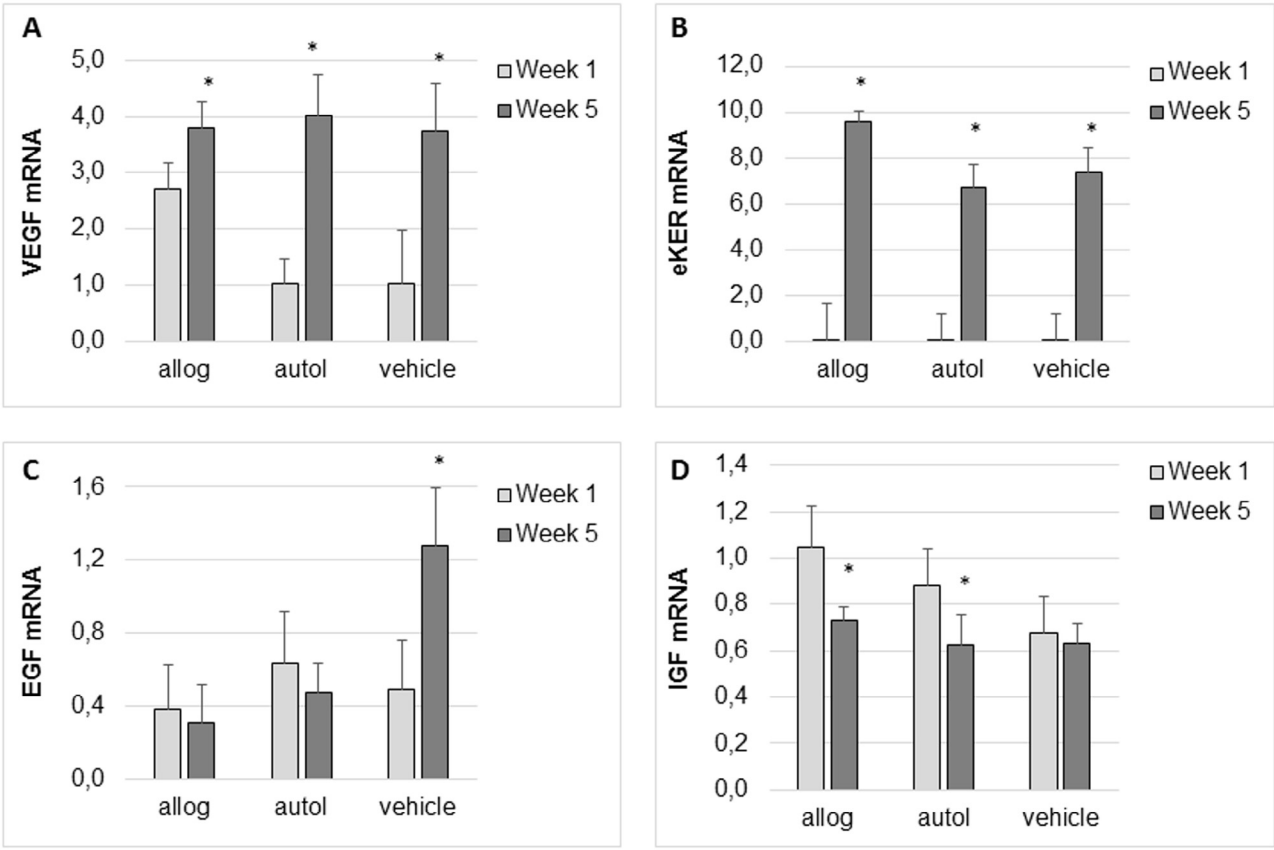

Figure 3. Tissue mRNA expression levels of *VEGF* (A), *eKER* (B), *EGF* (C) and *IGF* (D) at weeks 1 and 5 after treatments with autologous (autol) or allogeneic (allog) epithelial-like stem cells (EpSCs) or vehicle treatment. The expression levels were compared between the two time points (untreated wounds at week 5 were used as calibrator). Histograms indicate averages ± SEM. *Statistically significant difference over time, $P < 0.05$.

after treatment (Figure 2B), yet increased ($P < 0.001$) over time (Figure 3B) with a significantly higher *eKER* mRNA expression in all injected wounds compared with untreated controls at week 5 (Figure 2B). Although *EGF* mRNA expression remained relative constant over time in the autologous and allogeneic EpSC-treated wounds (Figure 3C), the latter wounds demonstrated significantly lower *EGF* mRNA expression at week 5 compared with vehicle-treated and untreated wounds (Figure 2C). At week 1, *IGF-1* mRNA expression was significantly higher in autologous and allogeneic EpSC-treated wounds compared with vehicle-treated and untreated wounds (Figure 2D) and only for the cell-treated wounds *IGF-1* mRNA levels significantly decreased over time (Figure 3D).

Discussion

The present study investigated the expression of growth factors and cytokines in skin wounds after treatment with autologous EpSCs, allogeneic EpSCs and vehicle compared with untreated wounds. The latter group served as a control group for all injections because it has been reported that just an injection might increase vascularization and T-cell response [5], which has to be taken into account.

For the present study, biopsies were taken from the same wounds of which protein analyses and wound-healing assessments were performed as previously reported [5]. In agreement with the *IFN-γ* mRNA expression analyses, no differences in wound fluid IFN-γ protein levels were detected between the treatment groups at 1 week after treatment, and a significant increase was visible over time. However, the increased *IFN-γ* mRNA and protein expression were both unrelated to EpSC addition. This is in contrast to a previous report in canine wounds in which allogeneic bone marrow–derived mesenchymal stromal cells (BM-MSCs) significantly decreased *IFN-γ* mRNA expression at 1 week after treatment compared with saline-injected groups, and the expression levels normalized at week 2 [21]. Although the authors used another cell type and did not show any data from later time points, the injection technique could have had an influence on the outcome of *IFN-γ* mRNA expression.

IL-6 mRNA expression was the highest in allogeneic EpSC treated wounds at week 1, whereas autologous EpSC treatment did not induce a significant increase. This is a remarkable finding because no differences in cellular immune response (CD3, CD20 and MHC II expressing cells) were noted between autologous and allogeneic EpSC-treated wounds at this time point [5]. However, at 2 weeks after treatment, allogeneic EpSC-treated wounds contained a slightly higher level of CD3 and MHC II–positive cells than autologous EpSC-treated wounds, which could result from up-regulation of *IL-6* in the allogeneic group the week before [22,23]. Because this increase was not significant, one might postulate that the temporary *IL-6* increase could be donor specific. Nevertheless, the same EpSCs were used in an autologous setting, and no *IL-6* mRNA increase was observed there. This means that an allogeneic setup initiates certain pathways that might not be directly correlated with the cellular immune response and that this is part of the modus operandi of allogeneic EpSCs. Indeed, *IL-6* mRNA has been associated with MSC communication before because these cells produce IL-6 and contain the IL-6 receptor [24]. In the present study, *IL-6* mRNA expression decreased in all wounds towards week 5, which, on the other hand, was in agreement with the cellular immune response evolution and IL-1 and IL-2 measurements from previous studies [5,21]. Further research is warranted to investigate the role of IL-6 in allogeneic EpSC treatment.

Another interesting finding was the significant increase in *VEGF* mRNA at week 1 in allogeneic EpSC-treated wounds only. Because increased vascularization was observed in both autologous and allogeneic EpSC-treated wounds at this time point [5], other wound healing aspects should be related with the increased *VEGF* expression. In this regard, epithelialization, granulation tissue deposition and minimizing scar formation have been correlated with the latter growth factor [25]. However, all the aforementioned parameters scored very similarly in autologous and allogeneic EpSC-treated wounds [5]. Nevertheless, an increase in *VEGF* mRNA is a desirable finding in any wound-healing study because it has been shown that topical VEGF application accelerates diabetic wound healing in mice [26], rats [27] and humans [28]. Although the present study was performed in healthy animals, these findings are promising and stimulate exploration of the influence of allogeneic EpSC treatment in diabetic patients who are immune compromised and suffer from microangiopathy. At week 5, all injected groups demonstrated a significant increase in *VEGF* and *eKER* mRNA compared with the untreated sample, demonstrating at least a favorable influence of the injection technique on the long-term epithelialization rate. Indeed, at this time point, almost complete re-epithelialization was obtained in 80% of the treated wounds, whereas only 60% of untreated wounds demonstrated this level of epithelialization [5]. Together with the findings on *IFN-γ* mRNA expression as mentioned earlier, investigating different injection routes and procedures might benefit future studies and generate valuable information for determining the most suitable application route for novel wound treatments.

