Mesenchymal Stromal Cell (MSC)-Based Control of Angiogenesis and Inflammation in Cartilage Formation

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von
Carolina Maria Medeiros da Cunha
aus Brasilien

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auf Antrag von

Prof. Dr. Markus Affolter
Prof. Dr. Ivan Martin
Prof. Dr. Marcy Zenobi-Wong

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Prof. Dr. Jörg Schibler
(Dekan)

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“A porta da verdade estava aberta,
mas só deixava passar
meia pessoa de cada vez.

Assim não era possível atingir toda a verdade,
porque a meia pessoa que entrava
só trazia o perfil de meia verdade.
E a sua segunda metade
voltava igualmente com meio perfil.
E os meios perfis não coincidiam.

Arrebentaram a porta. Derrubaram a porta.
Chegaram ao lugar luminoso
onde a verdade esplendia seus fogos.
Era dividida em metades
diferentes uma da outra.

Chegou-se a discutir qual a metade mais bela.
Nenhuma das duas era totalmente bela
e carecia optar. Cada um optou conforme
seu capricho, sua ilusão, sua miopia.”

Carlos Drummond de Andrade
“Verdade”

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Introduction

1. Cartilage

The human body is a formidable and complex structure. It comprises collections of various highly organized tissues, each being indispensable for the optimal functioning of the body. Throughout the outline of this thesis, we will focus on cartilage, a type of connective tissue that provides mechanical support and elasticity for the biomechanical aspects of human physiology.

1.1. Types of Human Cartilage

Cartilage is a specialized connective tissue that provides biomechanical support for many bodily structures, found mainly in the joints, rib cage, ear, nose, bronchi, and intervertebral discs. Cartilage is essentially avascular; it is comprised solely of chondrocytes, which are specialized cells that are confined to predetermined cavities (lacunae) dispersed throughout a collagen and proteoglycan-rich extracellular matrix (ECM).

The stability required for embryonic development is provided by cartilage. During this time, cartilage is also responsible for providing a template for myogenesis and neurogenesis to occur. Later in development and in adult life, most of the embryonic cartilage is replaced by bone, in a process denominated endochondral ossification (Horton *et al.,* 1988, Mackie *et al.,* 2008).

In order for endochondral ossification to occur, chondrocytes must progressively acquire a hypertrophic phenotype, no longer remaining quiescent and stable. Hypertrophy of chondrocytes is characterized by cell enlargement, type X collagen production onset (Castagnola *et al.,* 1986), increased alkaline phosphatase activity,

and the reduction of type II collagen and proteoglycan synthesis. Moreover, protease inhibitors, which prevent vascular invasion, also have their expression greatly reduced. The tissue becomes vascularized, and the hypertrophic chondrocytes undergo apoptosis followed by their replacement with osteoblast, which, in turn, will deposit bone matrix in the now unoccupied lacunae.

There are three major types of cartilage – hyaline, fibrous, and elastic – which can be distinguished depending on their biomechanical composition, mechanical properties and ECM structure (Buckwalter and Mankin, 1998). The main aspects of each type are briefly discussed in the next sections.

Hyaline Cartilage

Found in the nose, larynx, trachea, bronchi, articular ends of long bones, and ventral ends of ribs, hyaline cartilage is the most abundant type of cartilage. It provides flexible support in the nose and ribs and can sustain mechanical loading during motion. Hyaline cartilage is characterized by an arrangement of chondrocytes in multicellular stacks rich in type II collagen and proteoglycan matrix.

Fibrocartilage

Fibrocartilage is found in locations which are under mechanical stress (i.e. tendon, ligaments, between intervertebral discs), but that also require flexibility in body movements. It consists mainly of type I collagen fibers, which are aligned in thick bundles, and chondrocytes arranged in parallel rows between these fibers.

Elastic Cartilage

Consisting of type II collagen and elastin matrix, elastic cartilage is found in the pinna (ear), auditory and Eustachian tubes, larynx, and epiglottis. Due to its elastic properties, this type of cartilage maintains tube-like structures that are permanently open and provide intermediate mechanical stability.

1.2. Cartilage Development

Cartilage development is a well-defined, controlled, multistep differentiation process of mesenchymal cells (Cancedda *et al.,* 1995; Cancedda *et al.,* 2000; Olsen *et al.,* 2000). Precise and distinct patterns of cell differentiation and arrangement are required for the establishment of cartilaginous structures. Biomechanical factors (Heegaard *et al.,* 1999), cell-to-cell and cell-to-matrix interactions, and morphogen-mediated signaling (Ganan *et al.,* 1996, Vogel *et al.,* 1996) are among the main factors that guide and coordinate cartilage formation.

During development, mesenchymal precursor cells migrate from the lateral mesoderm towards the presumptive skeletogenic site and determine the cartilage anlagen (Hall & Miyake, 2000). Epithelial-mesenchymal interactions result in mesenchymal condensation. The pre-chondrogenic condensation is a prerequisite for the establishment of the limb skeleton (Thorogood & Hinchliffe, 1975) and is associated with an increased cell-to-cell contact, which facilitates the intercellular communication and the transfer of small molecules between cells (Coelho & Kosher, 1991). High cellular density is pivotal to allow chondrogenic development (Ahrens *et al.,* 1977). The level of condensation correlates with the stage of the chondrogenic development (DeLise *et al.,* 2000). Cell-matrix interactions are also key to

mesenchymal condensation (Dessau *et al.,* 1980), such as integrin-mediated binding of chondrocytes to collagen, which is essential for chondrocyte survival (Cao *et al.,* 1999; Lee *et al.,* 2004). The overt differentiation of immature cells to fully committed chondrocytes is observed by increased cell proliferation and by up-regulation of specific cartilaginous matrix components (i.e. type IIα1, IX, and XI collagen and aggrecan). To maintain the chondrogenic phenotype, chondrocytes reduce their proliferative activity and maintain their functional integrity.

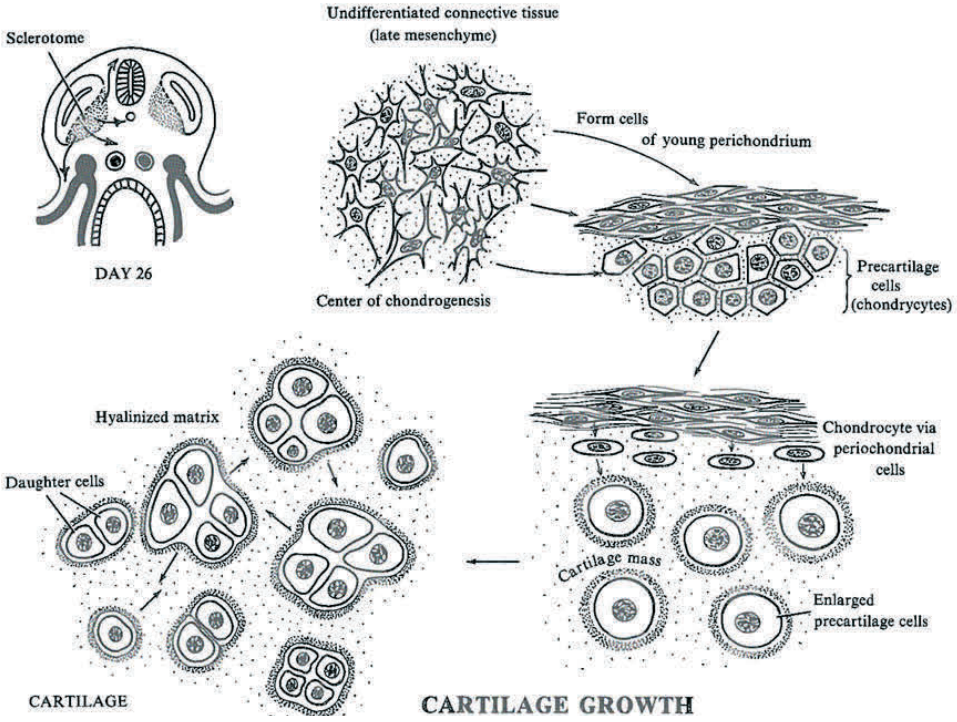

Figure 1. Cartilage development. Figure adapted from the book "Review of Medical Embryology" by Ben Pansky, Chapter 64: Development of the Skeletal and Articular Systems: Cartilage and Bone Histogenesis.

Soluble Growth Factors in Cartilage Development

A significant number of growth factors and morphogens are involved in cartilage formation. The most prominent factors belong to the TGF-β family of factors

(transforming growth factor), including TGF-β1 (involved in chondrocyte proliferation), TGF-β3 and BMP (bone morphogenetic protein), both involved in the terminal differentiation of chondrocytes (Thorp *et al.,* 1992). BMP is also necessary to promote cell-to-cell interactions in the early stages of chondrogenesis (Chen *et al.,* 2004). Cartilage formation is also governed by the IGF family of peptide hormones (insulin-like growth factor), mainly by IGF-1, which regulates various cellular functions, such as chondrocytic differentiation and proliferation (Oh & Chun, 2003; Phornphutkul *et al.,* 2004). IGF-1 promotes and maintains the anabolic synthesis of type II collagen and proteoglycans in mature cartilage (Martel-Pelletier *et al.,* 1998). The FGF (fibroblast growth factor) family of morphogens influence processes correlated with cell division and chondrocyte proliferation (Olney *et al.,* 2004). The orchestrated combination of defined concentrations of these morphogens, and other factors such as hypoxic environment (discussed further) at precise developmental stages are imperative for chondrogenesis to take place.

Oxygen Tension in Cartilage Development

Cartilage is essentially an avascular tissue. During development, chondrocytes are exposed to a low oxygen environment, termed hypoxia (Brighton & Heppenstall, 1971). Schipani and colleagues demonstrated that hypoxia-associated signals have a central role in chondrocyte differentiation and cartilage development (Schipani *et al.,* 2001). Importantly, hypoxia inducible factor 1α (HIF-1α) has been shown to be expressed in the growth plate and to have adaptive response to hypoxia. It can form an active transcriptional complex and up-regulate target genes, such as angiogenic factors and type II collagen (Pfander *et al.,* 2003). Hence, beyond conventional

growth factor mediated signals, hypoxia is critical in regulating chondrocyte differentiation and in increasing matrix deposition.

1.3. Mechanical Environment in Mature Cartilage

Cartilage tissue is exposed to stressful physiological and mechanical loading during joint movement. Specific arrangement of macromolecules in the ECM is required for the proper cartilage deformation during body movements. The viscoelastic properties of cartilage are due to its principal phases, which includes a solid phase composed of dense fibrous collagen network with negatively charged proteoglycan aggregates, an aqueous phase and an ionic phase consisting of ions, which neutralize the charged ECM components (Lai *et al.,* 1991; Mow *et al.,* 1999).

1.4. Defects and Self-Repair Capacity of Cartilaginous Tissue

Cartilage lesions can be caused by trauma, disease, or as a result of instability or abnormal loading. These factors are commonly associated with joint pain, reduced mobility, and loss of function, and can ultimately lead to severe conditions, such as osteoarthritis. Due to its avascular nature and low mitotic activity of chondrocytes, the self-repair capacity of cartilage is considerably limited. There are different grades of cartilage injury according to the size of tissue damage, namely partial and full thickness defects. According to the extent of the damage, different healing responses can be verified.

In partial thickness defects, the injured cartilage is capable of self-repair, first undergoing necrosis, followed by proliferation of chondrocytes that are found on the lesion site. In the short run, chondrocyte aggregates temporarily produce type II

collagen; however, in the long run, the newly formed tissue shows loss of hyaline cartilage characteristics. Hence, these lesions remain almost unchanged and can proceed towards osteoarthritic diseases (Hunziker *et al.,* 1999).

Full thickness defects are characterized by subchondral damage, in which the lesion gains access to the cells that reside in the bone marrow. The repair response elicited by this type of defect results in the formation of a fibrocartilaginous tissue in the defect area. The decreased deposition of extracellular matrix components and the formed tissue lack the strength and the mechanical properties necessary for optimal joint function.

Both mechanisms of spontaneous self-healing show great limitations. The quality of the healed tissue is suboptimal for its functions and can greatly increase the risk of tissue and joint degeneration (Buckwalter *et al.,* 2005). Therefore, regenerative approaches that are able of restoring the functional properties of cartilage are of crucial importance in order to hinder the progression of cartilage damage and initiation of joint disease.

1.5. Current Treatments for Cartilage Defects

The different strategies to treat cartilage defects vary from more conservative measures, i.e. physiotherapy and drug administration (corticosteroids, hyaluronic acid, growth factors), to more invasive procedures, which are further discussed in the next sections.

1.5.1. Arthroscopic Repair

Arthroscopic repair involves lavage and debridement. Lavage is the irrigation of the joint during arthroscopy; debridement is the removal of the damaged tissues from the joint (Chang *et al.,* 1993, Hubbard *et al.,* 1996). These procedures, however, do not promote cartilage repair, but only alleviate painful joint conditions. Other arthroscopic surgical procedures, such as drilling, abrasion, and microfracture, are used to induce repair. These techniques take advantage of the intrinsic repair response observed upon penetration of the subchondral bone in full thickness defects, relying upon the formation of a blood clot and mesenchymal progenitor cell invasion. The clinical outcome, however, is poor and varies with age and activity levels of the patient.

1.5.2. Osteochondral Transfer

As common standard practice for osteochondral defect repair, autologous and allogeneic tissue transplantation has been widely in clinical practice. Allogeneic material is of cadaveric origin and is indicated for large post-traumatic defects of joints (Beaver *et al.,* 1992). On the other hand, autologous graft implantation involves the removal of cylindrical plugs of osteochondral tissue from non-load bearing sites and its implantation into the defect. Functional cartilage surface is re-established, but it is limited due to poor tissue integration with the adjacent native tissues. The surgical intervention also damages intact host tissue and enhances donor morbidity (Hangody *et al.,* 2008), which imposes serious limitations to this type of tissue repair technique.

1.5.3. Autologous Chondrocyte Implantation (ACI) and Matrix-Induced Autologous Chondrocyte Implantation (MACI)

In 1987, Brittberg and colleagues introduced autologous chondrocyte implantation (ACI) (Brittberg *et al.,* 1994) as a clinically effective method proven to restore tissue structure of large full-thickness focal defects of the femoral chondyle (Peterson *et al.,* 2000). The technique consists of arthroscopic harvesting of a small cartilage biopsy from a non-load bearing area and subsequent transplantation of in vitro expanded autologous chondrocytes. ACI largely improves joint functionality and reduces the pain associated with tissue damage. However, due to complexity and morbidity associated with this procedure, ACI is correlated with several limitations. The in vitro expansion of chondrocytes is necessary to obtain sufficient amount of cells for transplantation purposes, but is often associated with de-differentiation and loss of chondrogenic phenotype.

Matrix-induced autologous chondrocyte implantation (MACI) derives from ACI, in which the expanded cells are seeded directly onto a biomimetic scaffold. This approach offers potential advantages, such as improved cell retention, even distribution of cells and easy graft handling (Behrens *et al.,* 2006; Pelttari *et al.,* 2009).

1.5.4. Cartilage Tissue Engineering

Tissue engineering is an innovative field of research, which has emerged with the needs for three-dimensional tissue repair. Patient cells are harvested, expanded, and cultured under optimal conditions for subsequent seeding onto scaffolds prior to in vivo implantation. Often, bioreactors allow for ideal incubation and culture

conditions. Several factors play a major role in the outcome of successful tissue engineering, namely the selection of the cell source to be used, biomaterial choice, and the use of bio-inductive molecules, such as growth factors, hormones, cytokines, glucose, and oxygen, which are to be supplemented to the culture media, or physical stimuli. These factors will be briefly discussed on the following sections.

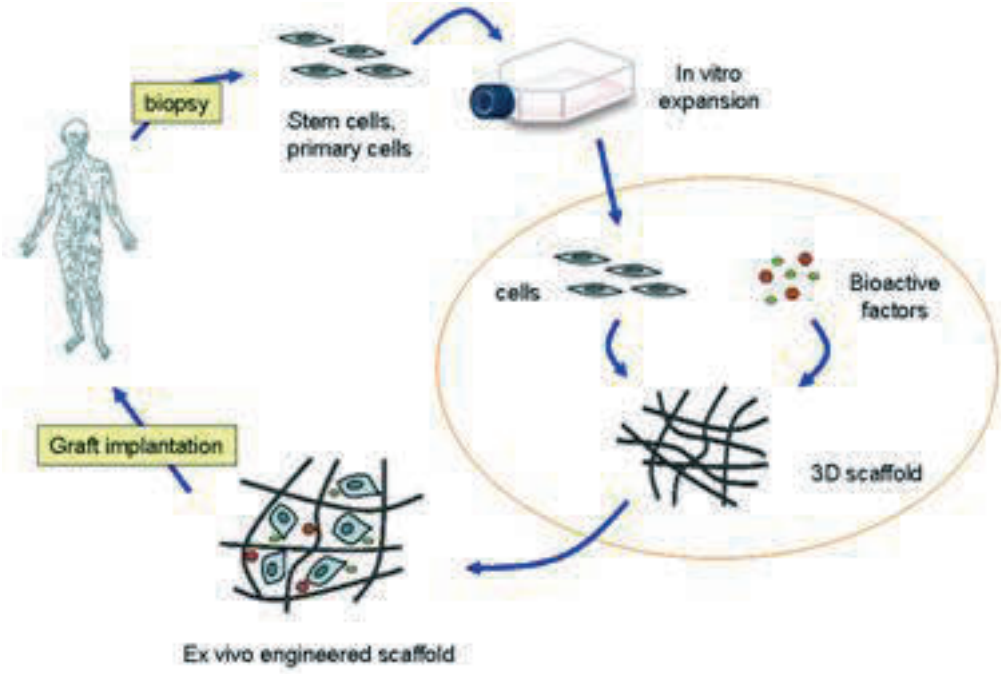

Figure 2. A combination of cells, scaffolds, and biological factors comprise the Tissue engineering approach. Figure adapted from the National University Health System, Singapore, on the website: http://www.nuhs.edu.sg/research/programmatic-research/som-registered-programmes/nus-tissue-engineering-programme.html.

Cell Sources

To be considered as an adequate cell source for cartilage engineering, the cell type of choice must not provoke host immune reaction, not induce tumor formation, be able to integrate itself within the site of implantation, provide sufficient number of cells (high cellular density) to improve cartilage repair, be associated with low donor

morbidity and, very importantly, be able to acquire or differentiate into a chondrogenic phenotype.

Xenogeneic or allogeneic cells constitute an almost unlimited source for cells but it is largely associated with unwanted immune effects (Platt *et al.,* 1996). To overcome this problem, autologous cell harvesting is the most reliable cell source of choice. Hence, autologous mesenchymal stem (or stromal) cells and chondrocytes are the first line of choice for cartilage tissue engineering.

Undifferentiated, multipotent mesenchymal stem or stromal cells (MSC) are a population of heterogeneous cells that derive from the mesoderm (Caplan *et al.,* 1991). They can be expanded in vitro and kept in their undifferentiated state when maintained under appropriate culture conditions (Pittenger *et al.,* 1999, Reyes *et al.,* 2001). Due to their ease of harvest, proven capability to undergo chondrogenesis (Pittenger *et al.,* 1999), and multistep possibilities to endure controlled differentiation under determined circumstances, MSC are a favorable and very attractive cell source for cartilage engineering purposes. In particular, MSC chondrogenesis occurs when these cells are cultured in 3D aggregates or "pellets" (Johnstone *et al.,* 1998; Yoo *et al.,* 1998) or cultured on porous polymers in the presence of TGF-β3 (Martin *et al.,* 2001). Moreover, it has been demonstrated that bone marrow-derived MSC migrate to a cartilage defect after micro-fracture of the subchondral bone and are able to differentiate in vitro into chondrocytes after specific culture conditions (Krame *et al.,* 2006).

Evidently, chondrocytes are also indicated for cartilage repair. Primary chondrocytes isolated from cartilage can be successfully maintained in vitro (Guerne *et al.,* 1995; Quarto *et al.,* 1997). The application of different growth factors during 2D expansion

enables the cells to proliferate and, once exposed to growth factors, to progressively lose their typical differentiated phenotype and acquire a fibroblast-like shape. The exposure of chondrocytes to a variety of growth factors (e.g. FGF-2, TGF-β1 and/or TGF-β3) can improve the capacity of chondrocytes in regaining their differentiated state (Barbero *et al.,* 2003). Chondrocytes produce type II collagen and aggrecan (Brittberg *et al.,* 2003), which is crucial for ECM reconstitution. Chondrocytes have limitations in supply, however, and must also be expanded in order for an adequate number of cells to be available for repair purposes. Chondrocytes are commonly harvested from the articular joint, ear, and nose.

Both MSC and chondrocytes undergo hypertrophy, which is detrimental for hyaline cartilage repair, as it conducts the cells to endochondral ossification, which ultimately remodels cartilage into bone. To maintain a cartilaginous phenotype of these cells, specific biomaterials and morphogens are used to circumvent the possibility of endochondral ossification transformation of chondrocytes and MSC.

Scaffolds for Tissue Generation

Biomaterials (scaffolds) mimic the 3D environment of the cartilage ECM, provide structural support to the forming tissue and appropriate surface area for cellular migration, adhesion, and differentiation (Capito *et al.,* 2003). The biomaterial must be biodegradable, biocompatible, have a defined 3D structure with appropriate porosity to allow for cell invasion, tissue growth, nutrient transport, and metabolic waste removal. It should also detain mechanical stability for in vitro handling and subsequent surgical implantation procedures and provide a suitable surface chemistry to allow for absorption of proteins to improve chondrocyte attachment,

proliferation, or differentiation (Bonzani *et al.,* 2006). Porous sponges are commonly used (Putnam *et al.,* 1996). Other biomaterials, such as collagen and fibrin-based scaffolds, agarose, alginate, chitosan, and hyaluronan have also shown promising results in its utilization as scaffolds (Getgood *et al.,* 2009).

Culture Media Supplementation

During in vitro culture of chondrogenic cells, specific growth factors, cytokines, hormones, and vitamins can enhance cell proliferation, migration and differentiation, and in consequence allow for the obtainment of cells fully capable of repairing cartilage.

Growth factors and cytokines are secreted molecules which, when bound to membrane receptors, can elicit intracellular signaling pathways responsible for cell adhesion, proliferation, and differentiation, through specific gene targeting.

FGF plays a key role in chondrocyte proliferation (Kato *et al.,* 1983). It also promotes its de-differentiation process (Martin *et al.,* 2001) and prevents its terminal differentiation. TGF-β, PDGF, and IGF-I are also commonly used supplements known for their ability to induce chondrogenic differentiation, either by aggrecan and type II collagen up-regulation, ECM synthesis, or chemo-attractant properties (Han *et al.,* 2005, Holland *et al.,* 2003). Alongside with the classical growth factors mentioned, ascorbic acid is commonly used as an enzymatic cofactor for the assembly and stabilization of collagen fibrils (Meier *et al.,* 1978).

2. Mesenchymal Stromal/Stem Cells

Stem cells are defined by their self-renewal capacity, producing at least one identical stem cell and one cell that differentiates further (McGuckin *et al.,* 2008). Stem cells can be identified as totipotent (able to produce all cells and tissues of an organism), pluripotent (produce cells and tissues from all 3 germ layers – ectoderm, mesoderm, and endoderm), multipotent cells (ability to produce more than one cell lineage), and unipotent (can differentiate only into a single cell phenotype) (Forraz *et al.,* 2011), regarding their potency to produce one or more lineages.