In our hands, *EGF* mRNA was significantly lower in both EpSC-treated groups compared with vehicle injection and untreated wounds at week 5. This is probably because untreated control wounds exhibited a lower epithelialization rate [5] and vehicle treatment results in less mature epithelial cell–containing follicles [6]. Therefore, a low *EGF* mRNA expression at week 5 probably corresponded to the low need for epithelialization after wound closure and a return to a more-or-less original status. In this regard, it has been reported that increasing EGF concentration in healthy human skin equivalents results in a decreased epidermal proliferation and increased epidermal stress [29]. Corresponding with the *EGF* expression levels, *IGF-1* mRNA expression was the highest 1 week after EpSC treatments, and these levels significantly decreased over time only in these wounds. The increase of this regenerative growth factor after autologous and allogeneic EpSC treatment, in contrast to vehicle-treated and untreated wounds, indicates an EpSC-associated finding. In this study, a clear correlation among EpSCs, early *IGF-1* expression and improved wound healing could be made. The latter was demonstrated by significantly reduced granulation tissue, faster wound closure and increased vascularization [5], which are all features that have been reported after MSC treatment as well [30]. This is in agreement with previous studies in which low IGF-1 levels were associated with non-healing wounds in diabetic patients [17] and exogenous IGF-1 application accelerated wound healing [31,32]. Interestingly, the correlation of IGF-1 with healing enhancement seems to be a process that is synergistic with other growth factors [33], and, together with the aforementioned findings, it can also be concluded that EpSC-associated wound healing improvement is a multifactorial process.

Our results suggest that allogeneic EpSC-treated wounds demonstrate the highest expression of *IL-6*, *VEGF* and *IGF-1* mRNA, whereas the injection itself caused a long-term increase in *IFN-γ*, *VEGF* and *eKER* mRNA expression, independent of the presence of cells.

Acknowledgments

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Disclosure of interest: SYB and JHS declare competing financial interests and Pell Cell Medicals declares a patent with number WO2014029778. S.Y.B. and J.H.S. are inventors of the aforementioned patent and shareholders of Pell Cell Medicals. The other authors have no commercial, proprietary, or financial interest in the products or companies described in this article.

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Mesenchymal stem cells improve the wound healing process of mammalian skin.

INTRODUCTION

Skin is a multilayer organ with primary function of protective barrier against external environment; in fact it prevents the body dehydration and the penetration of external microorganisms (Pereira RF et al., 2013). Loss of the integrity of large portions of the skin as a result of injury or illness may cause major disability associate to decreased bodily function, social problems and poor quality of life (Pereira RF and Bártolo PJ, 2014). The wound healing is a complex process that starts after injury and pass through three phases: hemostasis and inflammation, proliferation, and remodeling (Martin P, 1997; Singer AJ and Clark RA, 1999; Kondo T., 2007, McGavin MD and Zachary JF, 2007). Various cells, cytokines and growth factors regulate these phases. After the hemostasis, via vasospasm, fibrin the position and the coagulation process, in the inflammatory phase, leukocytes infiltrate the wound site, secret cytokines and through phagocytosis and the degenerative enzymes remove cell debris and pathogenic organisms. The proliferative phase consist in vessels neoangiogenesis, re-epithelialization and formation of connective tissue stroma to restore normal structure and function to injured tissue. In this phase, a new fibrovascular tissue, the granulation tissue, fills the wound. Its capillaries are arranged perpendicular to the surface of the skin and the proliferating fibroblasts are arranged perpendicular to them. The remodeling phase begins following injury and it includes remodeling of granulation tissue by conversion of immature connective tissue to mature connective tissue through new extracellular collagen formation. This connective tissue drives wound contraction and, if excessive, can cause fibrosis and hyperplastic scar (Diegelmann RF and Evans MC, 2004; Otero-Viñas M and Falanga V, 2016). The wound healing comprehends skin restoration and reestablishment of its tensile strength and natural barrier function (Singer AJ et al., 2000, Cerqueira MT et al., 2012). Dysfunctional healing causes frequently to lifelong disability, with significant consequences and economic impact. To optimize wound healing, cell therapy would be the perfect choice, particularly for extensive and chronic wounds. The presence of mesenchymal stem cells (MSCs) in normal skin (Sellheyer K and Krahl D, 2010; Maxon S et al., 2012) and their role in natural wound healing (Paquet-Fifield S et al., 2009; Maxon S et al., 2012) indicates that the use of exogenous MSCs might be a solution to treat wounds. MSCs are self-renewing and expandable cells in fact, they are able to differentiate into different cell lineages such as osteoblast, adipocytes, chondrocytes, tenocytes, and myocytes (Martinello T et al., 2010; Martinello T et al., 2011; Gomiero C et al., 2016).

Although bone marrow is one of the most frequently source used, there are few data in literature showing advantage of a particular tissue origin of MSC, for instance peripheral blood, adipose tissue, periosteum, synovial membrane, skin and others (Martinello T et al., 2010; Martinello T et al., 2011; da Silva Meirelles L et al., 2008; Maxon S et al., 2012). The involvement of MSCs in the wound-healing process is significant, in particular related to distant wound margin, vascular insufficient and inflammation grade. MSCs may regulate and improve the main phases of wound healing (Otero-Viñas M and Falanga V, 2016), contributing to the reduction of inflammation (Wu Y et al., 2007; Cerqueira MT et al., 2012), promoting the angiogenesis, reducing the wound contraction, attenuating the scar formation (Liu P et al., 2008; Cerqueira MT et al., 2012), and stimulating the cell movement during epithelial remodeling (Otero-Viñas M and Falanga V, 2016). Moreover, the immunosuppressive properties of MSCs allow their potential use in allogeneic therapy. Although the contribute of stem cell involvement in cutaneous wound healing is largely studied (Hu M et al., 2014; Jackson W et al., 2012; Cerqueira MT et al., 2016) this process has never been observed in extended wound and in large animal model. In this study a surgical wound model was developed in sheep and the effect of allogeneic MSCs treatment was evaluated.

MATERIALS AND METHODS

Animal model

Six female Bergamasca sheep homogeneous for size and age were used in this study. Sheep were acclimated to a box of the clinical science department (MAPS Department, University of Padua, Legnaro, Italy) of our University, 2 weeks prior to beginning of the experimental study. Parasitological and biochemistry examinations were carried out to ensure the good health of the subjects. In this study, sheep was chosen because they are less neurologically developed in comparison to carnivores/equines and have sufficient superficial space on their back for the experimental lesions. Moreover, sheep is also considered a possible animal model for human medicine. The experiment was approved by The Body for the Protection of Animals (OPBA) which deals with topics regarding animals used for scientific and educational purposes, in all facilities of the University which carry out scientific activity using animals, as per Legislative Decree no. 26/2014, and approved by article 9 of the executive order 116/92 and the ministerial decree n° 51/2015-PR released by the Health Department of Italy on January 29th, 2015. The number of sheep was chosen based on statistical models and on the “The 3Rs principles replacement-reduction-

refinement" (Russel WMS and Burch RL, 1959). On the basis of these principles, it is possible i) to make the replacement of animals with other experimental methods when possible (replacement), ii) the reduction of the number of employed subjects (reduction) and iii) the improvement of the techniques and procedures in order to eliminate or minimize stress and suffering of the animals (Refinement) (Russel WMS and Burch RL, 1959). At the end of project, the animals have not been sacrificed.

Sheep PB-MSCs isolation

MSCs used in the experiment were isolated from peripheral blood (PB) of different sheep that were not part of the experiment (homologous MSCs). A 100 ml of peripheral blood were taken from the jugular vein and collected in a vacutainer containing anticoagulant Li-heparin. The mononuclear cells were isolated using the protocol of Martinello et al (2010). Cultures were maintained at 37°C with 5% CO^2 and on the day of experiment, PB-MSCs were trypsinized with 0.25% trypsin-EDTA and used for the trial.

Induction of the surgical lesions

Six full-thickness square wound (4x4 cm) were performed under general anesthesia and analgesia on the back of the sheep using a scalpel and a sterilized square guide model. All lesions were used to analyze the effect of five different treatments and the distance of each lesion did not influence the result of trials. In this study, PB-MSCs treatment was compared to control, phosphate saline buffer (PBS) treatment.

At 15 and 42 days after the induction of the lesions, samples for histology, immunohistochemistry (IHC) and molecular analyses were collected by means of a 6-mm punch biopsy with appropriate sedation and analgesic drug administration of the sheep.

Application of treatments and management

Few minutes after wound induction, $1x10^6$ cells diluted in 1ml of PBS were injected in the margins of the lesions and $1x10^6$ cells diluted in 1ml of hyaluronic acid (Hyalgan®, Fidia) was topically applied. PBS was administered onto control wounds. Both PB-MSCs and PBS were applied only in the first day of experiment. After the application of the treatments, the lesions were bandaged with sterile gauze using the "wet-to-dry" method. Every day, wounds were cleaned with PBS and the bandage was changed.

Clinical Evaluation

The macroscopic aspect of the lesions was documented with photographs that were taken every day using a ruler for the evaluation of the process of healing of the wounds. Every week, the same operator performed a clinical evaluation without knowing the number of the subject and the type of treatment that was judging. The valuations obtained were catalogued using the model of Hadley (Hadley HS et al., 2012), giving different parameters such as presence, color and character of the exudate, the aspect of the gauze after removal and the hydration of the wound.

The score system is reported in Table 1.

Parameter	Score
Presence of exudate	1 absent 2 small 3 moderate 4 abundant
Color of exudate	1 clear 2 pink/red 3 brown 4 yellow 5 green
Character of exudate	1 serous 2 serosanguineous 3 sanguineous 4 purulent + 5 purulent ++ 6 purulent +++
Gauze	1 dry/clean 2 dry/stained 3 moist 4 wet
Hydration	1 Normal 2 Maceration + 3 Maceration ++ 4 Desiccation + 5 Desiccation ++

Table 1. Skin-healing parameters scored in the experiment.

The percentages of re-epithelization and wound contraction were measured at different time periods after 7, 14, 21, 28 and 42 days.

Microscopic evaluation

For the histological evaluation two sections were cut at different deepness from each biopsy punch (day 15 and day 42); sections have been examined for the presence of dermal and subcutaneous infiltrate, (immature) granulation tissue, undifferentiated mesenchymal tissue (mature granulation tissue) and the development of adnexa. The score system used was a scale from 0 to 4 (0 absence, 1 presence, 2 small amount, 3 moderate amount, 4 abundant amount).

RNA isolation and gene expression analysis

Total RNA extraction was performed using Trizol (Life Technologies) reagent and quantified on a Nanodrop spectrophotometer (Thermo Scientific). The complementary single strand DNA (cDNA) was synthetized to perform rt-PCR using ABI 7500 Real Time PCR system (Applied Biosystems) to evaluate Collagen 1α1 (Col1α1) and hair keratin (hKER) gene expression. All samples were tested in triplicate and untreated skin were used as a calibrator sample. The 2-ΔΔct method was used to analyze and normalize the RNA expression of the target genes with respect to the endogenous housekeeping gene RPS24 (ribosomal protein S24). PCR primers (Table 2) were designed using Primer Express 3.0 software (Applied Biosystems).

Gene	Abbreviation	5'-Forward primer-3'	5'-Reverse primer-3'
Collagen 1α1	COL1α1	GTACCATGACCGAGACGTGT	AGATCACGTCATCGCACAGCA
Hair Keratin	hKER	TGGTTCTGTGAGGGCTCCTT	GGCGCACCTTCTCCAGGTA
Ribosomal Protein S24	RPS24	TTTGCCAGCACCAACGTTG	AAGGAACGCAAGAACAGAATGAA

Table 2. Primers used for the quantitative Real time-PCR analysis.

IHC evaluation on tissue sections

In order to study inflammatory response, skin tissue was stained with polyclonal rabbit anti-human CD3 (Dako, 1:100), polyclonal rabbit anti-human CD20 (Thermo Fisher, 1:100) and monoclonal mouse anti-human MHCII (Dako, 1:40). To localize proliferating cells, monoclonal mouse anti-human Ki67 (Dako, 1:10) was used while to examine the neovascularization, tissue sections were stained with monoclonal rabbit anti-human vWF (Dako; 1:3200). Immunolabeling was achieved with a high-sensitive horseradish PO mouse or rabbit diaminobenzidine kit with blocking of endogenous PO (Envision DAB+kit; Dako) in an autoimmunostainer (Cytomation S/N S38-7410-01; Dako). An antibody diluent (Dako) with background-reducing components was used to block hydrophobic interactions. The average of three fields was used to evaluate different immunohistological parameters and all measurements were performed with a computer-based program (Leica microscope DM LB2 with Leica Application Suite LAS V4.0) using 20X magnification.

RESULTS

Macroscopic examination

Assessment of the trend of the healing process

Wound closure was observed between day 21 and day 28 in all sheep. During this period, the PB-MSC-treated group showed a faster closure rate than PBS-treated lesions (Fig. 1A).

Although there was no significant difference, mean wound closure time of the PB-MSC treated wounds (30,05 days) was slightly quicker than that of the PBS control group (31,80 days) (Fig. 1B).

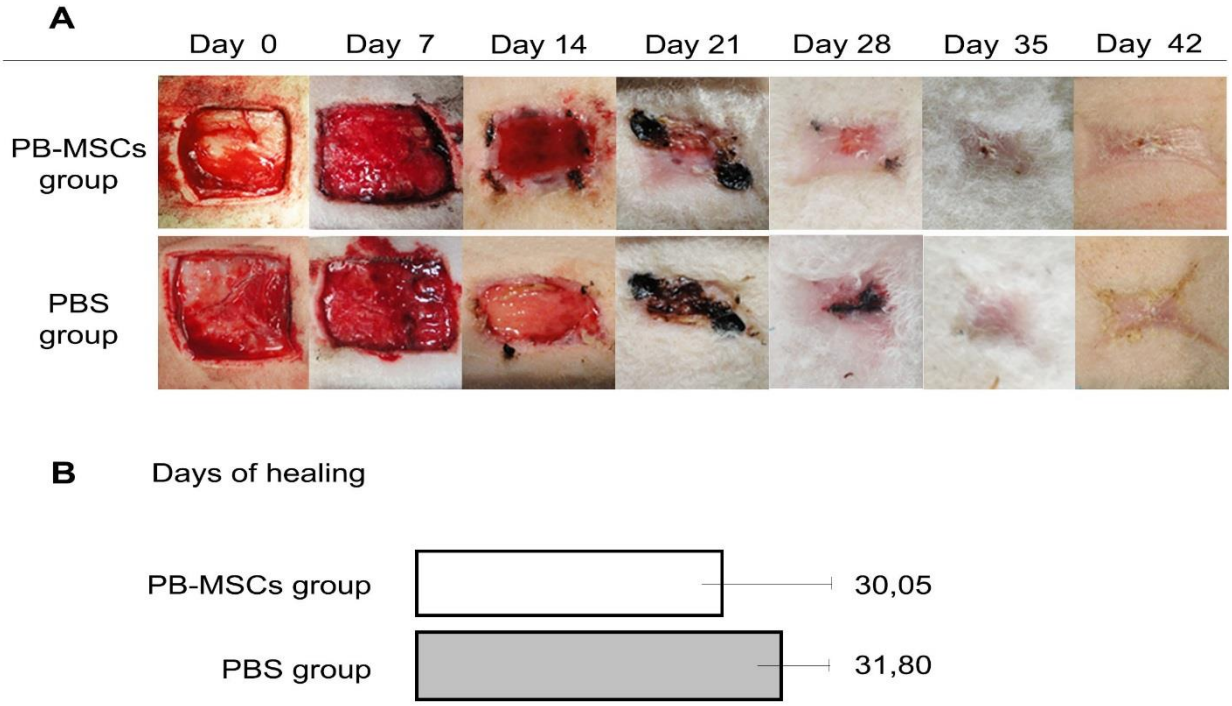

Fig. 1. (A) Serial macroscopic image of the wound site at different time points after PB-MSCs and PBS treatment. Between day 21 and 28, a smaller wound diameter and higher wound closure rate was observed in PB-MSCs-treated wounds. (B) The panel represents the percentage of days of healing. The wound closure time of the PB-MSC treated wounds (30,05 days) was slightly faster respect than the PBS-treated group (31,80 days).

Furthermore, the percentage of re-epithelization and contraction of the wound have been examined. Two weeks after the induction of injuries, PB-MSCs/PBS-treated wounds showed a percentage of re-epithelialization under the 40%. Between 14 and 28 days, PB-MSCs-treated lesions presented a higher percentage of re-epithelialization in comparison with PBS control group (58,69% vs 49,89% at 21 days and 93,5% vs 87% at 28 days). The data presented variability but not statistical significance. After 42 days of treatment, all wounds were healed presenting a 100% of re-epithelialization (Fig. 2A). After two weeks of treatments, PB-MSCs-treated wounds presented an 81% of percentage of contraction respect of 78% of PBS control group. These data revealed a small variability without statistical significance. All lesions presented a 100% of contraction after 42 days of treatment (Fig. 2B).