Mesenchymal stromal/stem cells (MSC) are a heterogeneous population of multipotent cells that can be found in most of the tissues in the human body. These cells display a fibroblastic morphology; they are also characterized by their capacity to rapidly adhere to plastic and by their rapid proliferative potential. MSC were first termed osteogenic stem cells by Friedenstein (Friedenstein *et al.,* 1987). In 1991, Caplan defined these cell populations as mesenchymal stem cells (Caplan *et al.,* 1991). Regarding isolation, availability, and clinical applications, the most relevant sources for MSC are bone marrow, skin, placenta, and adipose tissue (Chen *et al.,* 2008). The Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy have proposed three criteria to define MSC: (1) isolated cells should adhere on tissue culture plates; (2) more than 95% of adhering cells in the culture should express CD105, CD73, and CD90, and they should lack the expression of CD34, CD45, CD14, CD11b, CD79a, CD19, and HLA markers; (3) MSC should differentiate into osteoblasts, adipocytes, and chondrocytes in vitro (Dominici *et al.,* 2006).

The bone marrow is a highly vascularized tissue contained within the cavity of long bones and in the intra-trabecular spaces of spongy bones. Besides containing hematopoietic precursors, the bone marrow contains different non-hematopoietic cells including, reticular and endothelial cells, fibroblasts, osteoblasts, and mesenchymal progenitors. These cells constitute the bone marrow stroma, which provides structural and humoral signals, which regulate stem cell function.

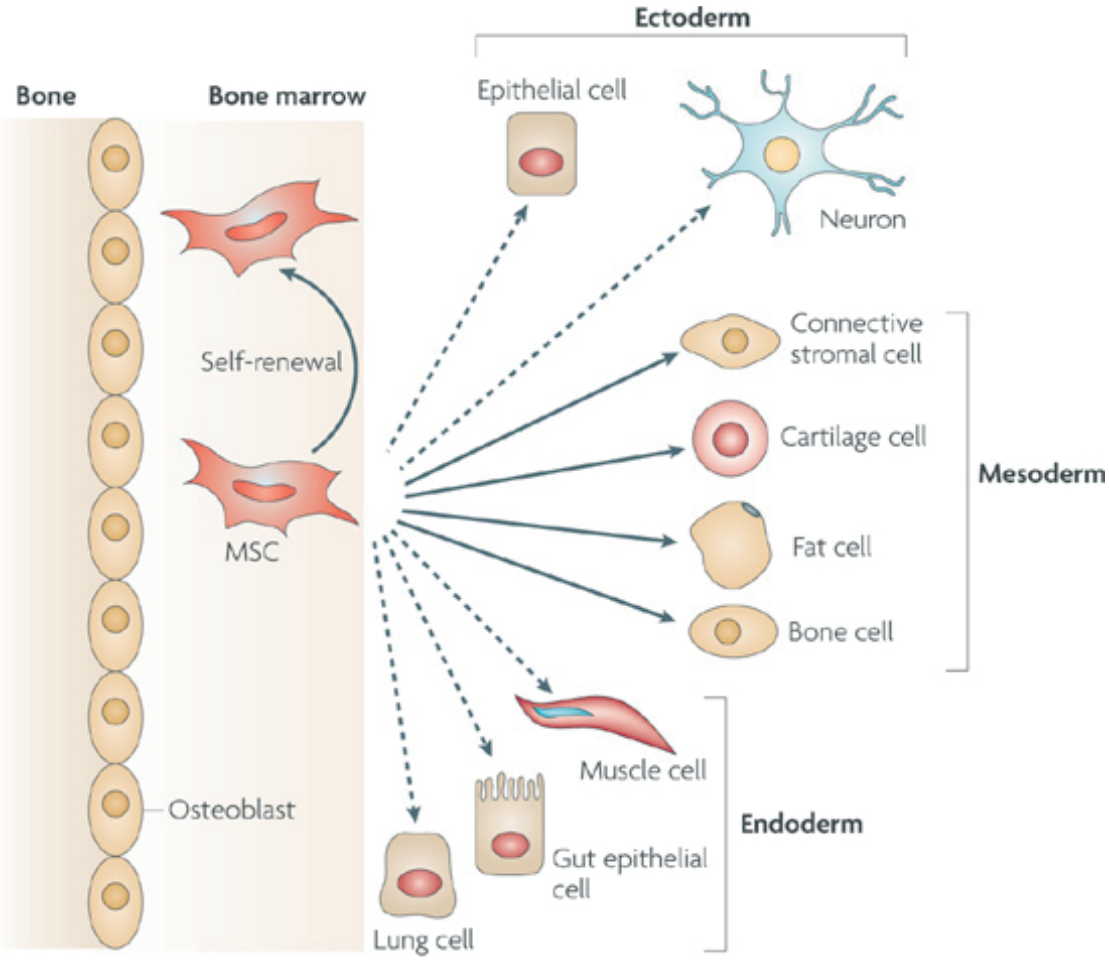

Figure 3. The multipotent aspects of mesenchymal stem cells (MSC). Figure adapted from Uccelli *et al.,* 2008.

The frequency of putative stromal cells, however, is less than 0.01% (Pittenger *et al.,* 1999), therefore requiring extensive *in vitro* expansion phases to be applied in tissue engineering applications. Various in vitro studies, preclinical and, more recently, human clinical trials have demonstrated the immunotolerance and immunomodulatory properties of allogeneic MSC (Patel *et al.,* 2008).

2.1 Clinical Applications of MSC

After the discovery of their biological property of differentiating into the common mesenchymal lineages, MSC were thought to be responsible for the normal turnover and maintenance of adult mesenchymal tissues (Caplan *et al.,* 2005). This led to the identification of MSC as an attractive cell source for therapeutic applications in different fields of regenerative medicine.

The most obvious application was to apply MSC for the regeneration of mesenchymal tissues, such as bone and cartilage. In fact, first among the others, tissue engineers tried to exploit MSC properties for the repair of bone defects and for the treatment of various bone disorders. Up to date, autologous bone graft still represents the first choice for site-specific bone defect repair, although it is associated with several complications such as donor site morbidity, infection, and loss of graft function (Hollinger *et al.,* 1996). The combination of a biomaterial and ex-vivo expanded MSC is thought to represent a valid alternative to functionally replace host bone tissue. Novel approaches have been developed to improve the performance of the engineered constructs: biomimetic material properties, including surface roughness and porosity, have been investigated to enhance MSC adherence, proliferation, and differentiation (Wilson *et al.,* 2004; Mante *et al.,* 2003; Behravesh

et al., 2003). Importantly, expanded MSC have been used as a potential treatment for cartilaginous injuries in humans (Wakitani *et al.,* 2004; Kuroda *et al.,* 2007). In summary, MSC represent a precious cellular source for tissue regeneration, showing strong regenerative potential in clinical applications.

3. Angiogenesis

3.1 The Importance of Angiogenesis

Vertebrates have very complex body structures that constantly require optimal delivery of nutrients, signaling cues, gases, and cells between the various tissues of their bodies. In order for this to happen, a highly organized network of branched blood vessels has appeared and progressed throughout evolution. Indeed, blood vessels constitute the first organ in the embryo to form, and, together, constitute the largest network in the adult body.

Two different mechanisms are accountable for the formation of new blood vessels. Vasculogenesis is the de novo assembly of vessels by endothelial precursor cells or angioblasts (vasculogenesis), which is largely confined to the formation of the first primitive vascular structures of the embryo, whereas angiogenesis involves enlargement of the vasculature through sprouting, proliferation and remodeling. Angiogenesis, unlike vasculogenesis, is the main process for the formation of the vast majority of blood vessels during development, tissue repair, or pathological processes.

Angiogenesis is of uttermost importance for normal tissue development, growth, maintenance, and repair. Most of the tissues in the human body require high levels

of oxygen and nutrient delivery in order to maintain its normal functioning. In some tissues, e.g. cartilage, angiogenesis is thought to be highly detrimental, since cartilage is essentially avascular and hypoxic in nature. In diseased states of cartilage, such as in osteoarthritic cartilage, angiogenesis starts to take place and vessel invasion progressively deteriorate the once healthy tissue. Hence, it is of utmost importance for angiogenesis to be controlled in order to establish homeostasis in the various tissues in the human body.

3.2. Mechanisms of Angiogenesis

For angiogenesis to occur in adults, local ischemia is required. In response to low oxygen tensions, the oxygen-sensitive hypoxia inducible factor 1α (HIF1α) upregulates many factors involved in angiogenesis, being vascular endothelial growth factor (VEGF) the most important. It was initially postulated that VEGF might play an important role in regulating the physiological and also pathological angiogenesis. VEGF was first identified in 1989 as the diffusible potent inducer of angiogenesis (Leung *et al.,* 1989; Keck *et al.,* 1989). Although VEGF is the key regulator of angiogenesis, other factor may also participate in the process; being angiogenesis a highly complex process, other factors must also coordinate the events in order to promote optimal blood vessel formation. Among other factors that participate in the regulation of angiogenesis, angiopoietins, tumor growth factor β (TGF-β), fibroblast growth factor (FGF), placental growth factor (PlGF) and hepatocyte growth factor (HGF) have been found to have pro-angiogenic effects (Korpisalo *et al.,* 2010).

VEGF is the key player in angiogenesis. It participates in the embryonic and early postnatal development of the vascular system. Besides, angiogenesis, VEGF also plays an important role in other physiological processes, such as corpus luteum development and endochondral bone formation (Ferrara *et al.,* 2003). VEGF dysregulation, on the other hand, is associated with various pathological conditions. VEGF also plays a role in tumor angiogenesis (Dvorak *et al.,* 1995). In vivo studies have shown that inhibiting VEGF activity in tumors results in suppression of their growth in animal models. Several clinical trials using small molecule inhibitors or humanized monoclonal antibodies (Presta *et al.,* 1997; Wood *et al.,* 2000) have confirmed these findings. Other diseases, such as retinopathy (Adamis *et al.,* 1994) and age related macular degeneration (Kvanta *et al.,* 1996) also correlate to uncontrolled vascularization.

3.3. VEGF Molecules and their Receptors

Five different polypeptides comprise the mammalian vascular endothelial growth factor (VEGF) family, namely VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placental growth factor (PIGF) (Tammela *et al.,* 2005). These secreted glycoproteins are homodimeric polypeptides that belong to the PDGF family of growth factors. In other non-mammalian species, other VEGF-related molecules can be found, such as VEGF-E, which is encoded by viruses of the Orf family, and VEGF-F, which has been isolated from the venoms of *Viperinae* snakes. VEGF-A, VEGF-B, and PIGF are predominantly required for blood vessel formation, while VEGF-C and VEGF–D are essential for the formation of lymphatic vessels (Cèbe-Suarez *et al.,* 2006).

The transcriptional control of VEGF is under the control of hypoxia-inducible factor-1 (HIF-1), which is a heterodimeric transcription factor composed of two subunits, HIF-1α and HIF-1β. HIF-1α is rapidly degraded under normoxic conditions through hydroxylation of proline residues, ubiquitination and targeting to the proteasome. Under hypoxia, HIF-1α is stabilized and dimerizes with HIF-1β. This complex binds and activates the hypoxia-responsive element (HRE) in the VEGF promoter (Nagy *et al.,* 2007).

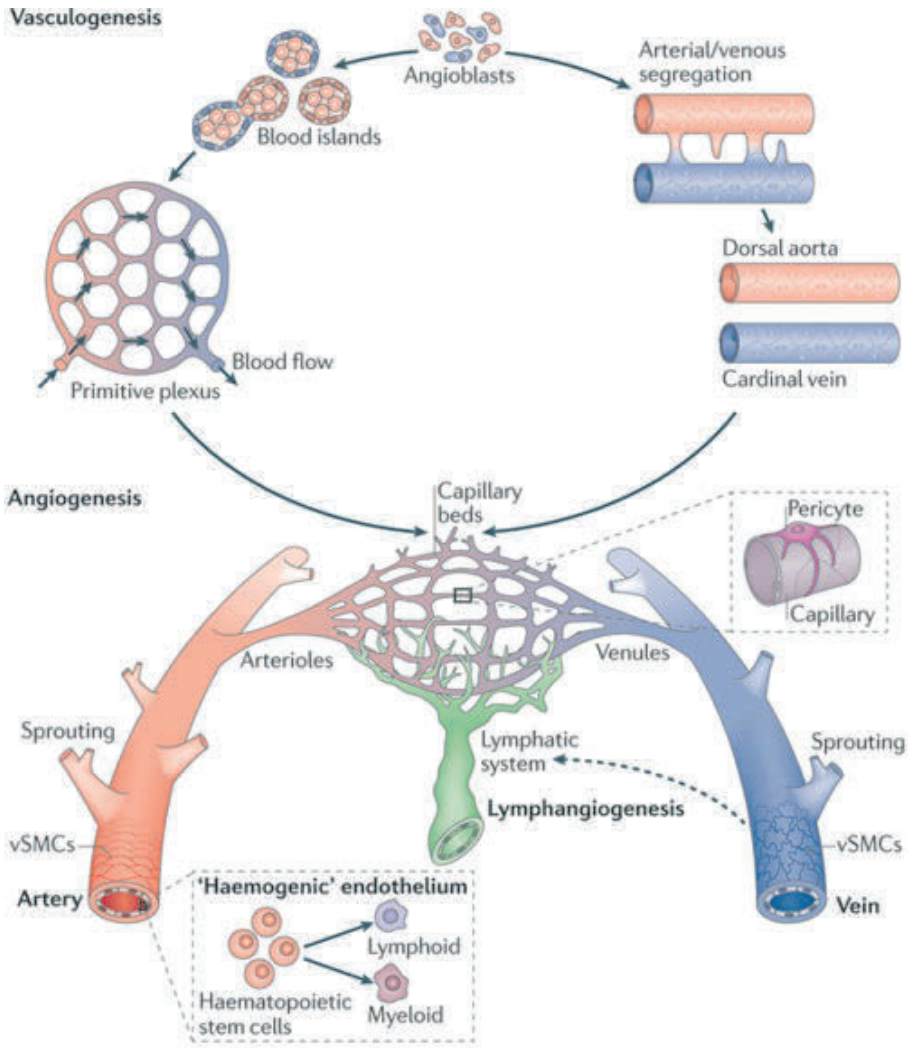

Figure 4. Vasculature development from endothelial progenitor cells. Figure adapted from Herbert *et al.,* 2011.

Among the mammalian VEGFs, VEGF-A (commonly reported as VEGF) is the most potent and best-characterized isoform of VEGF. It is a heparin-binding, disulfide-linked dimeric glycoprotein, which plays a pivotal role in both physiological and pathological angiogenesis. Alternative splicing provides further isoforms of VEGF-A. These isoforms differ from each other by the presence or absence of two domains with affinity for heparin and heparin sulfate. The length of the heparin-binding domain is crucial for determining the microenvironmental localization of VEGF.

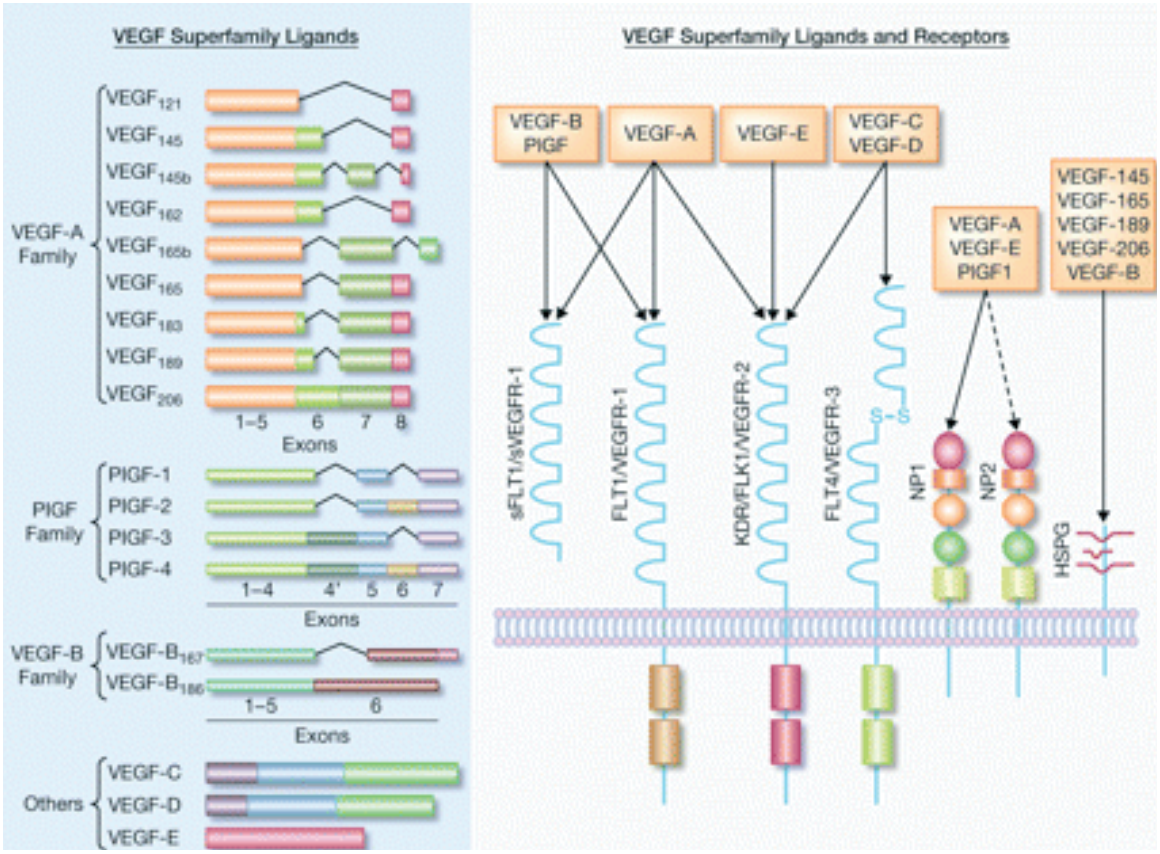

Figure 5. The VEGF family of receptors and ligands. Figure adapted from Rahimi, 2012.

Among the VEGF-A isoforms, VEGF183, VEGF189 or VEGF206 are longer and have strong heparin-binding domains characteristics, therefore attaching themselves

strongly to the ECM. Shorter isoforms, such as VEGF121 or VEGF145, lack these sequences and are therefore diffusible. The most biologically active VEGF-A is VEGF165 (VEGF164 in mice), which binds heparin with an intermediate affinity and thus sticks to the ECM with a frequency of 50-70% (Ferrara *et al.,* 2003; Houck *et al.,* 1992).

VEGF exerts its biological functions upon binding to type III receptor tyrosine kinases, VEGFR1 (Flt-1), VEGFR2 (Flk-1/KDR), and VEGFR3 (Flt4). These receptors are expressed on the cell surface or endothelial cells, vascular smooth muscle cells, bone marrow-derived hematopoietic precursors, macrophages, and malignant cells (Ferrara *et al.,* 2004). All VEGFRs share the same structure and are composed by an extracellular domain organized in seven immunoglobulin (Ig)-like folds, followed by a single transmembrane region, a juxta-membrane domain, a split tyrosine-kinase domain interrupted by a kinase insert and a C-terminal tail.

VEGFR1 and VEGFR2 are important in blood vascular endothelial cell proliferation, migration, and survival. Mice that are homozygous for either of the two receptors die during early development due to defects in vasculogenesis and angiogenesis. When lacking VEGFR2, embryos die without mature endothelial or hematopoietic progenitor cells. On the other hand, mice that are deficient in VEGFR1 have normal hematopoietic progenitor cells and endothelial cells that migrate and proliferate but do not assemble into tubes and functional vessels (Jussila *et al.,* 2002).

VEGFR1 binds VEGF-A, VEGF-B, VEGF-F, and PIGF and is expressed in two variants: a full-length, membrane-bound form and a soluble form (sFlt1), considered a decoy receptor. VEGFR1 has the highest affinity for VEGF; however, its kinase activity is weak, characterized by a lack of mitogenic response in endothelial cells upon VEGF

stimulation (Hoeben *et al.,* 2004). It is believed that VEGFR1 might act as a negative regulator of vascular development, since VEGFR1 knockout animals display embryonic lethality and increased cell proliferation. Thus, VEGFR1 activity may dampen the proangiogenic effects of VEGFR2 activation (Fong *et al.,* 1995; Kearney *et al.,* 2002; Dvorak *et al.,* 2002).

VEGFR2 is the first marker to appear during development. This receptor binds VEGF-A, VEGF-C, VEGF-D, VEGF-E, and VEGF-F. It binds VEGF-A with less affinity than VEGFR1, but shows a strong kinase activity that results in mitogenic, survival and chemotactic signals in several cell types. VEGFR2 is expressed in higher copy number than VEGFR1, but has an approximately ten-fold lower affinity for VEGF-A; however it is thought that VEGFR2 is the major receptor transducing VEGF-A signaling in endothelial cells (Ferrara *et al.,* 2004). VEGFR2 absence is lethal: embryos lacking VEGFR2 die and fail to form blood islands and to complete hematopoietic and endothelial development.

VEGFR3 binds VEGF-C and VEGF-D and it is expressed in lymphatic endothelial precursors and mature cells as well as in the blood vascular endothelial cells. It is considered an important regulator of lymphangiogenesis (Karpanen *et al.,* 2008). In embryos, VEGFR3 is present throughout the vasculature, but during development its expression becomes restricted to lymphatic vessels.

In addition to the VEGF receptor tyrosine kinases, two non-kinase receptors, neuropilin-1 and -2 (Nrp-1, Nrp-2) also interact with members of the VEGF family, namely VEGF-A and VEGF-B. These receptors are expressed not only on vascular endothelium but also on many types of normal and tumor cells. They have long been

known as receptors for the semaphoring/collapsing family of neuronal guidance mediators (Staton *et al.,* 2007).

4. Inflammation

4.1. Overview of the Inflammatory Response

The acute inflammatory response triggered by infection or tissue injury involves the coordinated delivery of blood components (plasma and leukocytes) to the site of infection or injury. The initial recognition of infection or injury is mediated by tissue-resident macrophages and mast cells, leading to the production of a variety of inflammatory mediators, including chemokines, cytokines, vasoactive amines, eicosanoids, and products of proteolytic cascades. The main and most immediate effect of these mediators is to elicit an inflammatory exudate locally: plasma proteins and leukocytes (mainly neutrophils) that are normally restricted to the blood vessels now gain access, through the postcapillary venules, to the extravascular tissues at the site of injury (or infection). The activated endothelium of the blood vessels allows selective extravasation of leukocytes while preventing the exit of erythrocytes. This selectivity is afforded by the inducible ligation of endothelial-cell selectins with integrins and chemokine receptors on leukocytes, which occurs at the endothelial surface as well as in the extravascular spaces.

A successful acute inflammatory response results in the elimination of the inflammatory agent (e.g. tissue damage) by a resolution and repair phase, which is mediated mainly by tissue-resident and recruited macrophages (Serhan *et al.,* 2005). If the acute inflammatory response fails to be resolved, the inflammatory process

persists and acquires new characteristics. The neutrophil infiltrate is replaced with macrophages, and in case of infection also with T cells. If the combined effect of these cells is still insufficient, a chronic inflammatory state ensues, involving the formation of granulomas and tertiary lymphoid tissues (Drayton *et al.,* 2006). Unsuccessful attempts by macrophages to engulf and destroy pathogens or foreign bodies can lead to the formation of granulomas, in which the intruders are walled off by layers of macrophages, in a final attempt to protect the host (Majno *et al.,* 2004; Kumar *et al.,* 2003).