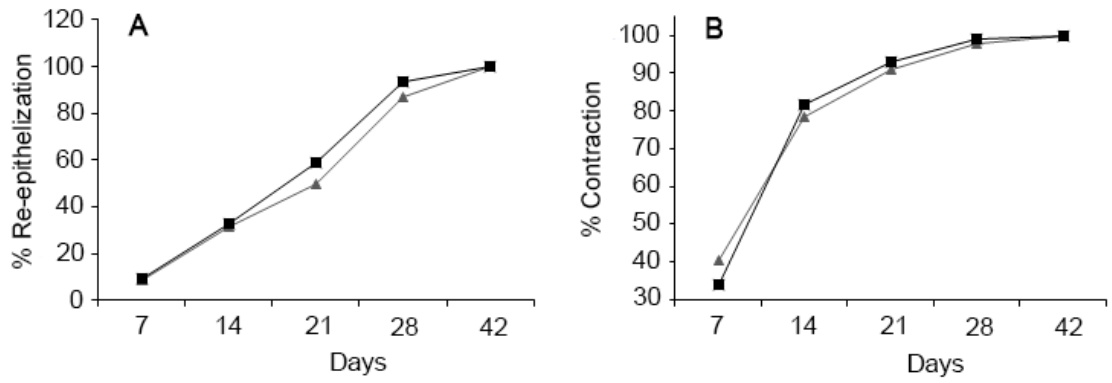

Fig. 2 the histogram shows the percentage of re-epithelization (A) and the percentage of concentration (B) after 14, 21, 28 days of treatment. PB-MSCs-treated wounds trend is represented by black lane while PBS control group is indicated in grey line.

Evaluation of the aspect of the wound

Presence, color and character of exudate: the wound exudate appears to be variable during the first week of experimentation and PB-MSCs-treated wound present a slight non-significant increase of exudate than PBS control group. From the second week, the exudate is absent in all lesions of sheep and they did not present variability or statistical significance. The color and the character of exudate were evaluated during the first week. For all lesions, the color of exudate is pink/red and the characters of exudate changed from the serosanguineous to sanguineous but the data did not present variability or statistical significance.

Aspect of gauze and hydration: the aspect of gauze of PB-MSCs-treated wounds were dry and clean while PBS control group were slightly moist than treated lesions. The data were not significant from the first week of the trial.

From the first week until the end of experimentation, the PBS control injuries and PB-MSCs-treated wounds showed a normal state of hydration.

Microscopic examination

Dermal inflammation: 33% of PB-MSCs-treated wounds presented a moderate amount of dermal inflammation, while 67% of them presented a small amount compared to PBS-treated wounds that contained 50% of moderate and 50% of small amount of dermal inflammation after 15 days of treatment. After 42 days of trial, in PB-MSC-treated wounds,

the inflammation is completely absent while the PBS control group presented a 60% of small amount.

Subcutaneous inflammation: after 15 days of treatment, the 83% of PB-MSCs-treated wounds contained a small amount of subcutaneous inflammation. The PBS control group showed a 17% of moderate and a 67% of small quantity of inflammation. After 42 days of treatment, subcutaneous inflammation was absent in all samples.

Immature granulation tissue: After 15 days of treatment, all PB-MSCs/PBS-treated wounds presented an abundant amount of immature granulation tissue and it was absent in all wounds after 42 days of trial (Fig 3).

Mature granulation tissue and cutaneous adnexa: They were observed in samples collected at 42 days only; the skin appendages were complete in all the components, such as hair follicles, sebaceous and apocrine glands. The cutaneous adnexa observed in PB-MSCs-treated wounds appeared more mature and densely disposed compare to those in PBS-treated wounds. Moreover, in PB-MSCs-treated wounds, vascular plexus were detected (Fig. 3).

Re-epithelization: After 15 days of treatment, all samples showed a 100% of ulceration and 100% of absent re-epithelization. Vice versa, after 42 days of trial all wounds contained 100% of absent ulceration and 100% of re-epithelization.

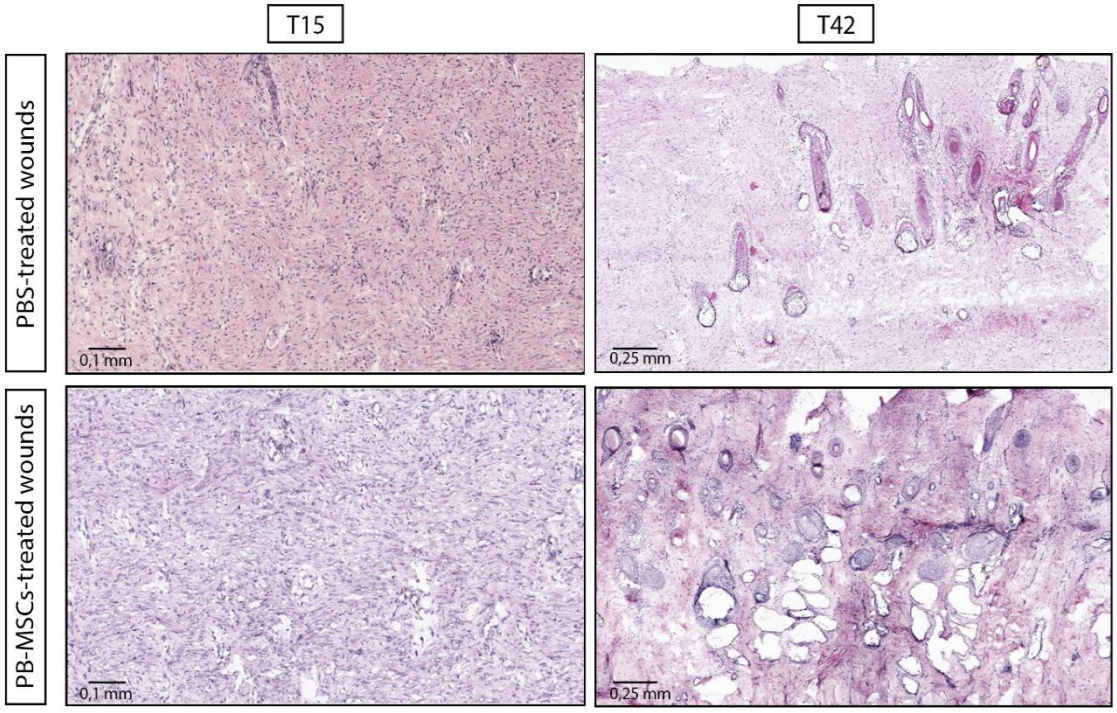

Fig.3 Sheep, skin, Hematoxylin-Eosin. Representative photomicrographs of PBS and PB-MSCs treated wounds analyzed after 15 and 42 days of treatments. The images show the presence of immature granulation tissue at 15 days, while mature connective tissue and developing cutaneous adnexa are present at 42 days.

Quantitative analysis of inflammatory, proliferative, vascular and structural factors

Histochemical staining revealed that skin lesions treated with sheep PB-MSCs (in black bars) presented, after 15 days, a significant increase in cells positive for MHCII respect the wounds treated with PBS only (in grey bars). After 42 days, the PBS-treated wounds showed a significant presence of inflammation cells (Fig. 4A, left).

Cell proliferation was studied with Ki67 marker: within the newly formed dermis, the lesions treated with PB-MSCs presented a higher cell proliferation compared to the PBS control group. After 42 days of trial, the expression of Ki67 in the PB-MSCs/PBS-treated skin injuries decreased over time showing areas with minimal Ki67 staining (Fig. 4A, center). Neovascularization was evaluated by means of vWF staining and a considerably higher average of dermal blood vessels was noticed in PB-MSCs-treated wounds compared with the PBS-treated lesions. Neovascularization decreased in the PB-MSCs/PBS-treated lesions during the healing, showing the same protein expression values (Fig. 4A, right) after 42 days of trial.

In the molecular analysis (rt-PCR) the expression level of gene Col1α1 and hKER were studied. Figure 4B indicates that after 15 and 42 days of trial, Col1α1 mRNA expression level was significant higher in the wounds treated with PB-MSCs compared to the PBS control group. The PBS treatment seems to not influence the expression level of the gene Col1α1.

After 15 days of treatment the hKER mRNA expression levels was already present in the wounds treated with PB-MSCs. Furthermore, the expression level of gene hKER was significantly increased only in the PB-MSCs-treated lesions after 42 days of trial. PBS treatment does not stimulate the cutaneous adnexa formation after 15 and 42 days of treatment (Fig. 4B).

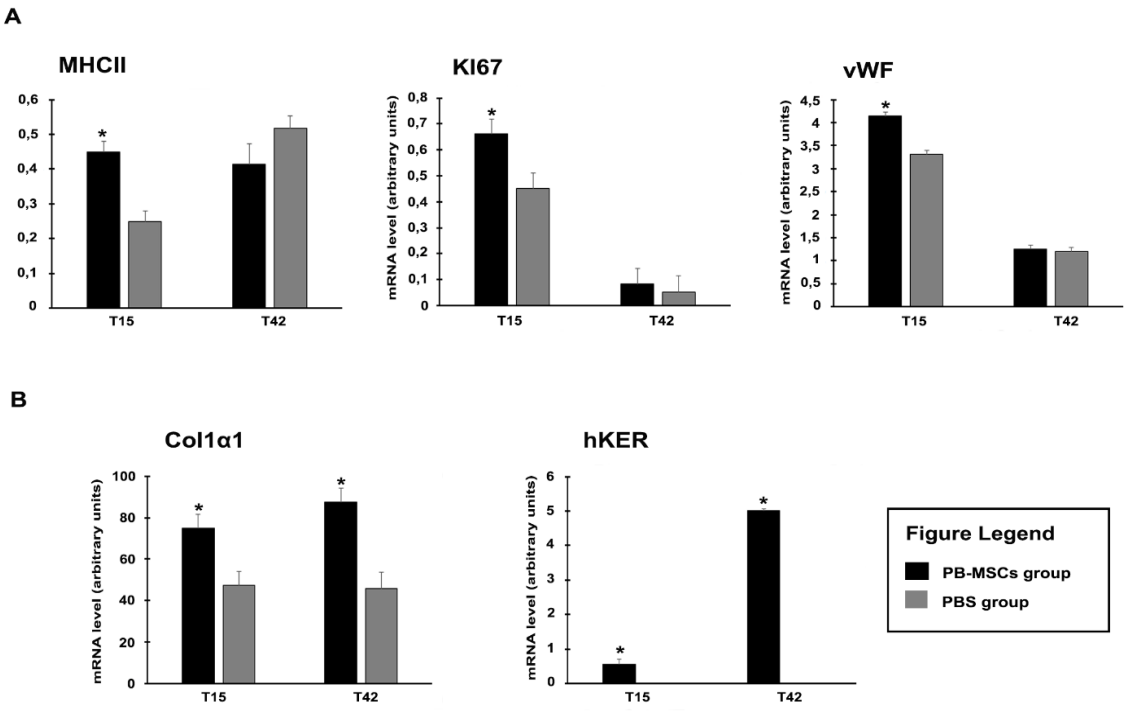

Fig.4 Immunohistochemistry analysis of MHCII, KI67, vWF and mRNA expression of Col1α1 and hKER in PB-MSCs-treated wounds (in black bars) and control group (PBS, in grey bars). Each graph represents the average ± SD of wound treated with PB-MSCs and saline solution PBS. Asterisk indicates significant differences between PB-MSC group and PBS control group (*P <0.05).

DISCUSSION

MSCs are a promising solution to promote wound healing. The presence of these cells in normal skin (Sellheyer K and Krahl D, 2010) suggests their important role in maintenance of skin; there are different types of stem cells in the epidermis, dermis, and hair follicle (Cui P et al., 2014), which preserve the dynamic state of tissue. Endogenous stem cells coordinate cell signaling of wound healing (Cerqueira MT et al., 2016) through different mechanism such as the targeting wound re-epithelialization, formation of granulation tissue and neovascularization, hair follicle formation. Several in vivo studies performed in small animals, have demonstrated that stem cells accelerate wound healing, hypothesizing their contribute in re-epithelization, vascularization and extracellular remodeling (Yoshikawa T et al., 2008; Kwon DS et al., 2008; Badillo AT et al., 2007; Stoff A et al., 2009; Fathke C et al., 2004). The present study investigated, in a large animal, the influences of a PB-MSC treatment in an experimental wound model evaluating their short/long-term effects on skin regeneration.

It is known that exists three modes of wound healing: primary, secondary and tertiary intention (Jeffcoate W et al., 2004). Primary intention refers to wound healing after a clean injury, such as an incision or a superficial burn, in which there is minimal epithelialization and the apposition of wound margins is needed to repair the skin defect (Iocono JA et al., 1998; Russell L, 1999; You HJ and SK Han, 2014) using sutures, staples or adhesive (Iocono JA et al., 1998; Russell L, 1999). Healing associated with a large and/or deep wound in which the tissue edges cannot be approximated is called of secondary intention (Iocono JA et al., 1998; Russell L, 1999). Wounds are left open to heal with the production of granulation tissue, followed by contraction and epithelialization (You HJ and SK H, 2014). Often, this type of healing can be associated with substantial scarring (Iocono JA et al., 1998; Russell L, 1999). Tertiary intention is a healing associated with wounds that are usually infected or dehisced surgical wounds. Healing is promoted by leaving the wound open for a prescribed period of time to treat the contamination or infection and to allow intention for growth of new tissue before approximating the skin edges for a primary closure (Iocono JA et al., 1998; Russell L, 1999). In literature, it is already known the capacity of stem cells seeded on nanostructured membrane to help healing of primary intention such as skin burned in a murine model (Souza C et al., 2014). This study suggests that MSCs might be used as a possible treatment also for wound healing of secondary intention.

After skin injury, the inflammatory phase starts immediately. During this process, it is observed a platelet aggregation at the injury site followed by the infiltration of neutrophils, macrophages and T-lymphocytes into the wound site (Martin P, 1997; Singer AJ, 1999). The data presented in this paper show, from a clinical perspective, a level of inflammation not significantly different between the PBS treatment and the PB-MSCs-treated wounds. The presence of exudate increases slightly after PB-MSC treatment, the color appears in all wound pink/red and the characters of exudate change not significantly from serosanguineous to sanguineous. Microscopic evaluations indicate the presence of inflammation phase 15 days after injury both in PBS control group and in PB-MSC group, and both at dermal level and at subcutaneous level. On the contrary, a considerable result obtained in our study is the complete absence of inflammation after 42 days in PB-MSC group whereas PBS control group still presented a 60% of dermal inflammation. These results corroborate data present in literature; for istance, Kim JW et al. (2013) showed that experimental full-thickness wounds treated with topical applications of allogeneic MSCs presented an increase healing and less inflammation probably because MSCs are able to release immunosuppressive factors in the wound bed that inhibit proliferation of immune

cells such as B cells, T cell and natural killers cells, therefore reducing the inflammation (Beyth S et al., 2005; Matthay MA et al., 2010; Hass R et al., 2011). Interestingly, Chen et al (2008) reported the influence of MSCs in the inflammatory response as an up-regulation of MIP-1α and β with an increase in the number of macrophages infiltrating the wound (Chen et al 2008). Moreover, in the last decade, it has been found that MSCs possesses also an antimicrobial effect, an important benefit in reducing excess inflammation from contaminants in the wound during injury and treatment (Mei SH et al., 2010) and in the scar formation (Nuschke et al., 2014).

After the inflammation phase there is the proliferative phase with newly formed granulation tissue that cover the wound area to complete tissue repair. This phase is characterized by angiogenesis indispensable for leading cytokines and sustaining the granulation tissue and re-epithelization (Singer AJ, 1999; Burnouf T et al., 2013). The granulation tissue, evaluated histologically in this study, is more abundant with PB-MSC respect PBS treatment and in both case it decrease with time. The newly granulation tissue is supported at 15 days both in PBS and in PB-MSC treatment by expression of vWF. vWF, a glycoprotein essential for normal hemostasis, plays multiple vascular roles and it is associated to angiogenesis. The evidence of the presence of a proliferative action is confirmed also by an increase of Ki67 expression. Ki67 is a cellular marker for proliferation; in fact, this protein is present during all active phase of the cell cycle. The PB-MSC treatment induces a significant increase of Ki67 expression respect PBS, correlated with the presence of more abundant granulation tissue. The increase of matrix and vessel formation could be probably attributed to the observed up-regulation of growth factors such as EGF, TGF-β1 and stromal-derived growth factor-1α (Chen et al. 2008). The more active proliferation induced by PB-MSC treatment reflected the percentage of re-epithelization and contraction. In fact if at 14 days PB-MSC and PBS treatment show the same level of re-epithelization, at 28 days the 93,5% of PB-MSC treated wound is re-epithelized, versus the 87% of PBS treated wound; moreover, the contraction of PB-MSC appears earlier respect PBS treated wounds. The last described parameters were observed clinically while histologically, none difference was noted between PB-MSC and PBS treatment. This could be explained by the sampling technique: small biopsies indeed have been taken at the healing edge of the wound and are not representative of the entire affected area. Furthermore, the proliferating and remodeling phases of the healing process could be present simultaneously in the healing wound (McGavin MD and Zachary JF, 2007). This leads to a variability of recorded observations in the different samples.