The mononuclear phagocytic system is generated from committed hematopoietic stem cells located in the bone marrow. Macrophage precursors are released into the circulation as monocytes, and within a few days they seed tissues throughout the body, including the spleen, which serves as a storage reservoir for immature monocytes (Geissmann *et al.,* 2010). When monocytes migrate from the circulation and extravasate through the endothelium, they differentiate into macrophages or dendritic cells. Thus, the primary role of monocytes is to replenish the pool of tissue-resident macrophages and dendritic cells in steady state and in response to inflammation. Monocytes, dendritic cells and macrophages, along with neutrophils, are “professional” phagocytic cells.

4.2. Monocytes

Circulating monocytes give rise to a variety of tissue-resident macrophages throughout the body, as well as to specialized cells such as dendritic cells and osteoclasts. Monocytes are known to originate in the bone marrow from a common myeloid progenitor that is shared with neutrophils, and they are then released into

the peripheral blood, where they circulate for several days before entering tissues and replenishing the tissue macrophage populations (Volkman & Gowans, 1965).

During monocyte development, myeloid progenitor cells sequentially give rise to monoblasts, pro-monocytes and finally monocytes, which are released from the bone marrow into the bloodstream. Monocytes migrate from the blood into tissue to replenish long-lived tissue-specific macrophages of the bone (osteoclasts), alveoli, central nervous system (microglial cells), connective tissue (histiocytes), gastrointestinal tract, liver (Kupffer cells), spleen and peritoneum (Gordon *et al.,* 2005).

The morphology of mature monocytes in the peripheral circulation is heterogeneous, and these cells constitute between 5-10% of peripheral blood leukocytes in humans.

In 1939, Ebert and Florey (Ebert & Florey, 1939) reported that monocytes emigrated from blood vessels and developed into macrophages in the tissues. Pro-inflammatory, metabolic, and immune stimuli all elicit increased recruitment of monocytes to peripheral sites (van Furth *et al.,* 1973), where differentiation into macrophages and dendritic cells occurs, contributing to host defense, and tissue remodeling and repair.

Peripheral blood monocytes show morphological heterogeneity, such as variability of size, granularity and nuclear morphology. Monocytes were initially identified by their expression of large amounts of CD14. Differential expression of CD14 and CD16 allowed monocytes to be divided into two subsets: CD14hiCD16- cells, which are often called classic monocytes, because this phenotype resembles the original description of monocytes; and CD14+CD16+ cells (Passlick *et al.,* 1989).

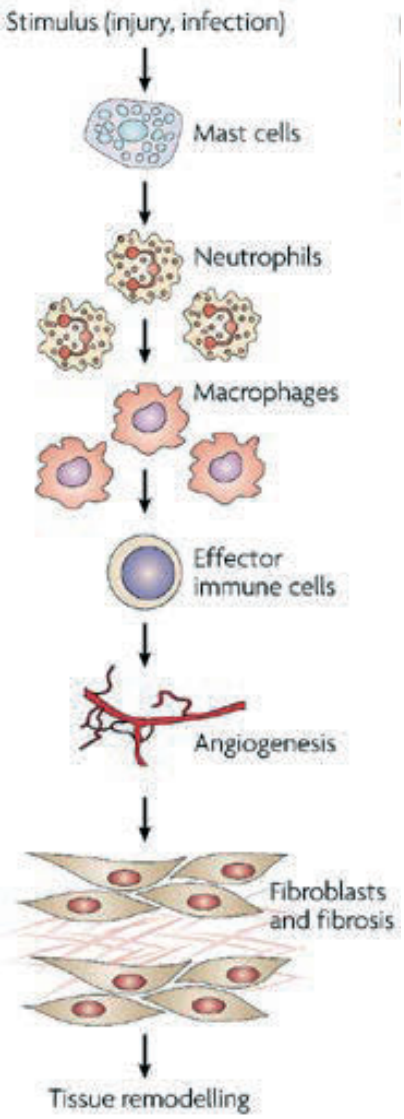

Figure 6. Immediate effects of inflammation on recruitment of mast cells, neutrophils, and macrophages to the lesion site. Figure adapted from de Visser *et al.,* 2006.

It was shown that the CD14+CD16+monocytes expressed higher amounts of MHC class II molecules and CD32, and it was suggested that these cells resemble mature tissue macrophages (Ziegler-Heitbrock *et al.,* 1993). An additional monocyte subset that is defined by the expression of CD14, CD16 and CD64 has also been reported (Grage-Griebenow *et al.,* 2001). Compared with CD14hiCD16- (classic) monocytes

(which are also CD64+), these CD14+CD16+CD64+ cells have a similarly high phagocytic activity and produce similarly large amounts of cytokines (such as TNF and IL6), and these phenotypes are not shared with the CD14+CD16+CD64- subset.

4.3. Macrophages

Macrophages are strategically located throughout the body tissues, where they ingest and process foreign materials, dead cells and debris and recruit additional macrophages in response to inflammatory signals. Tissue macrophages have a broad role in the maintenance of tissue homeostasis, through the clearance of senescent cells and the remodeling and repair of tissues after inflammation (Gordon *et al.,* 1986, 1998). Most macrophages in the tissues of an adult are considered to be derived from circulating monocytes, which constitutively replenish tissue-resident macrophage populations.

Macrophages, unlike monocytes, can be distinguished by their expression of CD11b and CD68. Macrophages are divided into subpopulations based on their anatomical location and functional phenotype. Classically-activated macrophages (M1 macrophages) mediate defense of the host from a variety of bacteria, protozoa and viruses, and have roles in antitumor immunity. M1 macrophages are also believed to participate in various chronic inflammatory and autoimmune diseases (Sindrilaru *et al.,* 2011). Therefore, pro-inflammatory M1 macrophage responses must be controlled to prevent extensive collateral tissue damage to the host. Alternatively-activated macrophages (M2 macrophages) have anti-inflammatory function and regulate wound healing. M2 macrophages exhibit potent anti-inflammatory activity and have important roles in wound healing and fibrosis (Wynn *et al.,* 2004; Xiao *et*

al., 2008). They also antagonize M1 macrophage responses, which may be crucial for the activation of the wound healing response and for tissue homeostasis to be restored (Sindrilaru *et al.,* 2011). Recent studies have also shown that M1 macrophages can "convert" themselves into an M2 wound healing phenotype (Biswas *et al.,* 2010; Arnold *et al.,* 2007).

Classically-activated macrophages (M1) arise in response to IFN-γ (O'Shea *et al.,* 2008), which can be produced during an adaptive immune response by T_H1 cells or CD8+ T cells or in response to TNF during an innate immune response by natural killer (NK) cells. The M1 chemokine secretion profile consists in CCL15, CCL20, CXCL9, CXCL10, and CXCL11 and cytokine profile IL1, IL6, IL12.

Similarly to classically activate macrophages, wound-healing (M2) macrophages can develop in response to innate or adaptive signals. Basophils and mast cells are important early sources of innate IL4 (Brandt *et al.,* 2000). This early IL4 production rapidly converts resident macrophages into M2 macrophages. The M2 chemokine secretion profile consists in CCL17, CCL18, CCL22 and cytokine profile IL10.

M2 macrophages produce growth factors that stimulate epithelial cells and fibroblasts, including TGFβ1 and PDGF (Barron *et al.,* 2011). M2 macrophages also produce matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) that control ECM turnover (Wynn *et al.,* 2008). They also engulf and digest dead cells, debris and various ECM components that would promote tissue-damaging M1 macrophage responses (Barron *et al.,* 2011; Atabai *et al.,* 2009) and secrete specific chemokines that recruit fibroblasts, T_H2 cells and T_{Reg} cells (Curiel *et al.,* 2004; Imai *et al.,* 1999).

It is generally believed that macrophages represent a spectrum of activated phenotypes rather than discrete stable subpopulations (Mosser *et al.,* 2008). At steady state, tissue macrophages have intrinsic anti-inflammatory functions. An initial level of macrophage activation occurs when early signals trigger monocyte recruitment and activation or when IL4 induces *in situ* macrophage proliferation (Jenkins *et al.,* 2011).

A key component of the next layer of the macrophage response is the production of anti-inflammatory feedback mechanisms that encompass cell-intrinsic signaling feedback loops and cell-extrinsic mechanisms, such as the production of IL10, which is an essential and non-redundant anti-inflammatory cytokine. The final layer of macrophage response is the least clear and involves the final decision between chronic inflammation and re-establishment of homeostasis. Tissue macrophages also suppress inflammation mediated by inflammatory monocytes, thereby ensuring that tissue homeostasis is restored following infection or injury.

4.4. Monocyte Differentiation

Several studies indicate a differentiation potential of monocytes. Monocytes are inherently programmed to differentiate into macrophages or dendritic cells. However, the differentiation course of monocytes can be altered once specific signals are provided to the monocytes. Many studies have shown that in vitro culture of monocytes with specific media conditions can induce monocyte differentiation into other cell types.

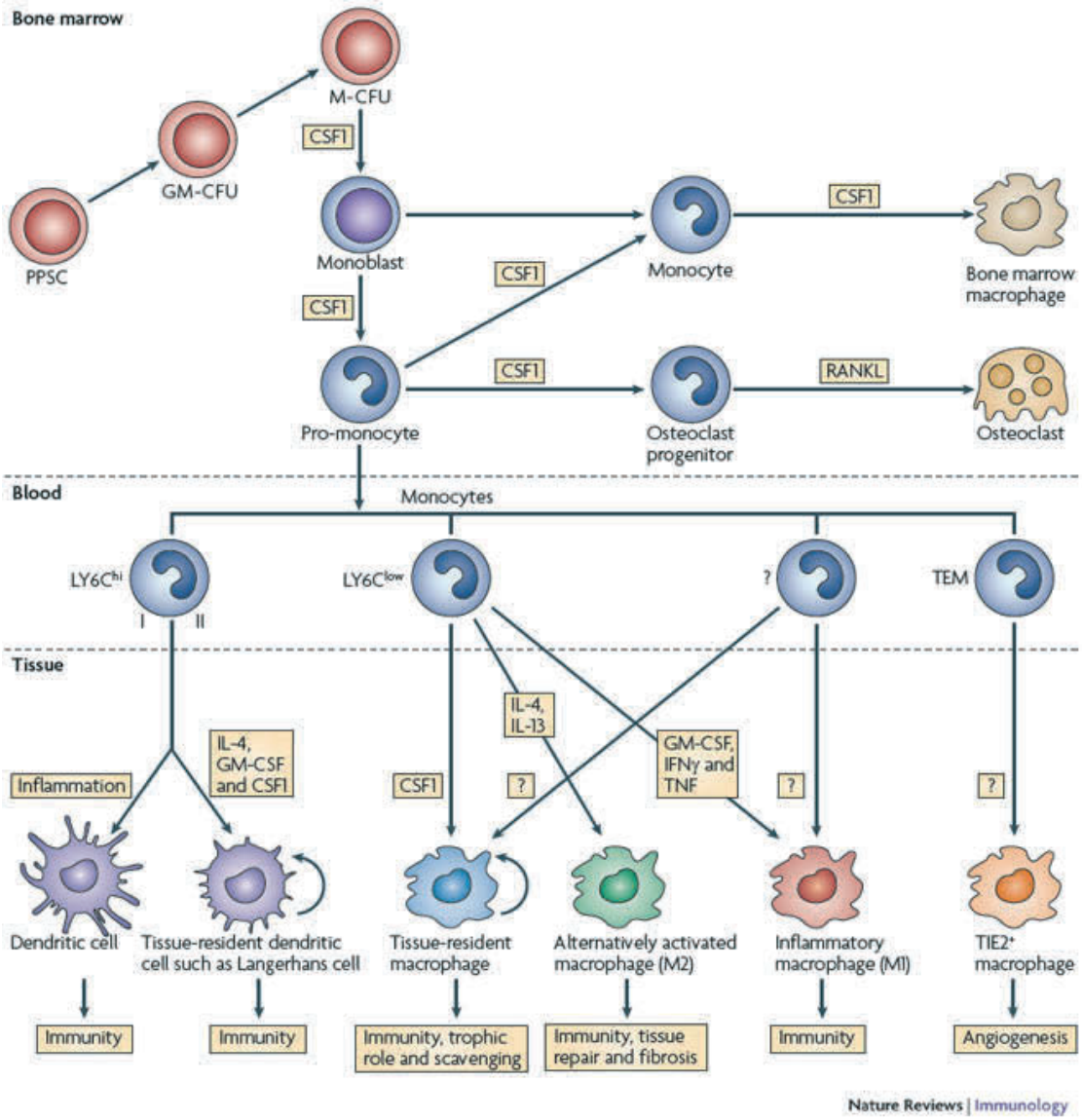

Figure 7. Monocyte differentiation according to function and location. Figure adapted from Pollard *et al.,* 2009.

Human monocytes have been found to be capable of differentiating into mesenchymal progenitors (Kuwana *et al.,* 2003), hepatocyte-like and pancreatic islet-like cells (Ruhnke *et al.,* 2005), keratinocyte-like cells (Medina *et al.,* 2009), endothelial cells (Fernandez-Pujol *et al.,* 2000; Schmeisser *et al.,* 2001), among several other cell types. On the other hand, claims have been made concerning the plausibility of these studies, regarding proper cell phenotype characterization and the need for artificial induction of monocyte differentiation. Hence, further

investigations are required to validate previous findings and to evaluate proper and controlled differentiation of monocytes into an intended cell and/or tissue.

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Aims of the Thesis

General Aim

The overall goal of my PhD studies was to analyze how the regulation of angiogenesis, a key factor that plays very important roles in tissue repair, could ultimately influence cartilage formation by mesenchymal stromal cells (MSC) and by differentiated nasal chondrocytes (NC) as a proof of concept.

Due to their multipotency and great self-renewal capacity, MSC is a highly attractive cell source for cartilage formation and regeneration. MSC have immunomodulatory properties and are in direct contact with inflammatory cells (monocytes) in the cartilage repair environment. By directly regulating the inflammatory response of monocytes and by regulating their phenotype, MSC could significantly benefit from their ability to control inflammatory events during cartilage repair processes. Thus, we also assessed whether MSC could modulate the properties and the phenotype of monocytes in order for monocytes to actively aid MSC in cartilage repair.

Aim 1

In chapter 1, we sought a proof of principle to confirm that the blocking of angiogenesis does indeed improve cartilage formation when using genetically-modified nasal chondrocytes (NCs) or antiangiogenic peptides associated with NCs. For this purpose, NCs were genetically-modified to express mouse soluble VEGF receptor-2 (sFlk-1) or were associated with an antiangiogenic peptide in order to have their chondrogenic capacity assessed *in vitro* and *in vivo*. Improved cartilage regeneration could be observed after *in vivo* implantation of NCs in an ectopic nude

mouse model. Whereas the anti-angiogenic approaches did not improve chondrogenesis *in vitro*, frank chondrogenesis occured *in vivo* only in the constructs generated by NCs expressing sFlk-1 or treated with the peptide. Blood vessel ingrowth was significantly hampered in the anti-angiogenic experimental groups when compared with naïve NCs, which correlated with chondrogenis improvement. Strikingly, the anti-angiogenic effect was even more evident when NC donors with low chondrogenic capacity were used, and no-preculture with chondrogenic soluble factors was performed prior to *in vitro* implantation of the constructs.

Aim 2

In chapter 2, we investigated how angiogenesis control by genetically-modified bone marrow-derived MSC could augment their chondrogenic potential *in vivo*. These MSC were genetically modified to release sFlk-1, the soluble version of VEGF receptor 2 (VEGFR2), which acted as a decoy receptor and could sequester VEGF from the immediate surroundings of MSC. Importantly, no external morphogens were supplemented in order for MSC chondrogenic differentiation to occur. Moreover, the *in vivo* chondrogenic capacity of sFlk-1-releasing MSC was assessed up to 12 weeks by an ectopic nude mouse model, in which collagen sponges seeded with MSC were implanted subcutaneously. Angiogenesis, as analyzed by blood vessel invasion, was markedly reduced in the constructs seeded with sFlk-1-releasing MSC. Frank and stable cartilage formation was only achieved once VEGF was blocked.

Aim 3

In chapter 3, we aimed at investigating whether MSC can instruct monocytes to acquire traits of mesenchymal progenitors or tissue repair macrophages when in direct or indirect contact with the latter. Thus, MSC could instruct monocytes to assist in the tissue-repairing process. MSC and monocytes were cocultured either in 3D collagen sponges or in transwells for a period of five consecutive days. We demonstrated that MSC instruct monocytes into acquiring a hybrid macrophage-mesenchymal phenotype, which varies greatly depending on which kind of contact with MSC the monocytes were exposed to (whether direct cell-to-cell contact or through the release of soluble factors by MSC). However, future studies will be needed to elucidate the mechanism in which MSC could instruct monocytes into differentiating into this hybrid state, and how one could better control this process in order for monocytes to optimally aid MSC in cartilage repair.

Chapter 1

Microenvironmental VEGF sequestration enhances in vivo cartilage formation

Microenvironmental VEGF sequestration enhances *in vivo* cartilage formation

Medeiros da Cunha CM[1], Perugini V[2], Bernegger P[1], Centola M[1], Santin M[2], Banfi A[1], Martin I[1], Marsano A[1†].

[1] Department of Surgery, Basel University Hospital and Department of Biomedicine, University Basel, Hebelstrasse 20, 4031, Basel, Switzerland

[2] School of Pharmacy and Biomolecular Sciences, University of Brighton, Brighton, BN2 4GJ, United Kingdom

Address correspondence to:

Anna Marsano Ph.D., Department of Surgery, Basel University Hospital and Department of Biomedicine, University Basel, Hebelstrasse 20, 4031, Basel, Switzerland, tel. +41-061-328-7448, Anna.Marsano@usb.ch

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Abstract

Autologous chondrocyte transplantation for cartilage repair has still unsatisfactory clinical outcomes because of inter-donor variability and poor cartilage quality formation. Re-differentiation of monolayer-expanded human chondrocytes is indeed not easy in the absence of potent morphogens. Since Vascular Endothelial Growth Factor (VEGF) plays a master role in angiogenesis and in regulating cartilage growth, we hypothesized that its sole microenvironmental sequestration by VEGF receptor-2 (Flk-1) could improve chondrogenesis of human nasal chondrocytes (NC) freshly seeded on collagen scaffolds. For this purpose, Flk-1 was either over-expressed in its soluble version by transduced NC, or mimicked by antiangiogenic peptides. Chondrogenesis of several NC donors with differential potential was assessed either *in vitro* or ectopically in nude mice. VEGF blockade appeared not to affect NC *in vitro* differentiation, whereas efficiently inhibited blood vessel ingrowth. After 8 weeks *in vivo* glycosaminoglycan (GAG) deposition was approximately two-fold higher in the constructs generated by sFlk-1 expressing NC or treated with the peptide when compared to the control group. The effect was even more pronounced when VEGF was blocked in low chondrogenic cell primaries. Our data indicate that VEGF sequestration, independently of the specific implementation mode, has profound effects on *in vivo* NC chondrogenesis, even in the absence of chondroinductive signals prior or at the implantation site.

Introduction

The treatment of focal articular lesions still remains an unresolved clinical issue, despite the significant improvements in the clinical outcomes of cell-based repair strategies. Autologous articular chondrocyte-based implantation techniques are indeed associated with long-term morbidity at the donor site (Dunkin *et al.,* 2013) and to the formation of non- durable, functionally competent and joint-specific cartilage (Pelttari *et al.,* 2006; Hunziker *et al.,* 2009). High inter-donor variability (Wolf *et al.,* 2008) and loss of phenotypic specificity during *in vitro* expansion (Dell'Accio *et al.,* 2001, Schnabel *et al.,* 2002) are critical issues which affect the chondrocyte potential to form *in vivo* stable cartilage resistant to blood vessel invasion and mineralization. Strategies focused on overcoming such limitations include supplementation of specific growth factors directly at the site of implantation or during expansion (Francioli *et al.,* 2007, Tay *et al.,* 2004) and identification of cell subpopulation with the highest chondrogenic potential (Dell'Accio *et al.,* 2001, Schnabel *et al.,* 2002, Hellingman *et al.,* 2011). Nevertheless, the autologous chondrocyte transplantation, direct after expansion without any cell pre-commitment, remains still a critical issue. An *in vivo* chondro-supportive environment is crucial to induce chondrogenesis and preserve the implant by blocking vessel and host inflammatory cell ingrowth, present usually at the implantation site. A biomimetic tissue engineering strategy aims at recapitulating some key features of the native tissue or mechanisms that trigger chondrogenesis during development. 3D pre-culture (Moretti *et al.,* 2005), use of biomaterials resembling key structural and biological cues of native extracellular matrices (Chuang *et al.,* 2012) or exposure to physiological mechanical loading (Responte *et al.,* 2012) or to specific culture conditions, such as hypoxia (Markway *et al.,* 2013), are also well known to promote chondrogenesis. Hyaline cartilage is avascular by nature, and the maintenance of

this avascular environment seems to be crucial for cartilage development and homeostasis (Carlevaro *et al.,* 2000). Vascular Endothelial Growth Factor (VEGF) is a crucial regulator of angiogenesis (Ferrara & Gerber *et al.,* 2001; Carmeliet & Collen *et al.,* 2000) and skeletal growth (Zelzer *et al*., 2002; Gerber *et al*., 1999); in particular, its downregulation triggers chondrogenesis and maintains chondrocytic differentiation and stability (Carlevaro *et al.,* 2000), and cartilage repair (Street *et al*., 2002). Block of angiogenesis by microenvironmental VEGF sequestration can be achieved in different ways: either by antiangiogenic factor delivery by cell-based gene approach or by functionalized biomaterials. Cells might be genetically modified to express soluble Flt-1 and Flk-1 (which are the extracellular cleaved forms of VEGFR-1 and VEGFR-2, respectively (Kendall *et al.,* 1996; Ebos *et al.,* 2004), which can act as decoy receptors resulting in blocking angiogenesis. Kubo and colleagues showed how muscle-derived stem cells overexpressing sFlt-1 can support cartilage repair, but only when combined with potent morphogens (Kubo *et al.,* 2009). Alternatively, block of angiogenesis can be achieved by scaffolds functionalized to release humanized VEGF antibody (Centola *et al.,* 2013) or with synthetic anti-angiogenic peptides (Foy *et al.*, 2011) competing with VEGF binding to VEGFR-2 (Erdag *et al.*, 2007), to inhibit endothelial cell proliferation and sprouting *in vitro* (Meikle *et al.*, 2011).