The data obtained in this study confirm that MSCs should produce a multiplicity of pro-angiogenic factors recruited in the site lesion to promote the stimulation of endothelial cell leading to novel blood vessels formation in the wound bed. The most notably is VEGF, a potent stimulator of angiogenesis in the wound bed (Herrmann JL et al., 2011). Revascularization of the wound bed is an important phase of the normal wound healing process and the new formation of vessels is necessary to carry blood in the wound area, which is in need of oxygen and nutrients (Morimoto N et al., 2012; Zhang Y et al., 2014).
The last phase that is observed during wound healing is the maturation of the tissue. In normal skin, a population of multipotent stem cells able of generating all of the components of hair as well as epithelial cells and is located in a specialized site, the hair follicle bulge (Oshima H et al., 2001). These cells do not contribute to preservation of the interfollicular epidermis but can differentiate into epidermal stem cells after a trauma (Levy V et al., 2007). The treatment of wound with PB-MSC demonstrated the ability of these cells of stimulating the appropriate production of matrix and developing new hair follicles. Collagen type 1 is the predominant collagen in normal skin and exceeds collagen type 3 by a ratio of 4:1. During wound healing, this ratio decreases to 2:1 because of an early increase in the deposition of collagen type (Fathke C et al., 2004). In our study, the expression of matrix protein collagen 1 is higher in PB-MSC treatment wounds respect than PBS control group at 14 and 42 days, indicating a correct and early process of wound healing. Our study conducted on large animals has led to the same results obtained with small animals. In fact, lesions created in rabbits (Borena BM et al., 2010) and in dogs (Borena BM et al., 2009) demonstrated significantly earlier vascularization, fibroplasia and premature maturation of collagen using autologous bone marrow-derived mononuclear cells. Another important result obtained in this study showed that the treatment with PB-MSC induce, in this last phase, a high and significant increase of mRNA level of hair Keratin, which expression is detectable already at 14 days. Furthermore, after 42 days, microscopic evaluation highlights the higher presence of skin appendages after PB-MSC respect PBS treatment; in particular, a higher percentage of treated sample present more mature adnexa such as hair follicles, sebaceous and apocrine glands and densely disposed. Formigli L et al. (2015) demonstrated as MSC-seeded on bioengineering scaffolds induced enhanced re-epithelialization (characterized by a multilayered epidermis, return of hair follicles, sebaceous glands and enhanced blood vessel formation); our study showed similar results in the presence of MSCs isolated from peripheral blood of sheep.

In conclusion, in the skin regeneration, PB-MSCs seem to have different roles but the most significant appears to be related to an improved quality of the healing process. Probably, the therapeutic benefit of these cells derives from the so-called bystander effects, although the molecular mechanisms are still under study (Savukinas UB et al. 2016). In fact, the PB-MSCs treated wound closed faster respect PBS control group, and the contraction area was larger. Moreover, the data presented in this paper indicate that the treatment with PB-MSCs might be advisable not only for superficial injuries, but even for deep lesions, such as burns or diabetic ulcers (Nuschke A, 2014; Falanga V et., 2007) supporting the stimulation of secondary intention wound healing. Indeed, PB-MSCs were able to speed up the appearance of granulation tissue, neovascularization, structural proteins and skin adnexa.

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DISCUSSION

Adult stem cells have been isolated from different tissues and their potential application is still under investigation in both human and veterinary medicine. Indeed, MSCs offer great promise in treating previously incurable disease for several reasons: i) they lack the ethical controversies that are associated to embryonic stem cells (ES) since are derived from adult tissues and do not require manipulation or destruction of embryo, ii) do not possess the risk of tumorigenicity if implanted *in vivo*. It has been generally demonstrated that iii) MSCs possess the ability to undergo toward different lineages of differentiation.
Current standard of care for tendon, muscle and other pathologies, is similar in all species and comprises a combination of physical therapy, reduced exercise and medical (systemic and local) and surgical modalities. Consequently, regenerative strategies including stem cell therapy have come into the focus not only in the human surgery but also in the veterinary medicine for dogs and horses (Brehm W et al., 2012). Despite the great advances made in the isolation, expansion and definition of the stromal cells population, several challenges remain open to fully understand the MSCs behavior of *in vivo* and *in vitro*. Improved understanding of MSCs function holds great promise for the application of cell therapy and also for the development of powerful cell-derived therapeutics for regenerative medicine (Spees JL et al., 2016).

This thesis has been divided in two sections: the first part focused on the *in vitro* mesenchymal stromal cells isolated from peripheral blood (PB-MSCs) reprogrammed towards musculoskeletal fate using i) growth factors (bFGF2 + TGF-β3) combined with the low level laser technology (LLLT), ii) innovative methods for transfection such as MyoD-TAT and iii) an “intelligent” colloidal nano-vector (SAMNs). The second part regarded two studies of *in vivo* application of autologous and allogeneic epithelial stem cells (EP-MSCs) and allogeneic PB-MSCs to improve skin wound regeneration respectively in horses and sheep.

The first section of this work investigates innovative methods to reprogram PB-MSCs towards tenogenic and myogenic fates.
Tendons are structures that present low cellularity and low vascularity and they are constituted of regulare dense connective tissue. These properties confer stiffness and elasticity to the tendons (Brehm W et al., 2012; Smith RK et al., 2013; Patruno M and Martinello T, 2014), which are essential in transmitting forces between muscles and bones (Freedman BR et al. 2014) but the not-vascularized nature and low number of tenocytes, reduce the self-healing and regenerative potential of tendons (Jiang D et al. 2014; Veronesi F et al. 2015). Autologous tenocytes can be used to repair injured tendons, (Cao Y et al., 2002) nevertheless tenocytes may not be an ideal source for tendon repair (Bi Y et al., 2007) because these cells have a limited proliferative potential and ethic committees from many countries prohibit invasive collection of tendon tissue to obtain tenocytes (Tan Q et al., 2012). For this reason, our first study tried to fulfill this problem: PB-MSCs were cultured in media

conditioned with different growth factors (GFs) such as TGFβ3 (transforming growth factor), EGF2 (Epidermal growth factor), bFGF2 (Fibroblast growth factor) and IGF-1 (insulin-like growth factor) in presence or without of the Low Level Laser Technology (LLLT). RT-PCR was used to study the mRNA expression levels of genes involved in the tenogenic induction such as Early Growth Response Protein-1 (EGR1), Tenascin (TNC) and Decorin (DCN). The singular addition of GFs did not show any influence on the mRNA expression of genes above mentioned, whereas the specific combinations bFGF2 + TGFβ3 and bFGF2 + TGFβ3 + LLLT arrested cell proliferation in favor of differentiation toward tenogenic fate. Indeed, the supplement of bFGF2 and TGFβ3 significantly upregulated the expression of EGR1 and DCN, while the use of LLLT induced a significant increase of TNC levels.

In literature, Gonçalves AI et al. (2013) showed that bFGF, EGF, PDGF-BB and TGF-β1 have a molecular influence on the tendon-related genetic expression of MSCs isolated from amniotic fluid (hAFSCs) and adipose tissue (hASCs), starting up the process of tenogenic differentiation and an extracellular matrix production. Moreover, Cai TY et al. (2013) indicated that bFGF2 is able to activate the MAPK pathway promoting the differentiation of MSCs into tendons with the expression of tendon matrix protein such as collagen type I, collagen type III and scleraxis. Furthermore, it was demonstrated that bFGF2, introduced with a viral vector, increased the gene level of TGF-β1, and VEGF and down-regulated IGF1 during the tendon repair process (Tang JB et al., 2014).

LLLT is a technique that usually is used *in vivo* as generate an anti-inflammatory effect, decrease the number of fibroblasts and neo vascularization in tendon lesions of sheep (Iacopetti I et al., 2015). *In vitro*, LLLT increase the bone morphogenetic protein 2 (BMP-2) because it stimulates cell proliferation in regenerative process (Pyo SJ et al., 2013).

It is known that bFGF2, TGF-β and LLLT possess stimulatory effects on cells: this study is a pivotal work where bFGF2 and TGFβ3 and LLLT were used together to differentiate PB-MSCs in tenoblasts *in vitro*.

The second paper highlighted the importance to have differentiated cells to regenerate muscle disease such as injuries, muscle degeneration (Duchenne dystrophy) and inflammation. Currently, is possible to use different methods to regenerate muscle lesions. Gene therapy may be an effective method by which to deliver high, maintainable concentrations of growth factor to injured muscle (Barton-Davis ER et al., 1998; Barton ER et al., 2002; Musaro A et al., 2001). Although IGF1 improved muscle healing, histology of the injected muscle revealed fibrosis within the lacerated site (Lee C et al. 2000). Another growth factor, VEGF, by favoring angiogenesis, is known to enhance skeletal muscle repair (Deasy BM et al., 2009; Frey SP et al., 2012; Messina S et al., 2007). By targeting simultaneously angiogenesis and myogenesis, it was shown that combined delivery of VEGF and IGF1 enhance the muscle regenerative process (Borselli C et al., 2010). In this direction, the use of platelet-rich plasma (PRP) is considered a possible alternative approach based on the ability of autologous growth factors to improve skeletal muscle regeneration (Hamid MS et al., 2014; Hammond JW et al., 2009).

Considered as safe products, autologous PRP injections are increasingly used in human and animal patients with sports-related injuries (Engebretsen L et al., 2010). Scaffolds are good helpers for muscle injuries: myogenic precursor cell survival and migration is greatly increased by using appropriate scaffold composition and growth factor delivery (Hill E et al., 2006; Boldrin L et al., 2007). Ideally, using an appropriate ECM composition and stiffness, scaffolds should best replicate the *in vivo* milieu and mechanical microenvironment (Gilbert PM et al., 2010; Engler AJ et al., 2006). A combination of stem cells, biomaterial-based scaffolds and growth factors may provide a therapeutic option to improve regeneration of injured skeletal muscles (Jeon OH and Elisseeff J, 2016). After an initial demonstration that normal myoblasts can restore dystrophin expression in mdx mice (Partridge TA et al., 1989), clinical trials, in which allogeneic normal human myoblasts were injected intramuscularly several times in dystrophic young boys muscles, have not been successful (Law PK et al. 1990; Mendell JR et al., 1995). Even recently, despite clear improvement in methodologies that enhance the success of myoblast transplantation in Duchenne patients (Skuk D et al., 2007) outcomes of clinical trials are still disappointing. These experiments have raised concerns about the limited migratory and proliferative capacities of human myoblasts, as well as their limited life span in vivo. Among all these non-satellite myogenic stem cells, human mesoangioblasts, human myogenic-endothelial cells and human muscle–derived CD133+ have shown myogenic potentials in vitro and in vivo (Sampaolesi M et al., 2006; Zheng B et al., 2007; Meng J et al. 2014).

As it is known that DNA does not possess the ability to translocate through the cellular membrane, various approaches are employed to accomplish this task (Ghosh PS et al., 2008) including physical manipulations (mechanical pressure, electric shock and hydrodynamic forces (Kamimura K et al. 2011), as well as the use of viral and non-viral vectors. In order, regarding viral vectors such as retrovirus, adenovirus, and herpes simplex virus, are typically selected (Kamimura K et al., 2011). Furthermore, viruses can be genetically modified in order to be highly effective. The promise of retroviral-mediated gene delivery to treat or even cure genetic diseases has been demonstrated in animal models and the clinic. Despite present accomplishments and vector design improvements such as the self-inactivating mechanism, testing in humans has not gone without serious consequences. Most notoriously, the emergence of cancer in several patients enrolled in a y-retroviral-mediated clinical trial for X-linked SCID (severe combined immunodeficiency) has forced current vector systems to be reconsidered (Papayannakos C and Daniel R, 2013).

Our study focused on the reprogramming of PB-MSCs towards myogenic progenitors using the small peptide TAT of HIV-1 that is a powerful transactivator of gene expression. It is able to translocate through the plasma membrane and when it is fused with other peptides may act as a protein transduction system. A short amino acid motif, highly enriched in basic amino acids, promotes the export of the protein from the expressing cells. Cellular internalization of TAT and TAT fusion proteins requires the integrity of cell membrane lipid rafts and when TAT basic domain attached to large protein cargos, also mediates their efficient cellular

internalization and can be thus utilized for transcellular protein transduction (Fittipaldi A and Giacca M, 2005). This ability appears particularly interesting to induce tissue-specific differentiation when the TAT protein is associated to transcription factors.
In our work, the potential of the complex TAT-MyoD in inducing equine PB-MSCs towards the myogenic fate was evaluated. Results showed that the internalization process of TAT-MyoD needs the absence of serum and the nuclear localization of the fused complex is observed after 15 hours of incubation. However, the supplement of TAT-MyoD only was not sufficient to induce myogenesis and conditioned medium, obtained coculturing PB-MSCs with C2C12 without a direct contact, was added.
To evaluate the myoblasts differentiation, RT-PCR analysis was performed to study Myf5 and Myogenin gene expression, and immunostaining experiments to estimate the expression protein of MyoD, Myf5 and Myogenin. It was interesting to note that the complex TAT-MyoD was able to remain in the cytoplasm after 2 and 6 hours after the cell transfection. Only after 15hrs of incubation, TAT-MyoD was localized in the nucleus and persists after 48hrs.
In the third paper, novel surface-active maghemite nanoparticles (SAMNs) are tested as vectors for eukaryotic cell transfection. SAMNs showed a higher efficiency respect lipofectamine in the transfection of coding gene in PB-MSCs without the application of external magnetic fields. The labelling efficiently, allows to informative data on redistribution, localization, and quantification of SAMNs nanoparticles in MSCs cells. In our study, SAMNs linked to rhodamine (SAMN@RITC) presented a cytoplasmic distribution in MSCs after 48hrs of incubation and no cytotoxicity effects was observed with XTT cell proliferation assay. The second step was to evaluate SAMNs efficacy as vector for pDNA coding GFP: equine PB-MSCs were incubated with SAMNs@pDNA and a consistent cytoplasmic green fluorescence light was originated in the cells after 24hrs of treatment. This novel pDNA vector offers a very good transfection efficiency even at low DNA concentration. Differently from complex TAT-MyoD, SAMNs@pDNA can be used in cell culture with medium containing serum and this peculiarity becomes particularly important with delicate cell lines.
In all studies reported in this section, equine MSCs isolated from peripheral blood were used: it is known that MSCs can be isolated from several types of tissues in adult mammalians (Martinello T et al., 2010; Martinello T et al., 2011; Zhu et al. 2013, Toupadakis CA et al., 2010). In terms of invasive MSCs collection, cells obtained from bone marrow require an offensive procedure associated with the risk of complications (Giovannini S et al, 2008). Another source is adipose tissue via lipectomy: this method is very hostile for human and animal patients because anesthesia is necessary to obtain abdominal fat. Peripheral blood seems to be a good alternative non-invasive source compared to bone marrow and adipose tissue. For this reason, PB-MSCs were chosen for our experiment and they can differentiate into mesodermal lineage cells, including osteoblasts, chondrocytes, adipocytes, cardiomyocytes, hepatocytes, endothelial cells, smooth muscle cells, and neuronal cells, under appropriate culture conditions (Parmar N et al., 2014).

To conclude, the three novel methods proposed in the first section have been used to overcome the problem of MSCs reprogramming towards musculoskeletal fate using viral vectors or gene therapy. These new non-viral transfection systems are able to reprogram PB-MSCs without biological risk and the cells can be used in complete safety for the treatment of tendon and muscle lesions.