In this study, we aimed at investigating the effects of blocking angiogenesis on the *in vivo* chondrogenesis of non-pre-committed nasal chondrocytes (NC) from donors with variable chondrogenic potential upon implantation in a non-chondrosupportive environment. Since culture in pro-chondrogenic conditions is well known to support the further *in vivo* cartilage maturation and preservation (Moretti *et al.,* 2005), we also investigated whether pre-culture alone can promote *in vivo* chondrogenesis of constructs generated by low chondrogenic donors. Nasal chondrocytes were chosen for their high clinical translation potential for

articular cartilage repair, due to their safety (Fulco & Miot *et al.,* 2014), ease of harvest, low donor morbidity, and capability to form stable hyaline cartilage - also in response to mechanical load - when compared to other cartilage donor sites (Chia *et al.,* 2004; Tay *et al.,* 2004; Barbero *et al.,* 2003; Rotter *et al.,* 2002). In order to block angiogenesis, we used two different methods to sequestrate VEGF from the microenvironment around the implanted cells, one relying on cell-based gene therapy and the other based on the synthesis of antiangiogenic peptides, as described above. Therefore, mouse naïve (not transduced) NC and sFlk-1-expressing NC were freshly seeded on a type I collagen scaffold; in addition, some scaffolds were pre-treated with antiangiogenic peptides (also called dendron) in order to investigate their effect on naïve cells. NC chondrogenic and anti-angiogenic potential were assessed directly after seeding in a subcutaneous mouse model, which is known for not supporting chondrogenesis (Scott *et al.,* 2012).

Methods

Cell isolation and transduction

Human nasal septal cartilage biopsies were harvested from different donors during orthopedic surgical procedures in accordance to the local ethical committee and following informed consent. Nasal cartilage was digested with a 0.15% type II collagenase solution (1mL/100 mg of tissue, Worthington Biochemical Corporation, Lakewood, USA) for 22 hours at 37°C. The samples were filtered (100 µm) and the collagenase activity was hampered by adding twice its original volume of complete media (CM), which consists of high glucose Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS (complete medium).

Nasal chondrocytes (NC) were cultured in a humidified incubator at 37°C and 5% CO_2 at a density of 5000 cells/cm^2 in CM supplemented with 5 ng/ml FGF-2 (BD Biosciences), and 1ng/mL of transforming growth factor β1 (TGFβ1, R&D Systems, Minneapolis, USA) in order to enhance NC proliferation (Tay *et al*., 2004).

NC were transduced with retroviral vectors carrying the gene for mouse sFlk-1 linked through an internal ribosomal entry site (IRES) to a mouse truncated CD8a version (Wolff *et al*., 2011). For control purposes, either NC transduced only with the truncated murine CD8a or naïve cells were used. Cells were cultured in 60-mm dishes and incubated with retroviral vector supernatants supplemented with 8µg/ml polybrene (Sigma-Aldrich) for 5 minutes at 37°C and centrifuged at 1100g for 30 minutes at room temperature, followed by fresh medium replacement. Cells were then expanded for two additional passages and re-plated at a density of 10^4 cells/cm^2.

In vitro 3D pellet culture

As previously established by Barbero and colleagues (Barbero *et al.,* 2003), the 3D pellet culture model was used for inducing NC towards chondrogenic differentiation using serum-free medium (SFM), which consists of DMEM (Gibco), supplemented with 1% Hepes (Gibco), 1% penicillin-streptomycin solution (Gibco), 1% sodium pyruvate (Gibco), 1% Insulin-transferrin-sodium selenite (ITS+1, Sigma-Aldrich, St. Louis, USA), 1% human serum albumin (HSA 100x, Invitrogen, Carlsbad, USA), 0.1 mM ascorbic acid 2-phosphate (Invitrogen) and dexamethasone 10^{-5}M (Invitrogen), then supplemented with 10ng/ml of TGFβ1 (Invitrogen). Briefly, 0.5x10^6 cells per 0.5mL of medium were placed into conical polypropylene tubes and spun down for 4 min at 1500 rpm to form cell aggregates. The NC cell pellets (control cells and sFlk-1 transduced cells) were cultured for 2 weeks at 2% or 20% oxygen tension. The

culture medium was changed twice a week. The supernatants of the pellet culture were collected and used for human VEGF quantification (described further).

Synthesis and characterization of peptide VEGF blockers

FFG3K $(WHLPFKC)_{16}$ was assembled on Tenta Gel S-NH_2 resin using an acid labile Rink amide linker (Iris Biotech GmbH, UK) by a modified microwave based solid phase peptide synthesiser (Biotage, UK) and then isolated using 94 % $^{v}/_{v}$ TFA (Fisher Scientific, UK), 2.5 % $^{v}/_{v}$ deionised H_2O, 1 % $^{v}/_{v}$ 1,2-ethandithiol (Sigma Aldrich Co. Ltd, UK) and 2.5 % $^{v}/_{v}$ trisopropyl silane (TIPS) (Sigma Aldrich Co. Ltd, UK). The dendron was characterized by analytical HPLC (Waters TM 717 plus Autosampler, UK), mass spectrometry (MS) (Bruker microTOF, UK) and gravimetric analysis as reported by Steve and colleagues (Meikle *et al.,* 2011).

sFlk-1 cell- based construct preparation and culture

A commercially available type I collagen-based sponge (Ultrafoam^{TM}, Bard, Davol Inc.) of 3 mm thickness was punched into 6 mm diameter disks and then seeded at the density of $1.7x10^6$ NC per scaffold. The constructs were cultured overnight with Dulbecco's modified eagle medium supplemented with 10% FBS and 1% penicillin-streptomycin before implantation.

VEGF blocker-functionalized Ultrafoam collagen scaffolds

Type I collagen sponges (6mmØ, 3-4 mm thickness, Ultrafoam^{TM}, Davol Inc.) were used to form NC constructs (1.7×10^6 cells/scaffold) in the presence of WHLPFKC at equimolar concentration of 1.62 nM of the blocking sequence. The generated constructs were initially let impregnating at room temperature for 20 minutes and then incubated with DMEM with

10 % FBS and 1% penicillin-streptomycin at 37 °C and 5 % CO_2 for 24 hours prior implantation.

In vivo ectopic implantation

Cell based constructs were implanted ectopically (subcutaneously) in nude mice 12 hours after cell seeding (CD-1 nude/nude, Charles River, Germany). Each animal had 6 implanted constructs. The mice were sacrificed with CO_2 at 1, 4, and 8 weeks after the implantation. Subsequently, the explants were further processed according to future analysis protocols.

Analytical methods

Flow cytometry

The percentage of population positive for CD8a in the sFlk-1-releasing NC and control NC was assessed by staining with a fluorescein isothiocyanate-conjugated anti-rat CD8a antibody (clone 53-6.7; BD Biosciences), using previously optimized staining conditions to ensure complete saturation of the CD8a molecules expressed on all cells in the heterogeneous transduced population (Mistelli *at al.,* 2010), that is, 2 µg of antibody/10^6 cells in 200 µL of PBS with 5% bovine serum albumin. Data were acquired with a FACSCalibur flow cytometer (Becton Dickinson) and analyzed using FlowJo software (Tree Star).

Quantification of human VEGF and murine sFlk-1

Supernatants were collected from pellets after two days of culture and were used for human VEGF and murine sFlk-1 quantification using the ELISA (enzyme linked immunosorbent assay, Quantikine immunoassay kits from R&D Systems). Data were presented as quantity in ng normalized by pellet and by time. Supernatants from monolayer expanded sFlk-1-expressing

cells were collected after 4 hours incubation and the quantified murine VEGF receptor 2 was normalized by the number of cells counted in each dish and the time of incubation. Data was assessed in duplicate for each donor (n=2).

Biochemistry

Engineered tissues cultured *in vivo* were digested with protease K for 15 hours at 56°C (Hollander *et al*., 1994). The glycosaminoglycan (GAG) and DNA contents were measured spectrophotometrically using dimethylmethylene blue (Farndale *et al*., 1986) and the CyQUANT Kit (Molecular Probes, Eugene, OR, USA). GAG contents were normalized to the amount of DNA.

HUVEC proliferation assay

Human umbilical vein endothelial cells (HUVEC) were cultured in 96-well plates overnight in growth medium (GM) (M199 supplemented with 20% fetal bovine serum (FBS), 100 mg/mL endothelial cells growth supplement, 50 IU/mL heparin, 100 IU/mL penicillin, and 100 mg/mL streptomycin) at a density of 5000 cells/well. The next day, GM was replaced with assay medium (AM) (consisting of M199 with 5% FBS, 10 IU/mL heparin, and 1% penicillin/streptomycin), supplemented with recombinant human VEGF (R&D Systems) at different titers between 0 and 50 ng/mL with the supernatant derived from either control or sFlk-1 expressing NC (Centola *et al.*, 2013). HUVEC metabolic activity was then assessed by using MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] (Promega, Madison, USA). HUVEC cells were incubates with MTS for 2 hours at 37°C and analyzed at 490nm.

HUVEC migration assay - after 18 hours of culture

HUVEC encapsulation, along with either sFlk-1-expressing or naïve NC were performed by using a synthetic MMP-degradable extracellular matrix (ECM) functionalized with RGD peptides to promote degradation and cell adhesion and migration, respectively (kindly provided by QGel® Matrix, Lausanne, Switzerland). HUVEC were first labelled with syto®13 in green and then seeded at a density of 130000 cells/well on collagen-coated culture plates. After HUVEC adhesion and proliferation overnight in growth medium, 100µL of QGel®with RGD was polymerized, followed by 100µL of QGel® without RGD and 100µL of sFlk-1-expressing or naïve NC embedded in QGel® with RGD. The next day, growth medium was replaced by assay medium supplemented either by 0, 0.25, or 0.75 ng/mL or recombinant human VEGF. HUVEC migration was analyzed for 12 hours, using Zeiss LSM 710 confocal microscope.

Safranin-O and immunohistochemistry for types II and X collagen

Explanted tissues were fixed with 4% formaldehyde and embedded in paraffin. Sections (5 µm thick) were stained for Safranin-O and for immunohistochemistry for types I, II and X collagen. Briefly, for collagen immunohistochemistry, a pre-treatment of the histological sections was performed with Hyaluronidase (Sigma) and Pronase (Roche). The primary antibodies used were mouse anti-collagen I (MP Biomedicals, clone I-8H5), mouse anti-collagen II (MP Biomedicals, clone II-4C11), and mouse anti-collagen X (Abcam). As a secondary antibody, goat anti-mouse IgG/Biotin (Dako) was used. The Vectastain ABC/AP kit (Linaris) and Vector Red Alcaline Phophatase kit with Levamisole (Linaris) were used to form the staining complex.

Human ALU in situ hybridization

Embedded paraffin tissue sections were deparaffinized and digested with pepsin (0.5mg/mL 0.01N HCL, Sigma) at 37°C for 10 min, washed with PBS, and incubated at RT for 10 min in 0.1 mol TEAC (triethanolamine containing acetic acid). The sections were incubated for 30 min at 42°C in pre-hybridization solution (consisting of 20% SSC (trisodium-citrate-2-hydrate, pH 7), Denhardt's solution 1% (ficoll, polyvinylpyrrolidone and BSA solution in distilled water), 10% dextransulfate, 1% salmon sperm DNA, 50% deionized formamide and 18% distilled water). The ALU probe (Microsynth, 200ng/mL) was applied to the slides and these were denatured at 95°C for 3 min, immediately cooled, and incubated overnight at 42°C. Sections were equilibrated with DIG-1 buffer at RT for 1 min (maleic acid and NaCl, pH 7.5) and blocked with 1% blocking reagent for 15 min at RT. Anti-digoxygenin staining was performed for 1 hour at RT (Roche). Successive washes with DIG-2 (DIG-1 buffer with 0.3% Tween 20) and DIG-3 (Tris HCl, NaCl, and $MgCl_2$, pH 9.5) were performed. The sections were finally incubated with NBT/BICP for 10 min, and the reaction was stopped with distilled water.

Immunofluorescence for CD31

Histological sections were deparaffinized for 20 min, and then washed 3x with PBS (5 min each wash). In a humidified chamber, the slides were incubated with 10% goat serum solution in PBS for 40 min. The sections were incubated with rat monoclonal anti-mouse F4/80 antibody (Caltag, RM2900, 1:200 dilution in PBS/0.05% Tween 20/ 1.5% goat serum) or rat anti-mouse PECAM-1 (CD31, BD 553370, 1:100 dilution in PBS/ 0.05% Tween 20/ 1.5% goat serum) for 60 min in a humid chamber at 37°C. After 3 successive washing steps with PBS/ 0.05% Tween 20/ 1.5% goat serum, the sections were incubated with donkey anti-rat

488-conjugated secondary antibody (Invitrogen, 1:200 dilution in PBS/ 0.05% Tween 20/ 1.5% goat serum) or goat anti-rat 647 secondary antibody (Invitrogen, 1:200 dilution in PBS/ 0.05% Tween 20/ 1.5% goat serum) and DAPI (48x) for 45 min in a humid chamber at 37°C, followed by the washing steps. The sections were mounted with faramount aqueous mounting medium (Dako, Glostrup, Denmark).

Vessel Quantification

CD31-stained vessels were quantified using cellSens (Olympus). Briefly, pictures were taken from the center and the periphery of the constructs (average of 5 pictures per construct) (BX63 microscope, Olympus). The pictures were then analyzed wih cellSens. Vessels had their length determined in (µm), and the vessel density was determined by dividing the total amount of vessel length by the total construct area.

Results

In vitro characterization of engineered nasal chondrocytes

Human nasal chondrocytes (NC) were transduced with a retrovirus to express murine sFlk-1. The retroviral construct also contained a truncated –non-functional- mouse version of the CD8a molecule, to allow for the cells to be sorted using flow cytometry. CD8a-only transduced cell populations (as control) and CD8a-sFlk-1-transduced cells were sorted for positivity in CD8a expression, as seen in Figure 1A. The transduction efficiency for NC was 86.5±9.2% (n=3). The transduction process did not impair the proliferation potential of NC, the total cell doublings for naïve, control and sFlk-1 transduced NC was indeed similar in 14 days: 9.5±1.3, 8.8±1.7, 9.2±2.0, respectively (data are presented as mean ±SEM, n=2),

consistently with previous findings (Helmrich *et al.,* 2012). Monolayer-expanded transduced NC released an amount of sFlk-1 equal to 1.07±0.41 ng/10^6 cells/day as quantified by ELISA.

HUVEC proliferation and migration assays were also performed in order to assess the activity of sFlk-1-releasing NC. Supernatants collected from monolayer expansion of sFlk-1-expressing NC significantly reduced human umbilical endothelial cells (HUVEC) proliferation induced by a concentration of VEGF up to 10 ng/mL (Figure 1B).

sFlk-1-releasing NC also hindered the migration of HUVEC under two different concentrations of VEGF (namely 2.5 and 7.5 ng/mL). On the other hand, control NC were permissive to the migration of HUVEC cells (Figure 1C). Taken together, this data shows that sFlk-1-transduced NC are actively blocking VEGF *in vitro*.

In vitro chondrogenesis

sFlk-1-, CD8a- expressing (CD8, control), naïve (Naïve) and mock-sorted (Naïve_S) cell populations were used to investigate the effect of the VEGF blockade and the transduction and sorting processes on the *in vitro* NC chondrocytic re-differentiation capacity by using naïve cells as positive control (n donors=3). Since hypoxic culture condition was shown to influence *in vitro* cell chondrogenic capacity, the pellet culture was performed at either 2 or 20 % of oxygen tension. Based on the ratio between the glycosaminoglycan (GAG) and DNA generated by pellets from naïve cells at 20% of oxygen, we distinguished between “low” and “high” chondrogenic donors (< and ≥ than 5 µg/µg of GAG/DNA, respectively).

For the low chondrogenic donors, the glycosaminoglycan content (GAG) (Figure 2C) was not affected by either the transduction or sorting processes. No significant difference was observed also when comparing unsorted naïve and sFlk-1-transduced cells. The different oxygen tensions seem to not play a significant role in chondrogenic differentiation for latter

conditions. Figure 2A shows the safranin-O staining for all conditions in 20% oxygen, and no substantial extracellular matrix can be observed (with slight variations among the experimental groups). As for the high chondrogenic donor, the different oxygen tensions seemed to exert no effect on the chondrogenic differentiation of naïve and CD8 NC. Although a slight improvement in GAG formation was observed under hypoxia for sFlk-1 pellets and under normoxia for sorted naïve pellets, no statistical significance was found (Figure 2D). Extracellular matrix deposition occurred in naïve, sorted naïve (naïve_S), and CD8 pellets, whereas lower deposition occurred in sFlk-1 pellets in 20% oxygen (Figure 2B).

Mouse sFlk-1 was quantified by ELISA in the supernatant of NC after pellet culture. sFlk-1-transduced NC pellets expressed approximately 30 ng/pellet/day of sFlk-1 under 20% and 2% O_2 (Figure 2E). Thus, sFlk-1 release was not affected by the different oxygen tensions used for pellet culture. As expected, murine sFlk-1 expression in naïve and CD8-only transduced cells (control cells, data not shown) remained undetected.

Moreover, both low and high chondrogenic NC were also cultured *in vitro* in constructs (cells seeded onto Ultrafoam® sponge) under normoxia for 2 weeks and no significant differences in GAG deposition could be identified among sFlk-1 and control groups (Figure 2F, 2G). Overall, sFlk-1 did not improve NC chondrogenesis *in vitro.* The i*n vitro* scaffold-based culture did not improve GAG deposition by low chondrogenic NC primaries, which had a GAG/DNA ratio similar to what obtained in the pellet system (Figure 2F).

In vivo blocking of angiogenesis

To verify whether sFlk-1 and the dendronized peptide WHLPFKC were capable of blocking VEGF *in vivo*, PECAM (CD31) stainings were performed in order to investigate vessel ingrowth within the implant. Figure 3 shows the PECAM immunofluorescence done for

control, sFlk-1, and dendron constructs. In the control samples, some CD31-positive vessels can be observed within the center of the implant, indicating vascular invasion. As for sFlk-1 constructs and dendronized WHLPFKC, CD31-positive vessels can be observed only in the host tissue surrounding the constructs, which translates into sFlk-1 and WHLPFKC being able to efficiently block angiogenesis within the implant area. Taken together, *in vivo* vessel invasion are effectively blocked by sFlk-1 and by the dendronized WHLPFKC peptide, which are able to block VEGF *in vivo.*

In vivo chondrogenesis

Naïve, dendron-associated, and sFlk-1 overexpressing NC from two different types of donors (low and high chondrogenic donors, n=2 for each experimental group) seeded onto collagen-I-based scaffolds (Ultrafoam®) were implanted ectopically in nude mice and assessed for their *in vivo* chondrogenic potential. Since it has already been shown that cell transduction and sorting did not influence the chondrogenic capacity of nasal chondrocytes, we used naïve cells as control group.

First, we sought to investigate whether a preliminary 2 weeks pre-culture in chondrogenic medium prior to the ectopic implantation would enhance *in vivo* chondrogenesis potential of low chondrogenic NC. As previously shown, the 2-week pre-culture did not enhance cartilaginous extracellular matrix deposition (Figure 2F) by low chondrogenic donors *in vitro*. In this regard, prior *in vitro* pre-culture of the constructs (sFlk-1 and control) did not enhance the *in vivo* cartilage forming capability of NC. Cartilage was formed only by sFlk-1-expressing NC (Supplemental Figure 1). Supplemental Figure 2 also shows the *in vivo* survival of the sFlk-1 antiangiogenic approach. In this regard, sFlk-1 greatly enhances NC survival *in vivo*, when compared to control NC. Constructs formed by control NC were mostly invaded by

host cells (red nuclei in Supplementary Figure 2). Limited murine cell infiltration was instead observed in general into the implant area and in particular in the region where cartilage was formed.

The further *in vivo* studies were only performed with direct implantation of freshly seeded cells on Ultrafoam® scaffolds, without any additional pre-culture. Low and high potential NC primaries were assessed for their *in vivo* capability to form cartilage. The low chondrogenic donor is represented in Figures 4, whereas the high chondrogenic donor is represented in Figure 5 for the genetically-modified NC approach. Figures 4A and 5A show the *in vivo* safranin-O comparative stainings for control NC and sFlk-1-NC at 4 and 8 week for low and high chondrogenic donors, respectively. Control NC were capable of forming cartilage *in vivo,* with sFlk-1 constructs showing a clear improvement in GAG deposition when compared to control constructs, especially at 8 weeks *in vivo.* For the low chondrogenic donor control NC, which were not capable of forming cartilage *in vivo*, the evidence of sFlk-1 improving cartilage formation was striking. For the high chondrogenic donor, sFlk-1 also improved cartilage formation, especially at 8 weeks *in vivo*.

Regarding collagen type II staining (Figure 4B and 5B) no prominent differences could be accounted for the high chondrogenic donor. However, a slight improvement of type II collagen production could be observed for the low chondrogenic donor. For the type X collagen, meanwhile, no differences can be seen between the control and sFlk-1 groups (Figure 4B and 5B).

Figure 4C shows a remarkable increase of GAG production by low chondrogenic sFlk-1-expressing NC both at 4 and 8 weeks *in vivo* when compared to control NC. In this aspect, sFlk-1 greatly augmented the GAG formation and deposition in nasal chondrocytes, while it did not affect cartilage remodelling by means of variances in collagen production. On the

other hand, Figure 5C shows that, despite the fact that the high chondrogenic potential naïve NC already display high GAG contents at both 4 and 8 weeks *in vivo*, sFlk-1 greatly increased GAG formation at 8 weeks *in vivo,* which indicates once more that preventing angiogenesis can greatly enhance chondrogenesis of nasal chondrocytes *in vivo.*

Figure 6 is representative of a high chondrogenic donor only associated with the dendronized antiangiogenic peptide. Figure 6A shows the *in vivo* safranin-O stainings for control and dendron-associated NC at 8 weeks, respectively. The enhanced cartilage forming capacity of the dendron construct is evident at 8 weeks *in vivo.* Biochemical analyses of GAG deposition corroborate the fact that anti-angiogenic dendronized peptide greatly enhances the *in vivo* cartilage forming capacity of high chondrogenic donors at 4 and 8 weeks (Figure 6B).

Figure 6A also shows collagen type II and type X stainings at 8 weeks *in vivo*. No prominent differences could be accounted for between the two groups. However, in regards to collagen type X, the dendron group displayed less collagen type X deposition, indicative of a more stable cartilaginous phenotype. In summary, both sFlk-1 and dendron approaches enhance the cartilage forming capacity of NC *in vivo.*

Discussion

In this study, we demonstrated that both tested methods to block angiogenesis have a great impact on the *in vivo* chondrogenic potential of non-pre-committed NC by significantly improving cartilage quality and enhancing glycosaminoglycan (GAG) deposition as compared to controls. Remarkably, only VEGF sequestration promoted *in vivo* cartilage formation by low chondrogenic cell primaries*,* thus resulting in a stronger inducer than *in vitro* pre-culture.

Based on our results, it appears that generating a permissive environment for NC is more relevant than *in vitro* induction by morphogen supplementation. Sequestration of VEGF microenvironmentally released by implanted human NC in order to inhibit blood vessel ingrowth within the implant area reduces the risk of resorption of non-mature constructs (Centola *et al.,* 2013) and supports chondrogenesis. Several studies showed indeed that anti-angiogenic approach support formation of stable cartilage while blocking hypertrophy and endochondral ossification (Klinger *et al.,* 2011, Kubo *et al.,* 2009) by either applying adeno-associated virus carrying chondromodulin 1 complementary DNA (Klinger *et al.,* 2011) or by injecting mouse muscle-derived stem cells genetically modified to overexpress the soluble version of sFlt-1 combined with morphogenic factors (Kubo *et al.,* 2009) in knee cartilage lesions. These previous studies differ from ours by the *in vivo* animal model adopted and by the target cell types: animal-origin native articular versus human de-differentiated nasal chondrocytes. While Kubo's study indeed aimed at investigating the effects of several growth factors, released by the genetically modified stem cells (which served mainly as delivery system), on cartilage repair, our goal was to generate a protected and chondro-permissive environment to allow sFlk-1-expressing NC re-differentiation. However, as in the previously mentioned studies, the enhanced chondrogenesis observed by adopting anti-angiogenic strategies appears to be associated only with paracrine signaling, since the *in vitro* chondrogenic potential was not affected by VEGF as well as other anti-angiogenic factors, such as endostatin (Jeng *et al.,* 2010). Paracrine VEGF signaling is crucial for angiogenesis to take place, including effects on EC proliferation, survival, migration, and endothelial differentiation (Lee *et al.,* 2007). Since one of the immediate consequences of inhibition of angiogenesis is the generation of a hypoxic environment, which is a key regulator of chondrogenesis during limb development (Araldi *et al.,* 2010), we also

investigated the *in vitro* effect of VEGF blockade at low oxygen tension. In the absence of the additional supplementation of a potent morphogenic factor (TGFβ3), reduction of the oxygen tension from 20% to 2% did not result in the deposition of cartilaginous matrix by NC in either experimental group. Thus, hypoxia alone was not sufficient to induce full chondrogenic differentiation of NC *in vitro*, which is in line with Scherer's study (Scherer *et al.,* 2004), who showed that while hypoxia does not prevent chondrogenesis differentiation in the presence of chondrogenic medium *in vitro*, it alone is not sufficient to induce chondrogenic differentiation of human articular chondrocytes. Moreover, TGβ3 supplementation did not improve in vitro cartilage formation by NC when a hypoxic stimulus was applied. Based on our *in vitro* results we can conclude that mechanisms other than the sole combined supplementation of hypoxia and morphogen are responsible for enhancing chondrogenesis *in vivo*. The lack of nutrients is also a direct consequence of the absence of blood vessel supply; low glucose culture has been shown to regulate the oxygen tension within engineered tissues (Heywood *et al.,* 2006) and the chondrogenesis capacity of chondrocytes (Heywood *et al.,* 2014).

The lack of vessels could prevent the infiltration of immune cells, such as macrophages, and the availability of other molecules necessary for the physiological maintenance of the NC phenotype. Macrophage infiltration can induce host reaction against the implant and bring about its premature resorption. It has been demonstrated by Luo and colleagues (Luo *et al.,* 2009) that a mild inflammatory response by the host tissue occurs when a 2-week *in vitro* pre-cultured chondrocyte-based construct is implanted *in vivo* compared to "no pre-culture group". Other studies have also reported that *in vitro* preconditioning can mitigate the post-implantation inflammatory reaction and how cartilage maturation is correlated with a different inflammatory chemokine production (Francioli *et al.,* 2011; Luo *et al.,* 2009). In our

findings, we could clearly distinguish macrophage invasion within the control groups, whereas the antiangiogenic groups (sFlk-1 and dendron) displayed a diminished macrophage invasion, improving the cartilage quality and hampering its resorption and inflammatory reactions (Centola *et al.,* 2013). Moreover, the antiangiogenic approach was capable of ameliorating chondrocyte survival when compared to control groups, thus augmenting cartilage maintenance.

Thus, vessel ingrowth and subsequent inflammatory reaction that takes place in cartilage engineering constructs appears highly detrimental for the cartilage being formed. Our antiangiogenic approach largely improves cartilage reproducibility, especially by low chondrogenic donors, and stability by hindering vessel formation and inflammatory host responses (Centola *et al.,* 2013). In this way, our strategy allows the standardization of cartilage formation, circumventing the inter-donor variability and the pre-culture period necessary for optimal cartilage formation, being therefore highly attractive for clinical applications. However, in the prospective of translating this approach into the clinic further investigations are needed such as assessing its efficacy in an orthotopic animal model. In our study, the effects of sFlk-1 and WHLPFKC on cartilage formation by NC were investigated in an ectopic environment, which is highly vascularized and permissive but not inductive to chondrogenesis (Dell'Accio *et al.,* 2001; Scott *et al.,* 2012). For this purpose, it was chosen as to obtain the proof of principle investigation of the effects of anti-angiogenesis in cartilage formation. However, further investigations in an orthotopic environment, where various physiological stimuli (e.g. mechanical loading and exposure of the NC to the cartilaginous milieu) can be taken into account in cartilage formation, are of imperative importance in the investigation of cartilage long-term stability. Although both the two approaches here investigated resulted to efficiently block angiogenesis and support chondrogenesis up to 8

weeks *in vivo* by using the same principle of sequestrating VEGF from the implant cell environment, they differ in the time of efficacy and for their clinical translational potential. Cell-based gene therapy indeed guarantees a sustained release of proteins whereas the scaffold functionalized with the antiangiogenic peptide might be limited in the Dendron reservoir capacity. The time, during which the anti-angiogenic effect needs to be maintained in order to induce chondrogenesis, needs still to be elucidated, however, it will be highly relevant to address this issue in an orthotopic model. Whether a sustained antiangiogenic effect will result to be essential, retroviral vectors might be substituted by lentivirus which is more compliant to clinical translation due to their lower oncogenic potential (Montini *et al.,* 2009).

Our study shows a clear advantage for potential therapeutic NC cartilage engineering, as it is deprived of any pre-culture mechanisms that could delay a clinical application setting. It also focuses solely on angiogenesis as a key component of NC cartilage formation, without the additional involvement of any growth factor.

Acknowledgements

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Figure Legends

Figure 1. *In vitro* characterization of human nasal chondrocytes expressing murine sFlk-1. A. Representative FACS plot shows that nasal chondrocytes (NC) had transduction efficiency above 80% (green-tinted plot) and populations of CD8-positive cells were purified after sorting (blue-tinted line). Not stained cells were acquired as control (in red) **B.** HUVEC metabolic activity assay showing that sFlk-1-containing supernatants efficiently blocks VEGF. CD8-expressing NC supernatants were used as control. **C.** HUVEC migration assay showing that sFlk-1-expressing NC hampered HUVEC migration by blocking different gradients of VEGF, whereas CD8-only expressing NC allowed HUVEC migration.

Figure 2. *In vitro* chondrogenesis of nasal chondrocytes. A, B. Safranin-O stainings of low (**A**) and high (**B**) chondrogenic donors, cultured in pellets at 20% of oxygen tension. Naïve, naïve mock-sorted, CD8 (control) and sFlk-1-releasing NC were cultured *in vitro* in pellets and analyzed for their chondrogenic potential. **C, D.** Glycosaminoglycan (GAG) content of low (**C**) and high (**D**) chondrogenic donors cultured at either 2 or 20% of oxygen tension. **E.** Amount of mouse sFlk-1 released by sFlk-1-expressing NC-based pellets cultured at different oxygen tensions. **F,G.** GAG content of collagen scaffold-based constructs generated by low (**F**) and high (**G**) chondrogenic NC donors. No statistical significant difference has been found.

Figure 3. *In vivo* blocking of angiogenesis. A Representative immunofluorescence pictures of naïve, sFlk-1 and dendron samples stained either in red for PECAM (CD31), an endothelial marker, and in blue for nuclei (DAPI), or in green for F4/80, a macrophage marker and in blue for DAPI. White arrows indicate the vascular structures, whereas white dotted lines mark the boundary between the implant and the surrounding host tissue. Asterisks indicate the implant area. **B** Quantification of the vessel length density in $\mu m/ \mu m^2$ at the center or edge of the implant area of naïve, sFlk-1 and dendron samples. * $p< 0.01$. Scale bars = 50µm.

Figure 4. *In vivo* chondrogenesis of low potential nasal chondrocytes – cell-based gene therapy approach. A. Safranin-O stainings for naïve and sFlk-1-releasing NC after 4 or 8 weeks *in vivo*. **B.** Types II and X collagen stainings for naïve (control) and sFlk-1 NC at 8 weeks *in vivo*. **C.** Total amount of GAG deposited *in vivo* at 4 and 8 weeks for naïve and sFlk-1 expressing NC. * $p< 0.01$. Scale bars = 50µm.

Figure 5. *In vivo* chondrogenesis of high potential nasal chondrocytes – cell-based gene therapy approach. A. Safranin-O stainings for naïve and sFlk-1-releasing NC after 4 and 8 weeks *in vivo*. **B.** Types II and X collagen stainings for naïve and sFlk-1 NC at 8 weeks in vivo. **C.** Total amount of GAG deposited *in vivo* at 4 and 8 weeks by naïve and sFlk-1 expressing NC. * $p< 0.01$. Scale bars = 50µm.

Figure 6. *In vivo* chondrogenesis of high potential nasal chondrocytes – anti-angiogenic peptide approach. A. Safranin-O, types II and X collagen stainings for naïve and dendronized peptide-associated NC at 8 weeks *in vivo*. **B.** Total GAG production in vivo at 4 and 8 weeks by naïve and dendron-associated NC. * $p< 0.01$. Scale bars = 50µm.

Supplemental Figure 1. Effect of pre-culture in *in vivo* chondrogenesis of low potential genetically-modified nasal chondrocytes. Safranin-O stainings for naïve and sFlk-1-releasing

NC construct after 2 week *in vitro* pre-culture and at 8 weeks *in vivo* after implantation of 2 week pre-cultured samples. Scale bar = 100µm.

Supplemental Figure 2. *In vivo* survival by cell-based gene therapy of low potential NC. Alu in situ hybridization images of *in vitro* pre-cultured or freshly seeded (without pre-culture) constructs generated by naive or sFlk-1-expressing NC after 8 weeks *in vivo*. Red nuclei are of murine origin, from the host, whereas blue nuclei identify human NC. Scale bar = 50µm.

FIGURE 1

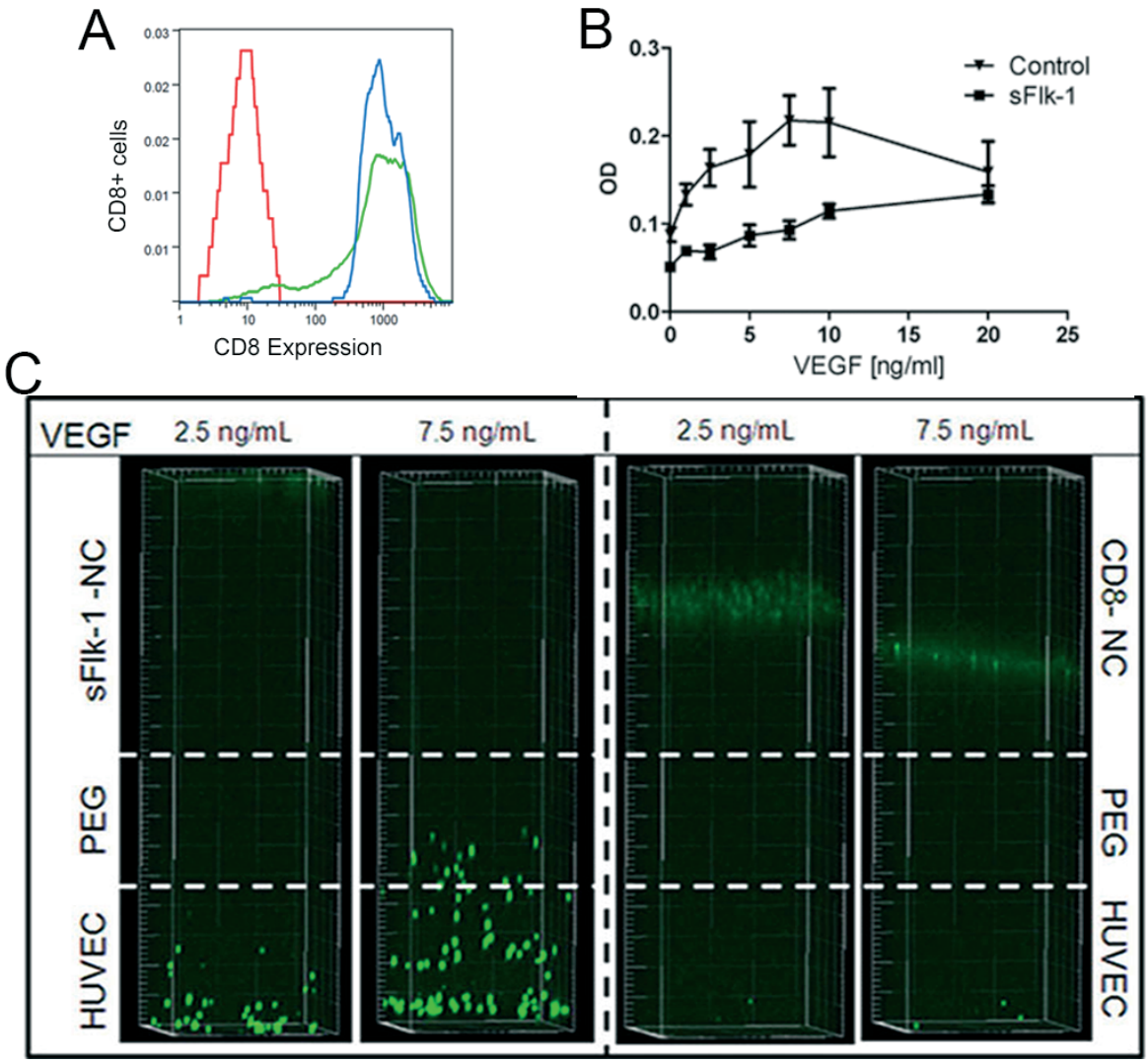
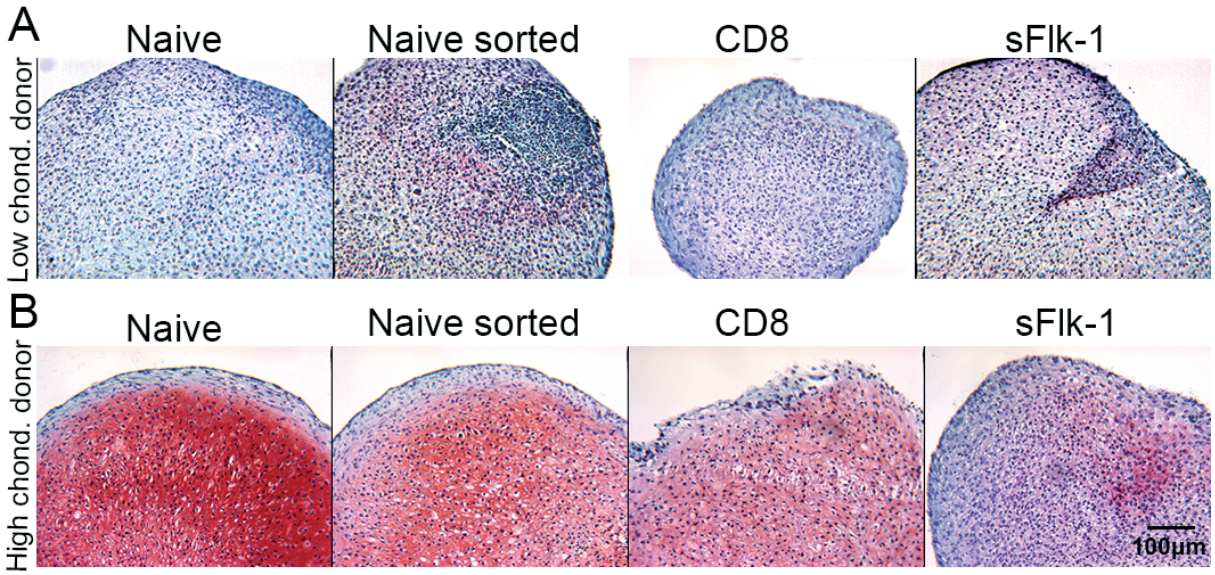
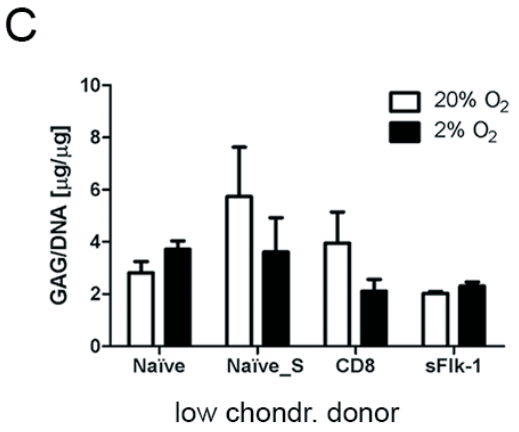
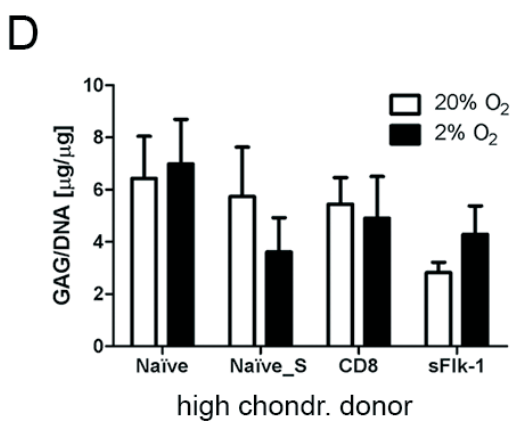

A
CD8+ cells
CD8 Expression
B
Control
sFlk-1
OD
VEGF [ng/ml]
C
VEGF
2.5 ng/mL
7.5 ng/mL
2.5 ng/mL
7.5 ng/mL
sFlk-1 -NC
PEG
HUVEC
CD8- NC
PEG
HUVEC

FIGURE 2


A
Naive
Naive sorted
CD8
sFlk-1
Low chond. donor
B
Naive
Naive sorted
CD8
sFlk-1
High chond. donor
100µm


C
GAG/DNA [µg/µg]
20% O2
2% O2
Naïve
Naïve_S
CD8
sFlk-1
low chondr. donor


D
GAG/DNA [µg/µg]
20% O2
2% O2
Naïve
Naïve_S
CD8
sFlk-1
high chondr. donor

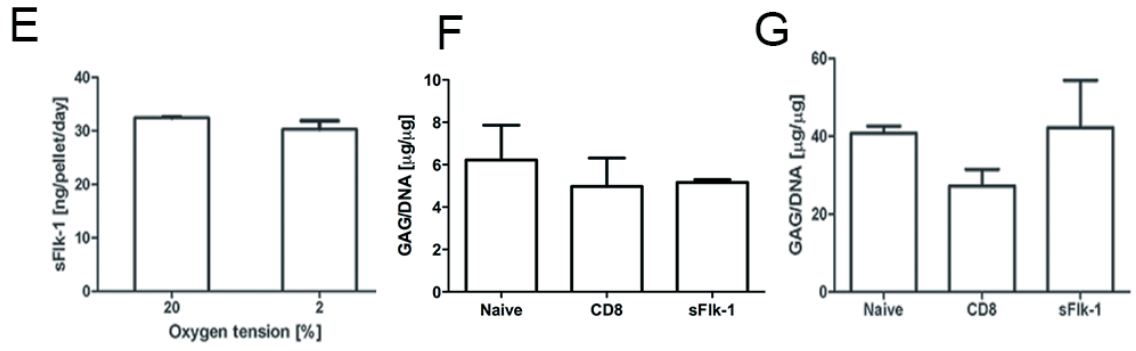

E
sFlk-1 [ng/pellet/day]
Oxygen tension [%]
F
GAG/DNA [µg/µg]
Naive
CD8
sFlk-1
G
GAG/DNA [µg/µg]
Naive
CD8
sFlk-1

FIGURE 3

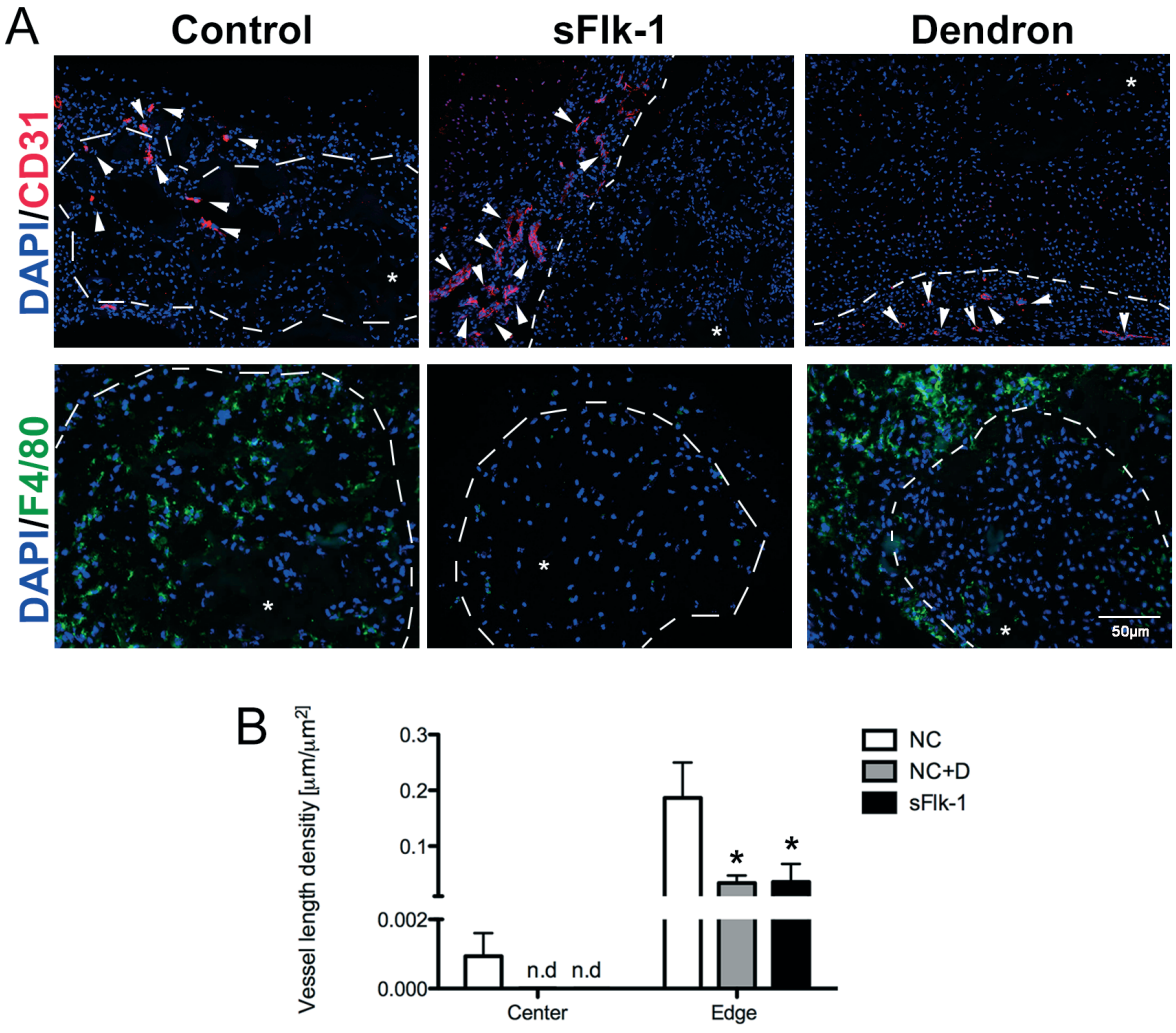

A
Control
sFlk-1
Dendron
DAPI/CD31
DAPI/F4/80
50μm
B
Vessel length densitiy [μm/μm²]
0.3
0.2
0.1
0.002
0.000
n.d
n.d
Center
Edge
NC
NC+D
sFlk-1