The second part of this work has been focused on application of stromal cells of mesenchymal origin in skin repair.
Wound healing is a complex multi-stage process that organizes the reconstitution of the dermal and epidermal layers of the skin. In many pathological circumstances such as diabetes or severe burns, the normal wound healing process fails to adequately restore function to the skin, leading to potentially severe complications from ulcers or resulting infections. As the incidence of obesity and resulting diabetes continues to increase in the western world (Beckles GL, Chou CF, 2006), the prevalence of chronic wounds related to these conditions continues to be a major focus of wound care research. In fact, non-healing wounds from these conditions have produced a multi-billion dollar advanced wound care market for technologies aimed at stimulating wound healing in patients that suffer from dysfunctional wound repair (Stuart M, 2007). Individuals with extensive skin lesions (e.g., full-thickness burns) suffer a substantial loss of dermis that does not regenerate spontaneously and may require a skin graft (Li X et al., 2015; Shen Y et al., 2015). Over the years, skin regeneration has been attempted with various types of transplantation, such as xenografts, allografts, or autografts. However, antigenicity (in allografts and xenografts) and the limited number of donor sites available (in autografts) mean that in many cases, these substitutes are unsuccessful in promoting skin regeneration (Freyman TM et al., 2001; Caliari-Oliveira C et al., 2015). Hence, there is a need to develop devices that can adequately replace the damaged tissue. Most current biological technologies for advanced wound care aim to provide antimicrobial support to the open wound and a matrix scaffold (collagen-based in many cases) for invading cells to reestablish the skin, with some focus on growth factor support of the healing process (Rees RS et al., 1999; Boateng JS et al., 2008).
MSCs are another important “device” able to orchestrate the three main phases of normal wound healing (inflammatory/proliferative/remodeling), directing inflammation and antimicrobial activity and promoting cell migration during epithelial remodeling (Maxson S et al., 2012). Nuschke A (2014) showed that MSCs used in a chronic or non-healing wound, promotes immunosuppression, angiogenesis stimulation, and scar reduction and the combination of matrix scaffold with MSCs-based cell therapy improves wound healing becoming a potential strategy in treatment of non-healing wounds (Huang S et al., 2012).
Based on this knowledge, our works present skin lesions performed *in vivo* in particular on the glutei of equines and on the back of the sheep and treated, respectively, with autologous and allogeneic Ep-SCs and allogeneic PB-MSCs.
In the first work of second section, autologous and allogeneic Ep-MSCs have been used to regenerate skin of equine glutei: the biopsies were collected after 1 and 5 weeks of treatments. Our results suggest that allogeneic EpSCs are able to increase

the expression of IL-6, VEGF and IGF-1 mRNA in the wound treated, whereas the injection itself caused a long-term increase in IFN-y, VEGF and eKER mRNA expression, independent of the presence of cells.
In the second project, six lesions performed on the back of six Bergamasca sheep were treated with allogeneic MSCs and two biopsies were obtained after 15 and 42 days of treatment. Clinical evaluation performed every day showed that the healing time of the cells treated group (30,05 days) was faster than placebo (31,80 days) and that the closure of the wound was better than the group control.
Dermal and subcutaneous inflammation, immature and undifferentiated mesenchymal tissue (mature granulation tissue) and skin adnexa were evaluated using histological analysis: PB-MSCs-treated wounds after 42 days have better healing than control group: dermal and subcutaneous inflammation were absent, undifferentiated mesenchymal tissue was slightly present and skin adnexa were perfectly regenerated. Molecular analysis confirmed the result obtained for skin adnexa in fact, the expression level of gene hKER increases significantly only in PB-MSCs-treated lesions after 42 days of trail. The Col1α1 gene expression increased after 15 days of treatment in the PB-MSCs-treated wounds raising further after 42 days.
Lastly, MHCII, vWF and KI67 were evaluated with immunohistochemical staining: PB-MSCs-treated wounds showed a higher protein expression of the major histocompatibility complex II (MHCII), neovascularization (vWF) and cellular proliferation (KI67) respect PBS-treated lesions confirming that i) MSCs naturally produce a variety of pro-angiogenic factors following recruitment to the wound bed that stimulate endothelial cell proliferation and tube formation in the wound bed, most notably VEGF, a potent stimulator for angiogenesis that is regulated by IL-6 and TGF-α in the wound bed (Herrmann JL et al., 2011). It has been shown that exogenous VEGF application to wounds can stimulate angiogenesis (Callaghan M et al., 2004); MSCs used in cell therapeutics also have been shown to stimulate EC recruitment and wound healing via VEGF secretion (Chen L et al., 2008; Wu Y et al., 2007) or via pre-differentiation into angiogenic precursors (Roura S et al., 2012). MSCs are able to secrete paracrine factors, including VEGF, EGF, keratinocyte growth factor, stromal cell-derived factor 1, insulin-like growth factor-1, and angiopoietin-1, which enhance the recruitment of macrophages, keratinocytes, dermal fibroblasts, and endothelial cells to the wound site, facilitate angiogenesis, stimulate collagen production from dermal fibroblasts, and reduce apoptosis, inflammation, and scar formation at the site of the wound (Ennis WJ et al., 2013; Satija NK et al., 2013; Hocking AM et al., 2010). ii) MSCs have several effects on fibrotic phenotypes in the wound, and thus play a major role in reducing scar formation following wound healing. The project about regeneration skin of sheep confirmed the results of Wu Y et al., (2013) (Huang S et al., 2013) that has seen an important decrease of scar tissue in a mouse model after the MSCs treatment. It is possible that MSCs can sense the degree of inflammation in the microenvironment and respond by releasing of growth factors, cytokines, and other mediators to reduce inflammation using real-time biochemical cues (Ennis WJ et al., 2013). Probably MSCs produce PGE2 that drives a variety of changes in the scarring

phenotype and increase secretion of IL-10 by T cells and macrophages involved in the wound environment (Németh K et al., 2009). iii) MSCs are able to reduce inflammation in the wound bed. Probably MSCs release immunosuppressive factors that inhibit proliferation of immune cells such as T cells, B cells, and natural killer cells (Matthay MA et al., 2010; Hass R et al., 2011). Moreover, the results obtained with allogeneic MSCs used to treat lesions performed on equine glutei confirmed the effects found by Li P et al. (2013) and Huang XP et al. (2010). Allogeneic MSCs do not induce a significant response in the host: these findings suggest that allogeneic cells may be used for chronic wound therapy and could be a useful strategy for situations when the host's endogenous MSC population may possibly be defective, as in diabetes (Li P et al., 2013 and Huang XP et al., 2010). This aspect is particularly important for treating chronic wounds in older individuals, patients with diabetes, and those with autoimmune diseases and compromised MSCs (Wikramanayake TC et al., 2014) and burns (Nuscke A, 2014).
In conclusion, it is known in literature that MSCs are currently used to improve the healing of primary intention such as burns and diabetic ulcers using MSCs seeded on bioengineering scaffolds (Nuschke A, 2014; Falanga V et. al, 2007). The second part of this tesis showed the complete regeneration of skin wounds using MSCs isolated from peripheral blood and suggesting their involment not only to improve the healing of primary intention but also for the stimulation of secondary intention wound healing.

Overall, and based on collected data during this research project, it should briefly point out that:

1. PB-MSCs can be used *in vitro* to be induced towards the tenogenic and myogenic fate with innovative methods such as combination of growth factors with laser, TAT peptide and SAMNs nanoparticles linked to cDNA or proteins. These experiments open the opportunity to have large amount of differentiated cells available to be used in wound heling without the use of viral vectors.
2. *In vivo*, MSCs have the ability to suppress excessive inflammation and decrease scarring while simulating *de novo* angiogenesis in the wound bed, all positive effects leading to promising outcomes in chronic wound repair. Although MSCs contribute to tissue regeneration and repair by modulating the host tissue via secreted cues, the therapeutic benefit of MSCs is thought to derive from the so-called bystander effects (Savukinas UB et al., 2016).
   Future study will have to focus on tracking of cells, e.g. using cells marked with GFP protein to enable tracking and investigation of homing as well as migration in the wound.

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[...] la tua Leggenda Personale. [...] è quello che hai sempre desiderato fare. Tutti, all'inizio della gioventù, sanno qual è la propria Leggenda Personale. In quel periodo della vita tutto è chiaro, tutto è possibile, e gli uomini non hanno paura di sognare e di desiderare tutto quello che vorrebbero veder fare nella vita. Ma poi, a mano a mano che il tempo passa, una misteriosa forza comincia a tentare di dimostrare come sia impossibile realizzare la Leggenda Personale. [...] Sono le forze che sembrano negative, ma che in realtà ti insegnano a realizzare la tua Leggenda Personale. Preparano il tuo spirito e la tua volontà. Perché esiste una grande verità su questo pianeta: chiunque tu sia o qualunque cosa tu faccia, quando desideri una cosa con volontà, è perché questo desiderio è nato nell'anima dell'Universo. Quella cosa rappresenta la tua missione sulla terra. [...]

l'Anima del Mondo è alimentata dalla felicità degli uomini. O dall'infelicità, dall'invidia, dalla gelosia. Realizzare la propria Leggenda Personale è il solo dovere degli uomini. Tutto è una sola cosa. E quando desideri qualcosa, tutto l'Universo cospira affinché tu realizzi il tuo desiderio.

Paulo Coelho "L'alchimista"

Grazie a tutti coloro che mi hanno appoggiata e aiutata a realizzare la mia Leggenda Personale!

Chiara