FIGURE 4

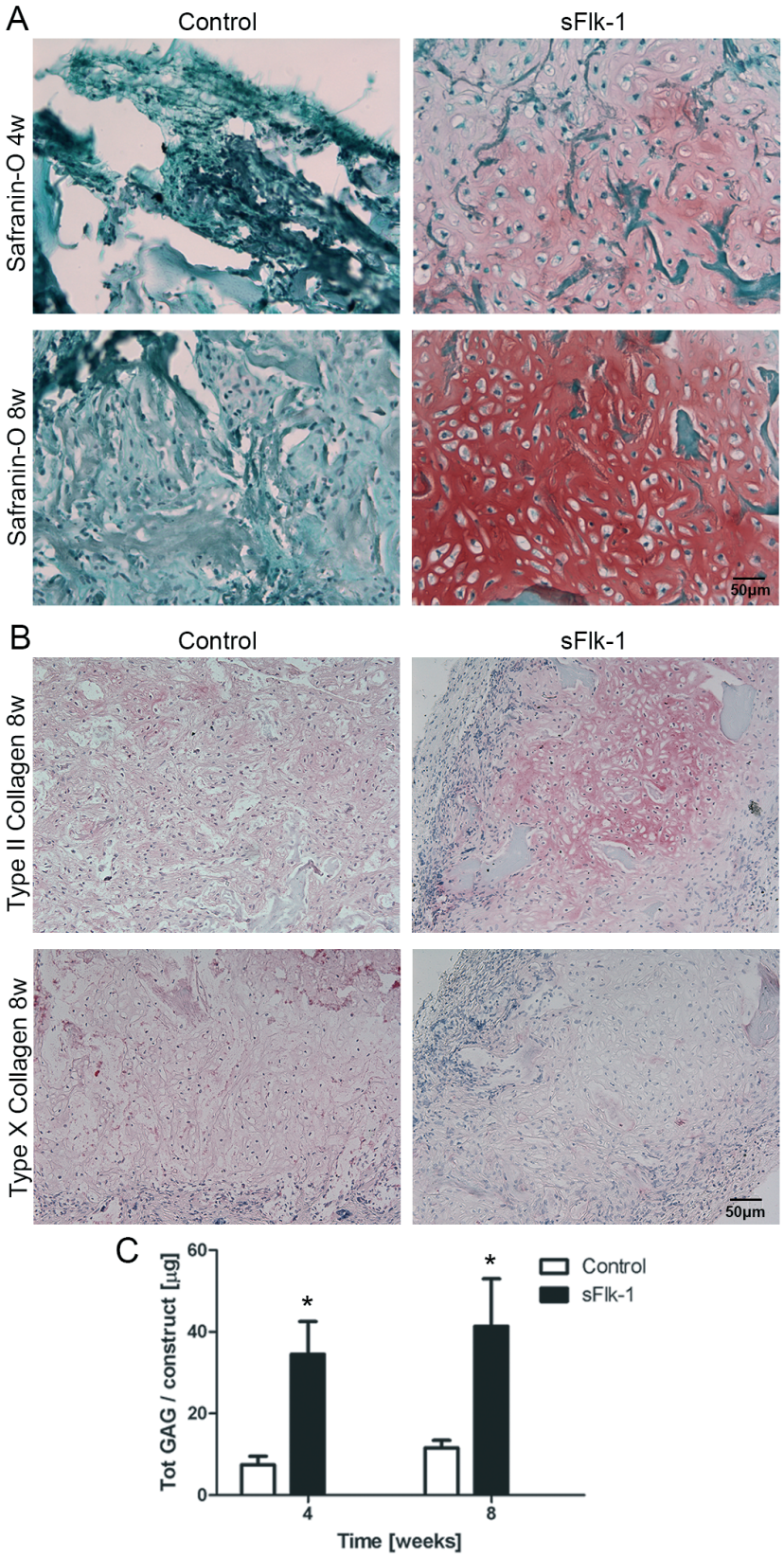

A
Control
sFlk-1
Safranin-O 4w
Safranin-O 8w
50µm
B
Control
sFlk-1
Type II Collagen 8w
Type X Collagen 8w
50µm
C
Tot GAG / construct [µg]
60
40
20
0
*
*
4
8
Time [weeks]
Control
sFlk-1

FIGURE 5

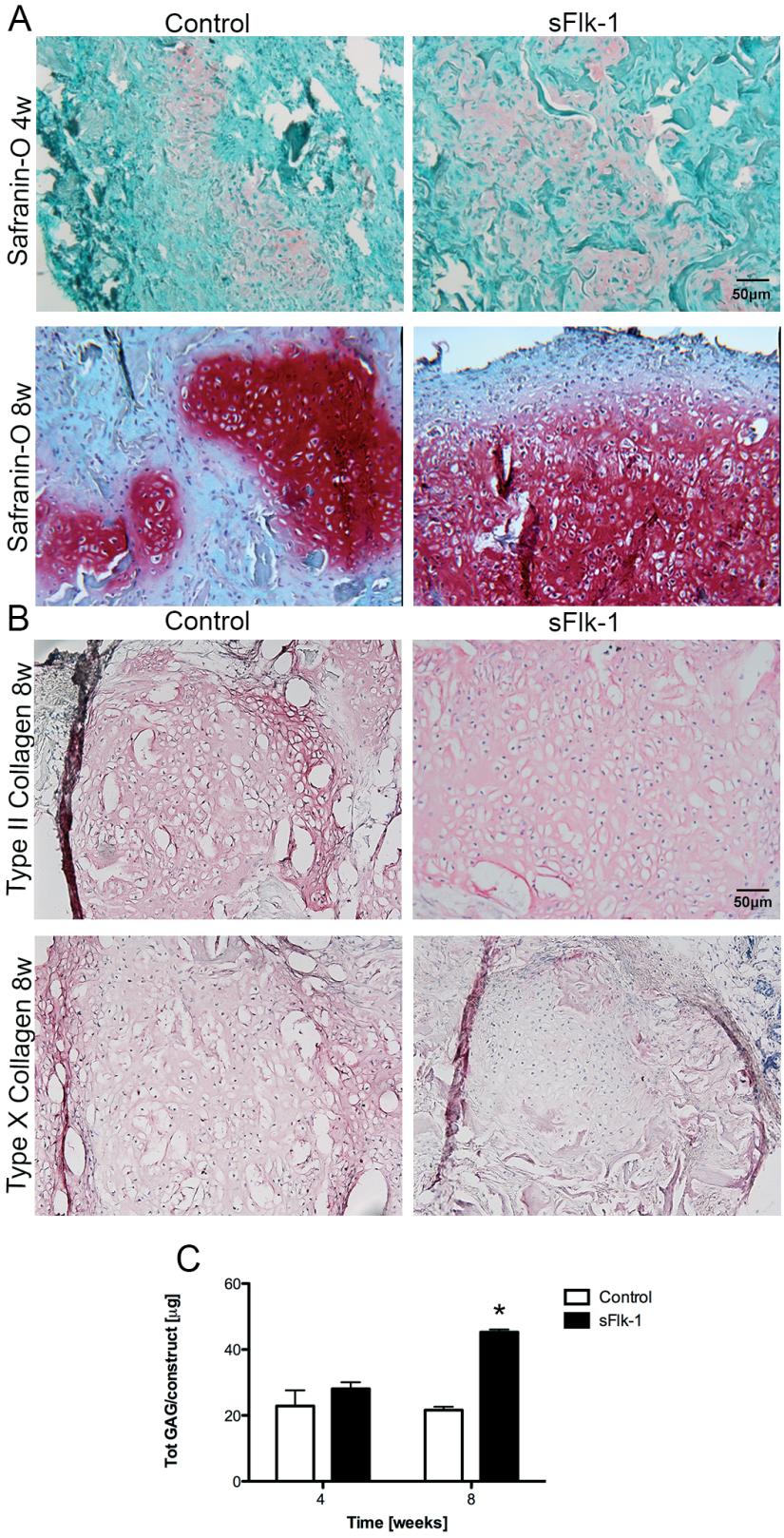
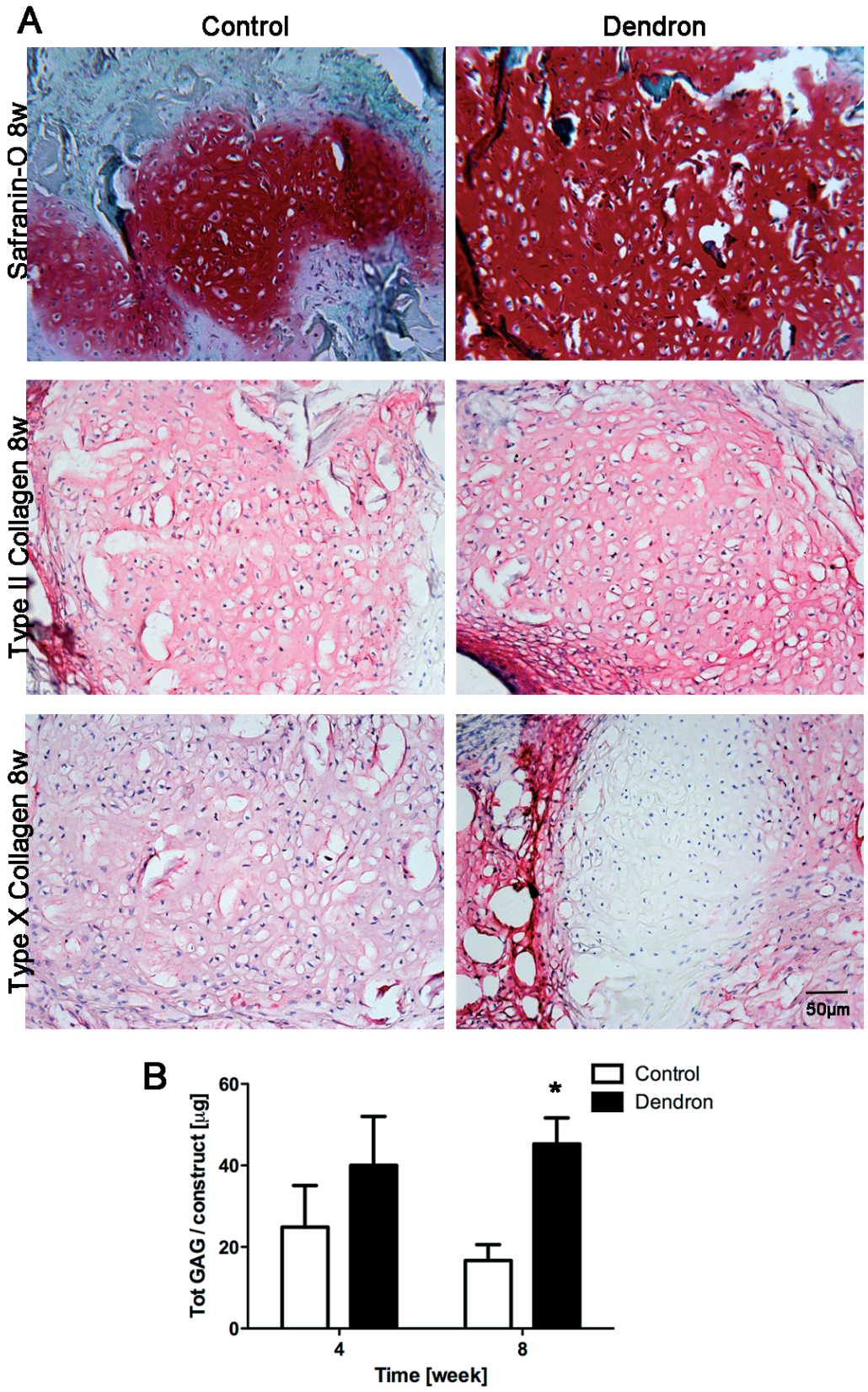
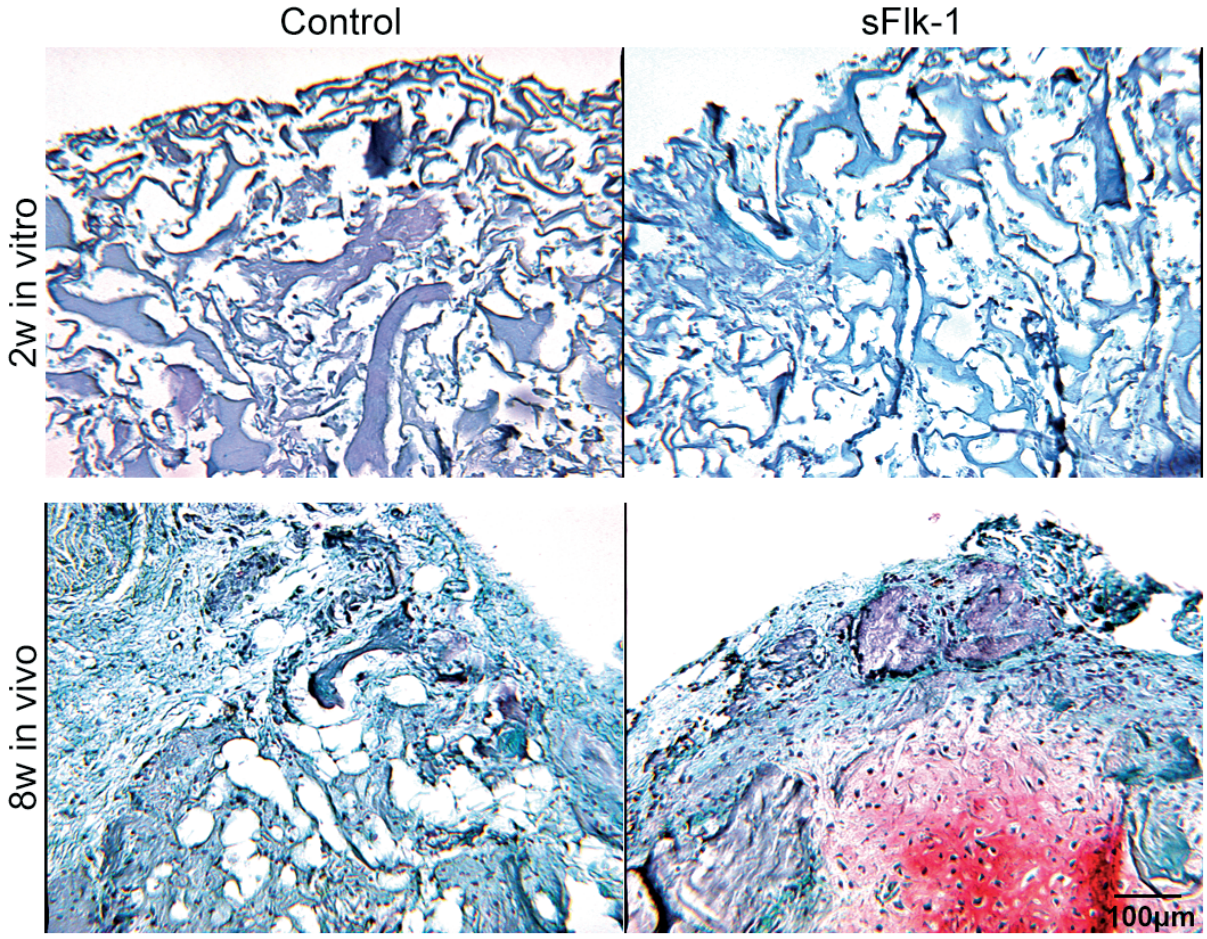

A
Control
sFlk-1
Safranin-O 4w
50µm
Safranin-O 8w
B
Control
sFlk-1
Type II Collagen 8w
50µm
Type X Collagen 8w
C
Tot GAG/construct [µg]
60
40
20
0
4
8
Time [weeks]
*
Control
sFlk-1

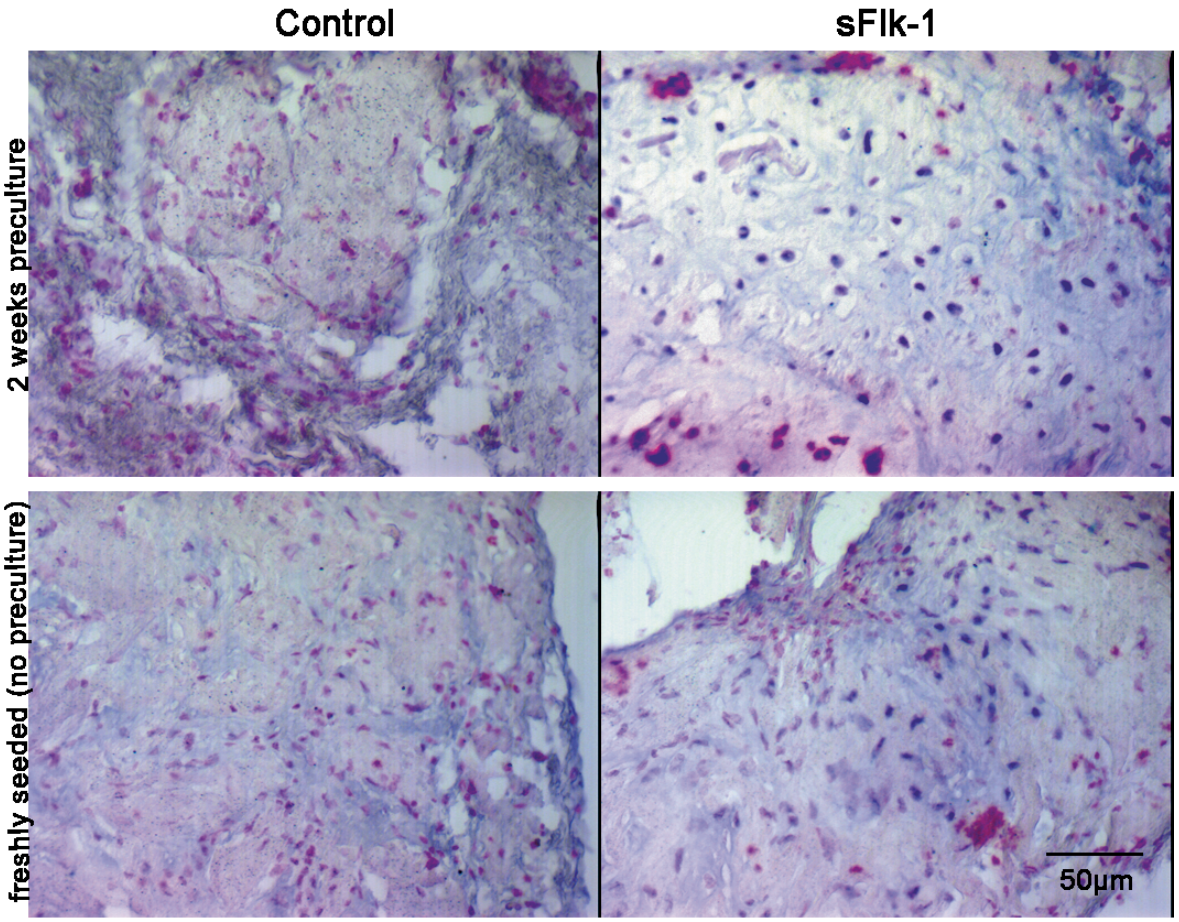
FIGURE 6


A
Control
Dendron
Safranin-O 8w
Type II Collagen 8w
Type X Collagen 8w
50µm
B
Control
Dendron
*
Tot GAG / construct [µg]
60
40
20
0
4
8
Time [week]

SUPPLEMENTAL FIGURE 1



SUPPLEMENTAL FIGURE 2



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Chapter 2

Spontaneous in vivo chondrogenesis of bone marrow-derived mesenchymal progenitor cells by blocking VEGF signalling

Spontaneous *in vivo* chondrogenesis of bone marrow-derived mesenchymal progenitor cells by blocking VEGF signalling

Marsano A[1†], Medeiros da Cunha CM[1†], Ghanaati S[2,3], Gueven S[1], Centola M[1], Tsaryk R[2], Barbeck M[2], Barbero A[1], Helmrich U[1], Schaeren S[1], Kirkpatrick CJ[2], Banfi A[1*†], Martin I[1*†].

[†] Equally contributing authors
* Corresponding authors

[1]Departments of Biomedicine and of Surgery, University Hospital of Basel, University Hospital Basel, Basel, Switzerland; [2]Institute of Pathology, University Medical Center Mainz, Germany; [3] Department for Oral, Craniomaxillofacial and Facial Plastic Surgery, University Frankfurt am Main, Germany.

For correspondence and reprint requests:

Prof. Dr. Ivan Martin Forschungsgruppenleiter Tissue Engineering; University Hospital Basel, ICFS, Hebelstrasse 20, CH-4031 Basel; Telefon +41 61 265 23 84; Telefax +41 61 265 39 90; E-Mail imartin@uhbs.ch

PD Dr Andrea Banfi Forschungsgruppenleiter Cell and Gene Therapy; University Hospital Basel, ICFS, Hebelstrasse 20, CH-4031 Basel; Tel. +41-61-265 3507; Fax +41-61-265 3990; E-mail: Andrea.Banfi@usb.ch

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Abstract

Objective. To induce ectopic chondrogenesis of human bone marrow-derived mesenchymal stromal cells (BMSC) by sequestering VEGF in the implant area, in the absence of any *in vitro* or *in vivo* commitment.

Methods. BMSC were transduced by using retroviral constructs to express soluble VEGF receptor-2 (sFlk-1). The effect of the transduction process and sFlk-1 release was investigated in an *in vitro* chondrocytic differentiation culture system. *In vivo* chondrogenic capacity of genetically modified BMSC was assessed by implantation for up to 12 weeks in an ectopic nude mouse model, immediately after seeding in a collagen sponge scaffold.

Results. *In vitro*, neither the transduction process nor the sFlk-1 expression affected the chondrogenic potential of BMSC. *In vivo*, blood vessel ingrowth was significantly reduced in the tissues engineered by sFlk-1-expressing as compared to control cells. Constructs generated by BMSC expressing sFlk-1 spontaneously developed into frank and phenotypically stable cartilage tissues, as assessed immunohistochemically and biochemically.

Conclusion. Overexpression of sFlk-1 and the associated reduction in blood vessel ingrowth can induce *in vivo* chondrogenesis by BMSC, even in the absence of chondroinductive signals during *in vitro* culture or at the implant site. The absence of such effect in the *in vitro* model suggests a predominant paracrine mechanism, related to the reduction of angiogenesis by host cells.

Introduction

Bone marrow-derived mesenchymal stromal cells (BMSC) have been proposed for cell-based cartilage regeneration due to their capacity to differentiate into chondrocytes and to their availability also in an autologous setting. However, the chondrogenic differentiation of BMSC typically requires the prolonged exposure to potent morphogens, which may not be available at the site of implantation (Johnstone *et al.,* 1998). On the other hand, the pre-culture of BMSC in the presence of chondroinductive molecules is generally associated with the onset of a hypertrophic chondrocyte phenotype, leading to mineralization, vessel ingrowth and remodeling into bone tissue upon *in vivo* implantation (Pelttari *et al.,* 2008; Scotti & Tonnarelli *et al.,* 2010).

During limb development, chondrogenesis of condensating mesenchymal cells occurs in the absence of vasculature. Moreover, the stability of the chondrocytic phenotype at the articulating surfaces of long bones as opposed to progression of differentiation towards endochondral ossification is associated with the maintenance of an avascular environment (Carlevaro *et al.,* 2000). Consistent with these observations, seminal experiments of marrow stromal cells implanted subcutaneously in diffusion chambers, not allowing blood vessel ingrowth, reported the spontaneous formation of cartilage matrix, along with bone-like and fibrous tissue (Ashton *et al.,* 1980).

Vascular Endothelial Growth Factor (VEGF) is the master regulator of angiogenesis during skeletal growth (Ferrara *et al.,* 2001; Carmeliet *et al.,* 2000). VEGF expression critically regulates cartilage ossification (Gerber *et al.,* 1999), as it is produced by hypertrophic chondrocytes at the start of endochondral bone formation, but not by quiescent and proliferating chondrocytes at earlier stages, and its inhibition during bone elongation prevents the vascular infiltration of hypertrophic cartilage and the ossification of the growth plate (Gerber *et al.,* 1999). Moreover,

VEGF has been involved in the onset of degenerative changes of cartilage tissue, such as osteoarthritis (Murata *et al.,* 2008).

The soluble variants of VEGF receptors, namely soluble Flt-1 and Flk-1 (which are the extracellular cleaved forms of VEGFR1 and VEGFR2, respectively (Kendall *et al.,* 1996; Ebos *et al.,* 2004) are capable of acting as decoy receptors, sequestering VEGF from the immediate chondrocyte environment. Kubo and colleagues have previously reported that articular cartilage repair by genetically modified skeletal muscle-derived stem cells in an osteochondral defect (Matsumoto *et al.,* 2009) or osteoarthritis model (Kubo *et al.,* 2009) could be improved by overexpression of both soluble Flt-1 and bone morphogenic protein BMP-4 (a potent chondrogenic morphogen).

In the present study, we investigated whether the simple inhibition of angiogenesis by VEGF blockade could be sufficient to direct *in vivo* chondrogenic differentiation of human BMSC, in a permissive environment and in the absence of any specific chondrogenic morphogens. We thus transduced BMSC to express sFlk-1, seeded them on type I collagen scaffolds (Sabatino *et al.,* 2012; Centola *et al.,* 2013), which do not induce chondrogenesis per se, and directly implanted the constructs subcutaneously in nude mice without *in vitro* pre-culture. This ectopic model provides a well-controlled environment to assess the cartilage-forming capacity of human cells, bypassing the biological (e.g., inflammatory component) and physical (e.g., mechanical loading) complexity of a joint environment (Dell'Accio *et al.,* 2001).

Methods

Cell isolation and transduction

Bone marrow aspirates were harvested from the iliac crest of 5 healthy donors (average age of 31 years, range 20-43) during orthopaedic surgical procedures, in accordance to the local ethical

committee and following informed consent. BMSC were isolated as described by Horn and colleagues (Horn *et al.,* 2008). Fresh bone marrow was plated at a density of 10^5 nucleated cells/cm^2 and cultured in high glucose DMEM (Gibco, Carlsbad, CA) supplemented with 10% FBS (complete medium) and 5 ng/ml FGF-2 (BD Biosciences, Basel, Switzerland), in order to maintain BMSC differentiation capacity (Bianchi *et al.,* 2003; Solchaga *et al.,* 2005). Six days after the first plating, primary BMSC were transduced with previously described retroviral vectors, carrying the gene for the mouse VEGF receptor-2 extracellular domain (sFlk-1) fused to the IgG2α Fc domain (kindly provided by Kuo CJ, Stanford University) (Kuhnert *et al.,* 2008), linked through an Internal Ribosomal Entry Site (IRES) to a mouse truncated CD8a version (Misteli *et al.,* 2010). Either naïve cells or BMSC transduced only with the truncated murine CD8a were used as controls. Cells were transduced according to a high-efficiency protocol we recently described (Helmrich *et al.,* 2012). Briefly, cells were cultured in 60-mm dishes and incubated with retroviral vector supernatants supplemented with 8µg/ml polybrene (Sigma-Aldrich, St. Louis, MO) for 5 minutes at 37°C and centrifuged at 1100g for 30 minutes at room temperature in the dishes, followed by fresh medium replacement. Transductions were repeated twice a day for a total of 6 rounds. Cells were expanded for two passages and replated at a density of 10^4 cells/cm^2.

In vitro pellet culture model

BMSC transduced for sFlk-1 and control cells were cultured in pellet systems as previously described (Barbero *et al.,* 2003). Briefly, $0.5x10^6$ cells per 0.5mL of serum-free medium (SFM), which consists of DMEM (Gibco), 1% Hepes (Gibco), 1% penicillin-streptomycin solution (Gibco), 1% sodium pyruvate (Gibco), 1% Insulin-transferrin-sodium selenite (ITS+1, Sigma-Aldrich, St. Louis, USA), 1% human serum albumin (HSA 100x, Invitrogen, Carlsbad, USA), 0.1 mM ascorbic acid 2-phosphate (Invitrogen) and dexamethasone 10^{-5}M (Invitrogen), were placed into conical

polypropylene tubes and spun down for 4 min at 1500 rpm to form cell aggregates. The SFM was either supplemented with 10ng/ml of TGFβ3 or without TGFβ3. The pellets (control cells and sFlk-1 transduced cells) were cultured for 2 weeks at 2% or 20% oxygen tension. The culture medium was changed twice a week and medium was first equilibrated to the specific oxygen tension.

Cell-based construct preparation and ectopic implantation

A type I collagen sponge (UltrafoamTM, Bard, Davol Inc., Warwick, RI) of 4 mm thickness was punched into 6 mm diameter disks, each of which was statically seeded with $1.7x10^6$ BMSC (density of $1,5x10^7$ cells/cm^3) (Helmrich *et al.,* 2012; Moretti *et al.,* 2005). The constructs were cultured overnight with DMEM supplemented with 10% FBS and 1% penicillin-streptomycin before implantation.

A total of 4 cell-based constructs was implanted subcutaneously for each nude mouse (CD-1 nude/nude, Charles River, Germany). The mice were sacrificed with CO_2 at 1, 4, 8 and 12 weeks after the implantation. All animals were cared for and processed according to the guidelines of the Cantonal Veterinary Office (Basel, Switzerland) for the care and use of laboratory animals. Each analysis was performed independently in four engineered tissues for each BMSC primary.

Analytical methods

Fluorescence-activated cell sorting (FACS)

Cells positive for CD8a (either sFlk-1-expressing or control CD8a-expressing BMSC) were sorted by staining with an allophycocyanin-conjugated anti-mouse CD8a (clone 53–6.7; BD Biosciences) as previously described (Misteli *et al.,* 2010) by using a FACSCalibur flow cytometer (Becton Dickinson, Allschwil, Switzerland) and analyzed using FlowJo software (Tree Star, Ashland, OR).

Enzyme-linked Immunosorbent Assay- *ELISA*

BMSC cultured in 60-mm dishes were incubated with 1 mL of culture medium for 4 hours. The supernatants were filtered through 0.45 µm syringe filters and frozen. Soluble mouse Flk-1 and human VEGF production were quantified in cell culture supernatants using species-specific Quantikine immunoassay ELISA kits (R&D Systems, Abingdon, UK) according to the manufacturer's instructions. Data were assessed in duplicate for each donor and results were normalized by the number of cells counted in each dish and the time of incubation.

Biochemistry

Engineered tissues cultured *in vitro* for three weeks were digested with protease K for 15 hours at 56°C (Hollander *et al.,* 1994). The glycosaminoglycan (GAG) and DNA contents were measured spectrophotometrically using dimethylmethylene blue (Farndale *et al.,* 1986) and the CyQUANT Kit (Molecular Probes, Eugene, OR) (Barbero *et al.,* 2003), respectively. GAG contents were normalized to the amount of DNA.

Quantitative real time RT-PCR

Total RNA of cells was extracted using Trizol (Life Technologies, Basel, Switzerland) and the standard single-step acid-phenol guanidinium method. RNA was treated with DNAseI using the DNA-free™ Kit (Ambion, Applied Biosystems, Carlsbad, CA). cDNA was generated from 3 µg of RNA by using 500 µg/ml random primers (Catalys AG, Wallisellen, Switzerland) and 1µL of 50 U/ml StratascriptTM reverse transcriptase (Stratagene, Agilent Technologies, Basel, Switzerland), in the presence of dNTPs. Real-time RT-PCR reactions were performed and monitored using the ABI Prism 7700 Sequence Detection System (Perkin- Elmer/Applied Biosystems, Rotkreuz, Switzerland), and the gene expression data for all tested genes were normalized to GAPDH or

18S. The primers used were the following: GAPDH (reverse – ATGGGGAAGGTGAAGGTCG; forward – TAAAAGCAGCCCTGGTGACC; probe - CGCCCAATACGACCAAATCCGTTGAC); 18S (reverse – GCTGGAATTACCGCGGCT; forward – CGGCTACCACATCCAAGGAA; probe - TGCTGGCACCAGACTTGCCCTC); type II collagen (reverse – GGCAATAGCAGGTTCACGTACA; forward – CGATAACAGTCTTGCCCCACTT; probe – CCGGTATGTTTCGTGCAGCCATCCT); type X collagen (reverse – CAAGGCACCATCTCCAGGAA; forward – AAAGGGTATTTGTGGCAGCATATT; probe – TCCCAGCACGCAGAATCCATCTGA); RUNX-2 (reverse – GCCTTCAAGGTGGTAGCCC; forward – CGTTACCCGCCATGACAGTA; probe – CCACAGTCCCATCTGGTACCTCTCCG). Taqman Assays on Demand (Applied Biosystems) were used to evaluate the expression of Indian Hedgehog (IHH, Hs654504).

HUVEC proliferation and HDMEC tube formation in vitro assays

Human umbilical vein endothelial cells (HUVEC) were cultured in 96-well plates (5 x 10^3 per well) in growth medium, consisting of medium199 supplemented with 20% FBS, 100 µg/ml endothelial cell growth supplement (ECGS, Sigma-Aldrich), 50 U/ml heparin and 100 U/ml penicillin and 100 µg/ml streptomycin. After one day of culture, the growth medium was replaced by assay medium, consisting of medium199 (Invitrogen, Basel, Switzerland) with 5% FBS, 10 U/ml heparin, 100 U/ml penicillin and 100 µg/ml streptomycin (Witzenbichler *et al.,* 1998), that was conditioned by 6-hour incubation with either control or sFlk-1 expressing BMSC and supplemented with increasing levels of human VEGF (up to 50 ng/mL). HUVEC proliferation was then assessed after three further days of culture by using [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] (MTS, Promega, Madison, WI).

Human dermal microvascular endothelial cells (HDMEC) were used in an *in vitro* tube formation assay as previously described (Peters *et al.,* 2002). Briefly, HDMEC (1.2x10^6 cells per 1 ml gel)

were resuspended in a solution containing type I collagen (0.77 mg/ml, MP Biomedicals, Basel, Switzerland) and fibrinogen (1 mg/ml, Sigma-Aldrich) and the gel was polymerized for 45 min at 37°C by the addition of double concentrated IMDM medium (Invitrogen) and thrombin (0.5 U/ml, Sigma-Aldrich). Control or sFlk-1-expressing BMSC conditioned media (Endothelial Cell Basal Medium MV (PromoCell, Heidelberg, Germany) with 5% FBS and 1% penicillin-streptomycin solution) were extracted after 48 h. Human VEGF (Biomol, Hamburg, Germany) at different concentrations (up to 50 ng/mL) was added to the conditioned media 1 h before the media were used in the tube formation assay. The formation of tube-like structures by HDMEC was visualized 48 h after embedding in collagen/fibrin gel via calcein-AM (Invitrogen) staining. Number and length of tubules, as well as network characteristics (number of branching points and of loops, defined as the closed areas inside the tubular structure) were quantified with web-based WinTube Image Analysis (Wimasis, Munich, Germany).

Alternatively, tube-like formation was assayed in a direct co-culture of control or sFlk-1 expressing BMSC with HDMEC (Unger *et al.,* 2002). $1x10^5$ HDMEC were seeded on gelatin pre-coated 35 mm dishes and after 24 h $0.3x10^5$ BMSC were added. The cells were co-cultivated for 14 days in Endothelial Cell Basal Medium MV (PromoCell) with 15% FBS, 1% penicillin-streptomycin solution, sodium heparin (10 µg/ml) and bFGF (2.5 ng/ml). Tube-like structures were visualized with CD31 staining (Dako, Glostrup, Denmark) and a secondary goat anti-mouse antibody (Alexa Fluor 488, Invitrogen); nuclei were stained with Hoechst 33342 (Sigma-Aldrich).

Histology and Immunohistochemistry for Types II and X Collagen

Engineered tissues were fixed with 4% formaldehyde and embedded in paraffin. Sections (5 µm thick) were stained for Safranin-O and for immunohistochemistry for types II and X collagen. Briefly, for collagen immunohistochemistry, a pre-treatment of the histological sections was

performed with 2 mg/ml hyaluronidase (Sigma-Aldrich) for 15 minutes followed by 1 mg/ml Pronase (Roche Diagnostics, Mannheim, Germany) for 30 minutes. The primary antibodies used were mouse anti-collagen II (clone II-4C11, MP Biomedicals, 1:1000), and mouse anti-collagen X (monoclonal clone COL-10, Abcam, Cambridge, UK, 1:5000). As a secondary antibody, goat anti-mouse IgG/Biotin (Dako, 1:200) was used. The Vectastain ABC/AP kit (Linaris, Dossenheim, Germany) and Vector Red Alcaline Phophatase kit with Levamisole (Linaris) were used to form the staining complex.

Human Cell Engraftment

Explants were fixed in 4% paraformaldehyde (Sigma-Aldrich), cryoprotected in 30% sucrose in PBS overnight and frozen in freezing isopentane (Sigma-Aldrich) after embedding in OCT. Cryosections (10 µm thick) were incubated with the primary antibodies pan human anti-nuclei (BD Pharmingen, Franklin Lakes, NJ) and with a secondary antibody labelled with Alexa Fluor 546 (Invitrogen/Life Technologies, Carlsbad, CA). DAPI was used as nuclear staining. Fluorescence images were acquired using an epifluorescence microscope (Olympus DP80) (Olympus, Muenster, Germany).

Histomorphometry and Quantification of Vascularization

Immunohistochemical processing was applied as previously described (Ghanaati *et al.,* 2011). Briefly, an initial quenching of endogenous peroxidase activity using 3% H_2O_2 in demineralized water was performed after deparaffinizing and rehydrating the tissue sections. Heat-induced epitope retrieval was then applied using citrate buffer with a pH of 6.0 for mouse-specific CD31 (PECAM-1, Dako) staining. Histological sections stained for CD31 were analyzed for vessel

quantification using the NIS-Elements 4.0 software. The vessel number was then normalized to the total implantation bed area, reporting values as vessels/mm^2.

Statistics

Data were analyzed with the statistical software Prism 5.0 (GraphPad Software Inc., La Jolla, CA), using one-way analysis of variance (ANOVA) with Bonferroni correction. Differences were considered significant if their P-values were less than 0.05 (*P < 0.05) and highly significant if their P-values were less than 0.01 (**P < 0.01). Data are presented as mean ± standard deviation.

Results

Genetically modified BMSC

The efficiency of BMSC transduction to express CD8a alone or in combination with sFlk-1 was 82±7% and 89±3%, respectively. Genetically modified BMSC released 1.37±0.14 ng/10^6 cells/day of mouse sFlk-1. The transduction process did not impair the proliferation potential of BMSC, despite a slight reduction in the doubling rate in monolayer culture when compared to control BMSC (total cell doublings of 5.6±0.5, 4.5±0.8, 3.9±0.2 for naïve, control and sFlk-1 transduced BMSC, respectively, in 14 days, data are presented as mean ±SEM, n=2), consistently with our previous findings (Helmrich *et al.,* 2012). The transduction process also did not affect the chondrocytic re-differentiation capacity of BMSC in transwell culture. In fact, the GAG per DNA ratio of the tissues formed by CD8- or CD8a/sFlk-1-expressing BMSC (18.8±9.1 and 27.6±8.9, respectively) was similar or even higher than that by naïve cells (14.5±1.8). Based on these *in vitro* results, most of the subsequent analyses were performed using only naïve cells as control.

Secreted sFlk-1 was biologically active, as shown by its ability to impair *in vitro* angiogenesis by HDMEC in a 3D collagen/fibrin gel-based culture model (Figures 1 A-F). Comparing the 3D

cultures with control or sFlk-1 supernatants in the presence of up to 50 ng/mL of VEGF, sFlk-1 substantially reduced endothelial cells' organization into tubular structures, as shown by the decreased tube number and length, as well as the maturation of capillary-like networks, as shown by the lower branching points and number of loops (Figures 1 I-L). The inhibition of *in vitro* capillary-like structures formation was also observed by the direct co-culture of HDMECs with sFlk-1-releasing cells (Figure 1H) as compared to control cells (Figure 1G). sFlk-1 released by BMSC was shown to significantly reduce HUVEC proliferation induced by VEGF concentrations up to 10 ng/mL (Figure 1M).

In vitro pellet culture model

We then tested whether VEGF blockade would influence BMSC chondrogenesis *in vitro*. Since hypoxia is known to enhance VEGF expression (Liu *et al.,* 1995) and has been postulated to play a role during chondrogenesis (Malladi *et al.,* 2007; Kanichai *et al.,* 2008), the experiments were performed at different oxygen tensions and chondrogenesis was assessed by Safranin-O staining and biochemical analysis of GAG deposition (Supplementary Figure 1). No differences in chondrogenesis could be observed among the sFlk-1-expressing and control BMSC under the different oxygen tensions. Cartilaginous tissues formed in the presence of TGFβ-3 at either oxygen tension by control and sFlk-1 BMSC were morphologically similar (Supplementary Figure 1 A-D), with no differences in the amount of GAG (Supplementary Figure 1E).

Analyses at the mRNA level confirmed the lack of effects of VEGF blockade on BMSC differentiation: sFlk-1 constructs at 2% or 20% oxygen did not display an increased expression of chondrogenic genes, such as SOX9 or type II collagen, or a regulation of hypertrophy, assessed by type X collagen expression (data not shown). These data indicate that VEGF sequestration does not exert a direct effect on BMSC chondrogenesis.

VEGF expression at mRNA level was similar in both control and sFlk-1 constructs, for all culture conditions (data not shown). However, as shown in Figure 2F, while significant amounts of VEGF protein were released by naïve BMSC when cultured both at 20% and 2% oxygen tension, in the supernatants of sFlk-1 constructs VEGF protein was almost undetectable both under hypoxic and normoxic conditions, confirming the potency of sFlk-1 in sequestering released VEGF.

Since sFlk-1 is not responsible for promoting chondrogenesis *in vitro,* we sought to investigate whether other factors would contribute to cartilage formation *in vitro*. The pellet culture was repeated using only control BMSC with and without the supplementation of TGFβ3. When in the presence of TGFβ3, no major differences in BMSC chondrogenesis were observed among the different oxygen tensions. No significant differences were detected histologically or at the mRNA level (Figure 2 A-E). However, once TGFβ3 was removed from the pellet culture, chondrogenesis only occurred under hypoxia, which can be observed by Safranin-O (Figure 2F) and type II collagen (Figure 2H). Furthermore, type X and IHH, typically overexpressed in hypertrophic cartilage, are downregulated in the absence of TGFbeta 3 at both oxygen tension culture conditions.

In vivo block of angiogenesis

Control or sFlk-1-expressing BMSC were loaded into collagen sponges and immediately implanted subcutaneously in nude mice to assess their vascularization potential. Quantification of vessel ingrowth was performed on CD31 stained cross sections on the total area of the implant. Constructs based on control BMSC displayed an ingrowth of vessels already after 1 week, up to the center of the implant (Figure 3 A-D). Instead, no vessel infiltration within the implant area was observed in the center of sFlk-1 constructs up to 12 weeks, such that the angiogenic process was confined at the periphery of the implant (Figure 3 E-H). Explants

generated by control or sFlk-1 expressing BMSC displayed a clear difference in macroscopic vascularization at 12 weeks (Figure 3I). Control constructs appeared smaller and vascularized as compared to the sFlk-1-expressing BMSC-based implants, which had a white cartilage-like appearance. Quantitative analysis further indicated a significantly higher vessel density in the total implant area (comprising both centre and periphery) when control cells were used as compared to sFlk-1-expressing BMSC (Fig. 3J, p=0.0015).

In vivo chondrogenesis

No cartilage could be observed in collagen sponges loaded with control BMSC at any time point and in any of the 5 donors investigated in this study (Figures 4A, 4C, 4E). Remarkably, instead, implants based on sFlk-1 engineered BMSC displayed cartilaginous appearance and a positive staining for GAG after 4 weeks (Figure 4D), which increased in intensity up 12 weeks (Figure 4F). The collagen-based scaffold was almost completely remodelled by the cells and only few traces were still visible (Figure 4A-F).

Gene expression analysis was performed 1 week after *in vivo* implantation in order to investigate the initial commitment of the implanted cells toward a chondrocytic lineage. Type II collagen, a hyaline cartilage marker, was expressed at undetectable level in expanded BMSC (data not shown) and was 210–fold higher expressed in the BMSC expressing sFlk-1 as compared to control naïve cells (Figure 4G). On the contrary, the expression of osteogenic (Runx2) and hypertrophy (type X collagen) markers was markedly reduced by at least 10-fold in sFlk-1 constructs as compared to control BMSC.

Immunohistochemical analysis indicated that type II collagen deposition increased over time in the sFlk-1 implants (Figure 5 E-F), whereas type X collagen remained predominantly negative at

all time points (Figure 5 G-H). In the control group a loose connective tissue was formed and neither type II nor type X collagen could be detected by immunostaining (Figure 5 A-D).

Immunofluorescent assessment of human cell nuclei in the explants indicated that sFlk-1-expressing human BMSC persisted in the formed cartilaginous tissue up to 12 weeks after *in vivo* implantation (Figure 6). In contrast, control BMSC could be detected *in vivo* only up to 4 weeks *in vivo*, even though at a much lower frequency than sFlk-1 cells, and a negligible fraction survived by 12 weeks (Figure 6). Therefore, VEGF blockade was essential to induce the long-term engraftment of human BMSC and cartilage formation.

Discussion

In this study, we demonstrated that blocking VEGF signaling by sFlk-1 overexpression, resulting in efficient inhibition of angiogenesis, could reproducibly induce *in vivo* chondrogenesis by uncommitted BMSC. Mature hyaline cartilage was already formed after only 4 weeks and maintained up to the last time of observation (12 weeks) with features of phenotypic stability, namely abundant deposition of type II collagen and GAG, as well as undetectable type X collagen staining.

Although it is well established that one of the hallmarks of hyaline cartilage during development and adulthood is the avascular nature, our findings provide the unprecedented proof that the sole regulation of angiogenesis, without the delivery of any additional morphogens, may be sufficient for inducing mesenchymal progenitors into full chondrogenic differentiation. The effects of sFlk-1 on cartilage formation by BMSC were investigated in an ectopic environment, which is permissive but not inductive to chondrogenesis (Dell'Accio *et al.,* 2001; Scott *et al.,* 2012). Previous studies, using murine muscle-derived stem cells (MDSC) engineered to sequester VEGF by overexpressing soluble VEGFR-1 (sFlt-1), failed to achieve enhanced cartilage healing in

osteochondral defect (Kubo *et al.,* 2003) or osteoarthritis (Matsumoto *et al.,* 2003) models, unless a potent chondrogenic morphogen (BMP-4) was additionally delivered. The apparent discrepancy may imply (i) the yet uncontrolled complexity of biochemical or biomechanical regulatory signals at an orthotopic joint environment and/or (ii) a fundamental biological difference between the two species and classes of progenitors, with BMSC – as opposed to MDSC - being physiologically responsible for cartilage formation (Yoo *et al.,* 1998).

In our study, overexpression of sFlk-1 by BMSC appeared to be essential (i) to induce a chondrogenic commitment, as assessed by the 210-fold higher type II mRNA expression as early as 1 week after implantation, (ii) to enhance implanted cell survival up to 12 weeks, and (iii) to preserve a stable chondrocyte phenotype, as the formed cartilage did not undergo the expected process of endochondral ossification (Scotti & Tonnarelli *et al.,* 2010). Mechanistically, these findings could be explained by the indirect effect of inhibition of angiogenesis, or by a direct autocrine regulation by sFlk-1, as VEGF is expressed by human BMSC (Kaigler *et al.,* 2003), and immature (Maes *et al.,* 2004) as well as hypertrophic (Carlevaro *et al.,* 2000) chondrocytes.

Our *in vitro* experiments indicate that blocking VEGF by sFlk-1 expression did not regulate the chondrogenic capacity of BMSC, consistent with a previous report using muscle-derived stem cells releasing sFlt-1 (Kubo *et al.,* 2009). Since one of the immediate consequences of inhibition of angiogenesis is the generation of a hypoxic environment, and hypoxia is a key regulator of chondrogenesis during limb development (Araldi *et al.,* 2010), we next investigated the effects of hypoxia in promoting *in vitro* chondrogenesis. Strikingly, in the absence of TGFβ3, reduction of the oxygen percentage from 20% to 2% resulted in BMSC chondrogenesis, whereas under normoxic conditions without TGFβ3 no cartilage formation was observed. To our knowledge, this is the first time that *in vitro* chondrogenesis of BMSC is achieved without a soluble member of the TGFβ/BMP superfamily as process initiator.

Interestingly, when differentiated in the hypoxic environment in the absence of TGFβ3, BMSC seem to acquire an articular chondrocyte-like phenotype as shown by the downregulation of collagen type X and Indian Hedgehog.

Thus, we have shown that hypoxia alone is capable of (i) triggering full chondrogenic differentiation of BMSC *in vitro* (Robins *et al.,* 2005, Kanichai *et al.,* 2008), (ii) inducing a BMSC-derived hyaline chondrogenic phenotype *in vitro*, and (iii) maintaining a long-term stable phenotype of the neo-engineered cartilage *in vivo* upon VEGF blockade.

Furthermore, the effect of inhibition of angiogenesis on BMSC could have also been mediated by a reduced delivery of specific nutrients. For example, limited availability of glucose could have forced its reduced consumption, which physiologically parallels chondrocytic differentiation by mesenchymal progenitors (Pattappa *et al.,* 2011). Alternatively, our findings could be explained by the fact that BMSC would not be exposed to possible inhibitors of chondrogenesis present in serum or released by platelets (Zhu *et al.,* 2013; Boilard *et al.,* 2010), as well as to pro-inflammatory factors (e.g., IL-1b) produced by ingrowing granulation cells (Kapoor *et al.,* 2011) and known to be detrimental for chondrocyte differentiation and cartilage formation (Ousema *et al.,* 2012). The more efficient survival of sFlk-1 expressing cells *in vivo* could also be due to the limited ingrowth of specific cell populations, including pro-inflammatory macrophages exerting a phagocytic function. Similarly, the absence of remodeling of the resulting cartilage into bone tissue would likely be mediated by the impairment to monocytic/osteoclastic cells, whose recruitment, differentiation and activity is known to be dependent on VEGF (Aldridge *et al.,* 2005; Niida *et al.,* 2005).

In order to develop towards a clinical relevance the here described strategy to induce BMSC chondrogenesis, our investigation needs to be extended to a cartilage repair model, with a longer-term observation time. Moreover, safety issues related to the use of genetically-modified

BMSC need to be addressed. For example, sustained stable expression of a transgene could be obtained by using self-inactivating lentiviral vectors, with the great advantage of decreasing the oncogenic potential as compared to retroviral ones (Montini *et al.,* 2009; Zychlinski *et al.,* 2008). Alternatively, the therapeutic approach could be implemented by a scaffold-based delivery of humanized monoclonal VEGF antibodies, as recently described for the enhanced formation of cartilage by human nasal chondrocytes (Centola *et al.,* 2013). Thus, the current study establishes that two of the main obstacles towards BMSC-based cartilage repair, namely the efficiency and stability of their chondrocytic differentiation, can in principle be bypassed by control of local angiogenesis.

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Figure Legends

Figure 1. Inhibitory effect of sFlk-1 expressed by BMCS on *in vitro* endothelial organization and proliferation. Representative images of tubular structure formation of endothelial cells (stained with calcein-AM in green after 48 hours) cultured in medium conditioned by control (**A-C)** or sFlk-1 expressing BMSC **(D-F**) supplemented with 0 (**A**,**D**), 10 (**B**,**E**) or 50 (**C**,**F**) ng/mL of VEGF. Total tube number (**I**), length (**J**), branching points (**K**) and loops (**L**) were quantified with the image analysis (10 images per sample were analyzed, the data are presented as mean + SEM, n=3-4; *p<0.05, **p<0.01). Immunofluorescence staining for CD31 in green showing the capillary-like structure formation in the direct co-culture of either control (**G**) or sFlk-1-expressing (**H**) BMSC with HDMEC. Scale bars=300 µm. HUVEC proliferation assay (**M**) performed by using supernatants collected by either control, namely naïve, or sFlk-1 expressing BMSC.

Figure 3. *In vivo* blocking of angiogenesis. Immunohistochemistry for mouse CD31. Representative images at 20x magnification of the implants generated by naive (**A-D**) and sFlk-1 expressing (**E-H**) BMSC at 1 (**A**, **E**), 4 (**B**, **F**), 8 (**C**, **G**) or 12 weeks (**D**, **H**). Representative macroscopic picture of the explants of engineered tissue generated by naïve (*on the left*) or sFlk-1 expressing (*on the right*) BMSC after 12 weeks *in vivo* (**I**). Vessel density quantified within the total area of the implant generated by control (naïve) or sFlk-1- expressing BMSC at different time points by histomorphometric analysis (**J**). Arrow heads indicate the blood vessels. Scale bar=100 µm.

Figure 4. *In vivo* chondrogenesis. Histological staining of Safranin-O for glycosaminoglycans (GAG) of engineered tissue generated by naive (**A**, **C**, **E**) or sFlk-1-expressing (**B**, **D**, **F**) BMSC after 1 (**A**, **B**), 4 (**C**, **D**) or 12 (**E**, **F**) weeks *in vivo*. Analysis at mRNA level of types II and X collagen, and Runx-2 genes of engineered constructs generated by naïve (control) or sFlk-1 expressing cells after 1 week *in vivo* (**G**). Delta Ct values normalized to levels of GAPDH are shown (n=3-4; *p<0.05). Scale bar=100 µm.

Figure 5. *In vivo* collagen deposition. Immunohistochemistry for types II (**A**, **B**, **E**, **F**) and X (**C**, **D**, **G**, **H**) of constructs generated by naïve (**A-D**) or sFlk-1 expressing (**E-H**) BMSC at 4 (**A**, **C**, **E**, **G**) or 12 (**B**, **D**, **F**, **H**) weeks after implantation. Scale bar = 50 µm.

Figure 6. *In vivo* cell engraftment. Immunofluorescence for DAPI in blue and human specific nuclei antibody in red of constructs generated by naïve (**A**, **B**, **E**, **F**) or sFlk-1 expressing (**C**, **D**, **G**, **H**) BMSC after 4 (**A**-**D**) or 12 (**E**-**H**) weeks *in vivo*. Representative images at 20x (**A**, **C**, **E**, **G**) and at 40x (**B**, **D**, **F**, **H**) magnification of the center of the implants. Rectangles identify the area at higher magnification. Dashed lines distinguish the implant from the surrounding host tissue (identified with *). Empty and full arrow heads indicate human (both positive for human nuclei antibody and DAPI) and mice (stained only with DAPI) cells, respectively. Scale bar = 50 µm.

Supplementary figure 1. *In vitro* chondrogenesis. Histological staining of Safranin-O for glycosaminoglycans (GAG) of cartilaginous tissue engineered by control (naïve) (**A**, **C**) or sFlk-1 expressing (**B**, **D**) BMSC at either 20 (**A**, **B**) or 2 (**C**, **D**) % of oxygen. Graph displaying the ratio of GAG and DNA quantified in constructs generated by control (naïve) and genetically modified BMSC (**E**). Quantification of human VEGF released in the supernatant by engineered constructs during *in vitro* chondrocytic differentiation (**F**). Scale bar = 100 µm.

FIGURE 1

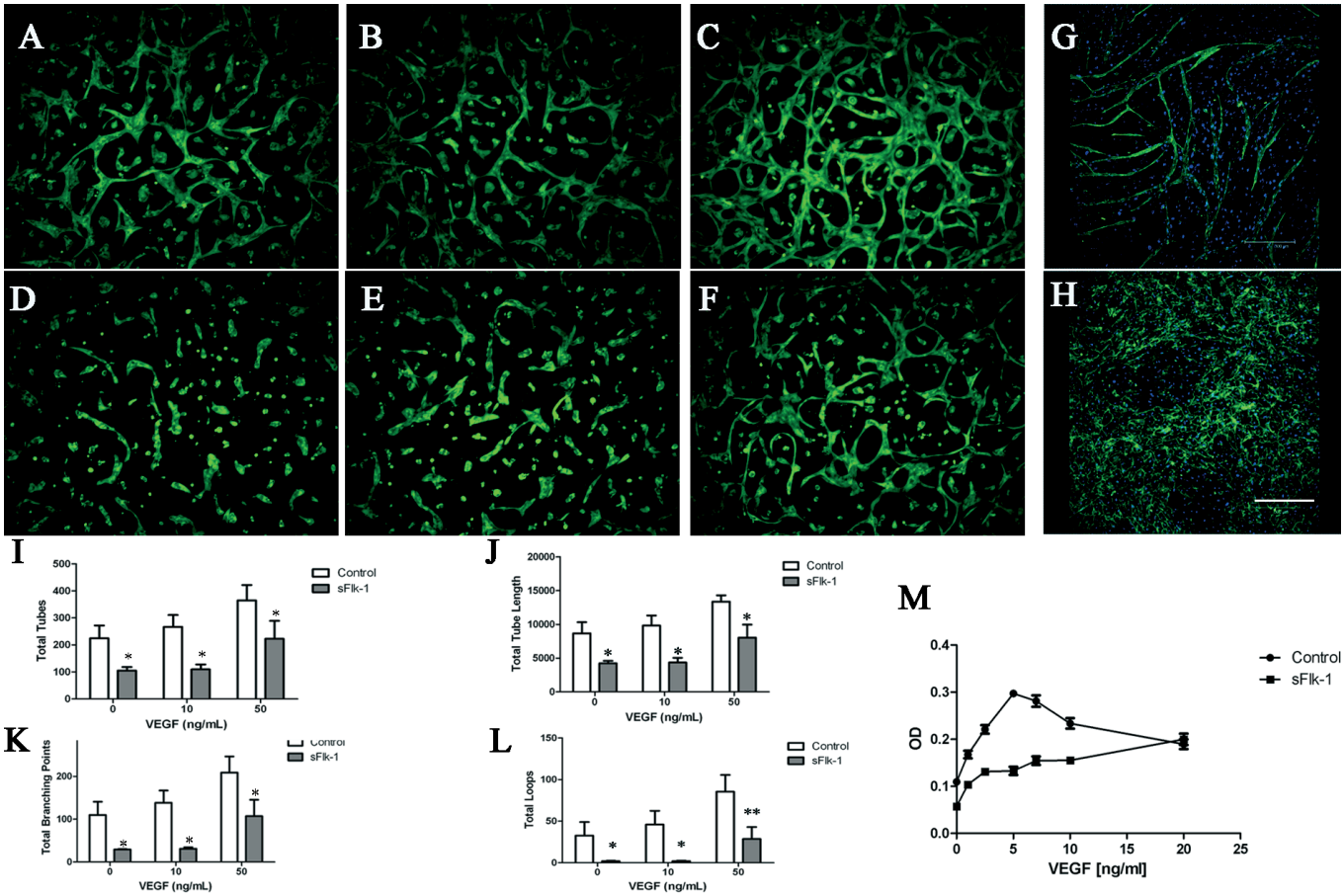

A
B
C
G
D
E
F
H
I
Total Tubes
Control
sFlk-1
VEGF (ng/mL)
J
Total Tube Length
Control
sFlk-1
VEGF (ng/mL)
M
OD
Control
sFlk-1
VEGF [ng/ml]
K
Total Branching Points
Control
sFlk-1
VEGF (ng/mL)
L
Total Loops
Control
sFlk-1
VEGF (ng/mL)

FIGURE 2

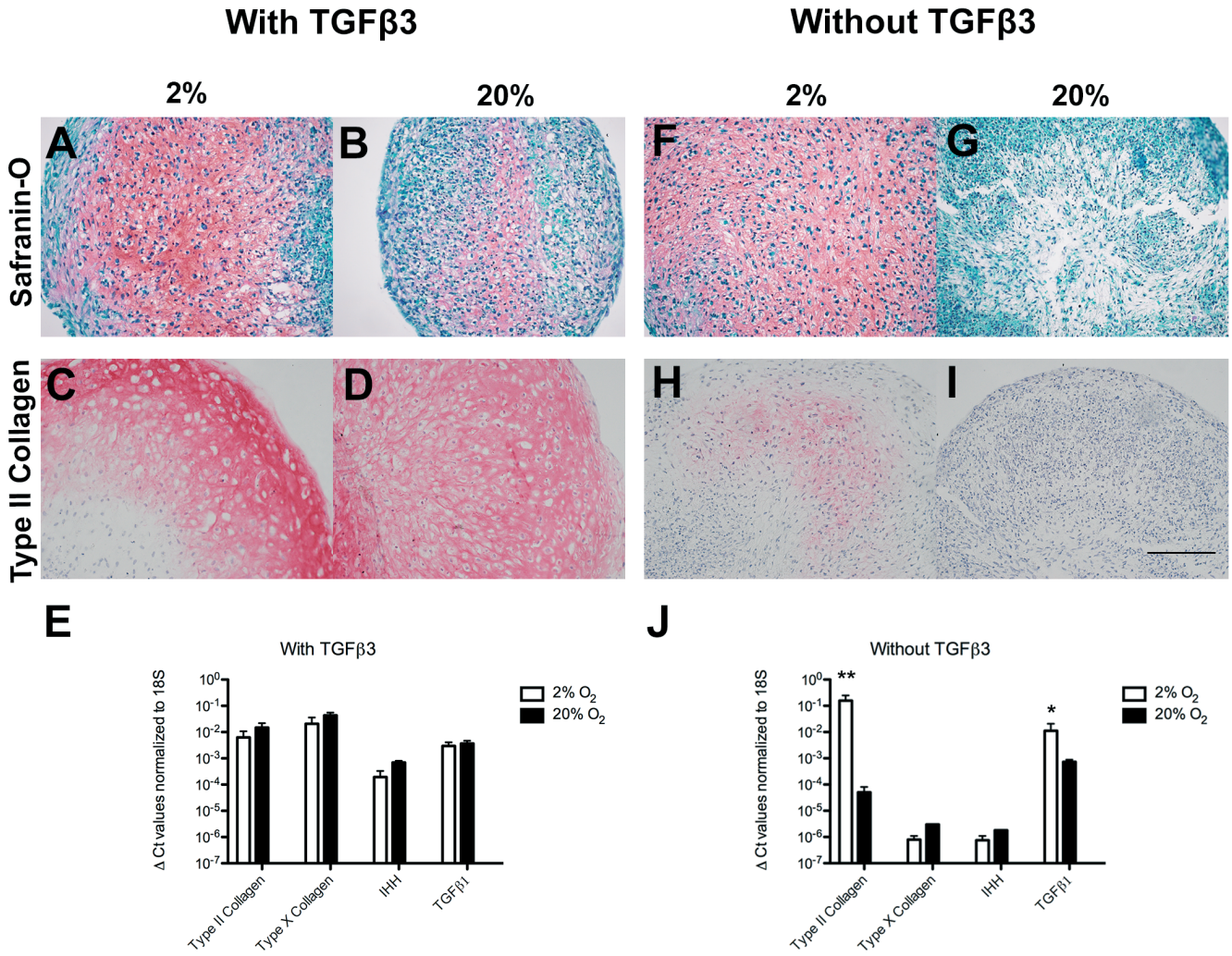

With TGFβ3
Without TGFβ3
2%
20%
2%
20%
Safranin-O
Type II Collagen
A
B
F
G
C
D
H
I
E
With TGFβ3
Δ Ct values normalized to 18S
2% O2
20% O2
Type II Collagen
Type X Collagen
IHH
TGFβ1
J
Without TGFβ3
**
*

FIGURE 3

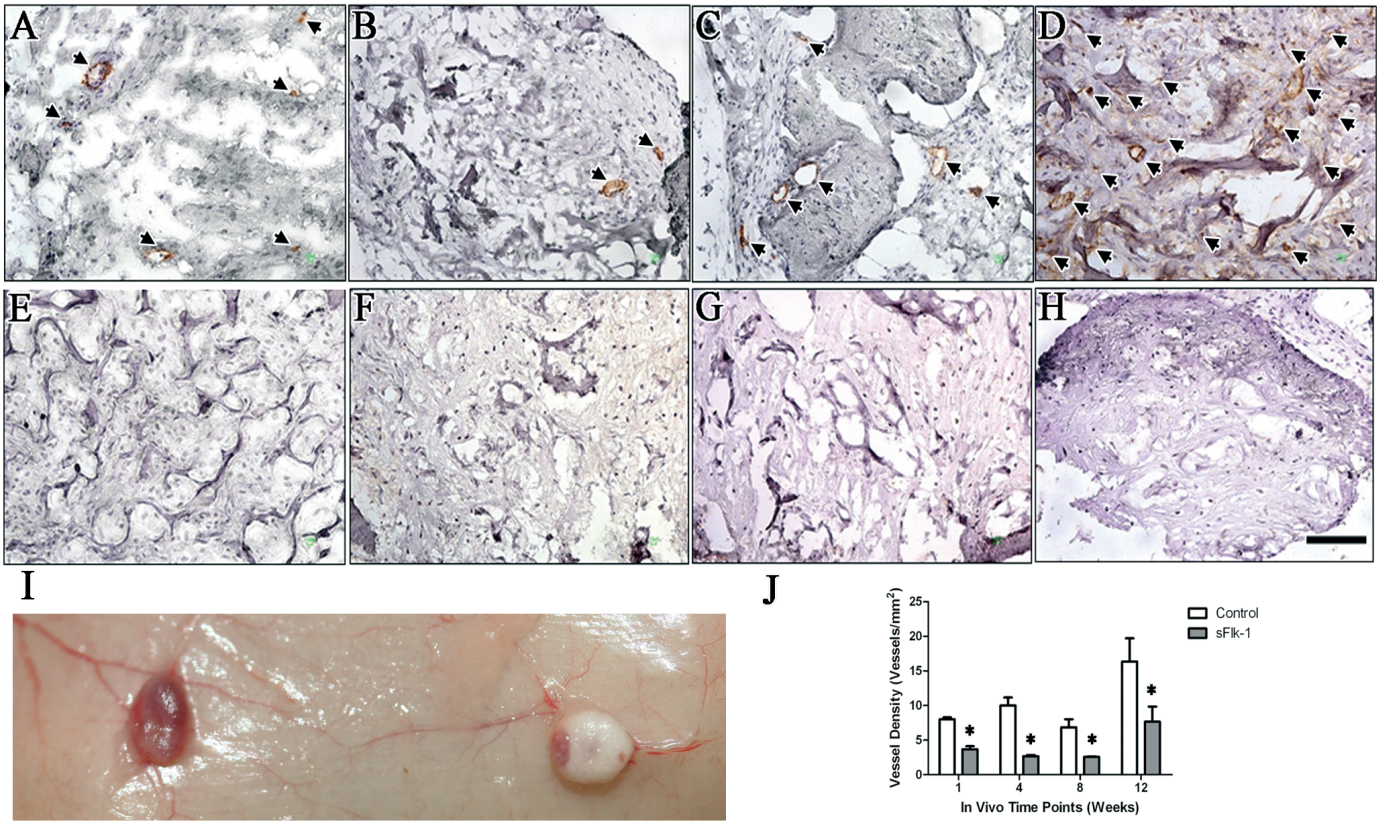
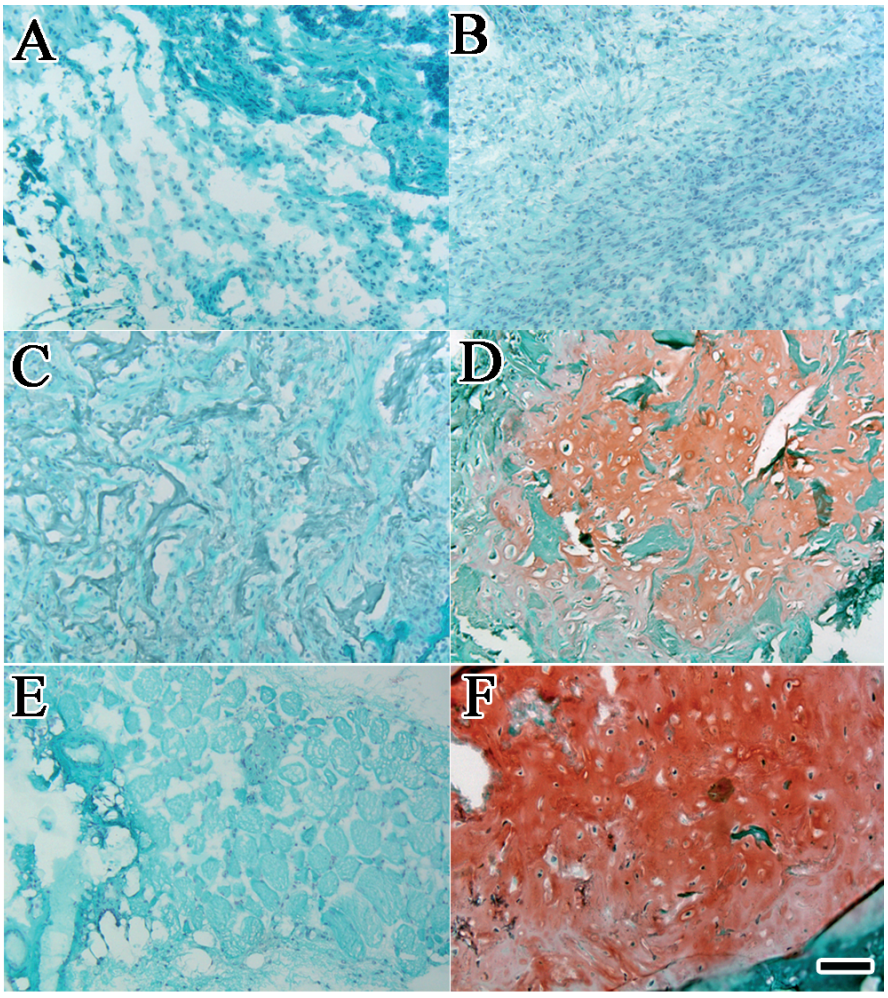

A
B
C
D
E
F
G
H
I
J
Vessel Density (Vessels/mm^2)
25
20
15
10
5
0
1
4
8
12
In Vivo Time Points (Weeks)
Control
sFlk-1
*
*
*
*

FIGURE 4


A
B
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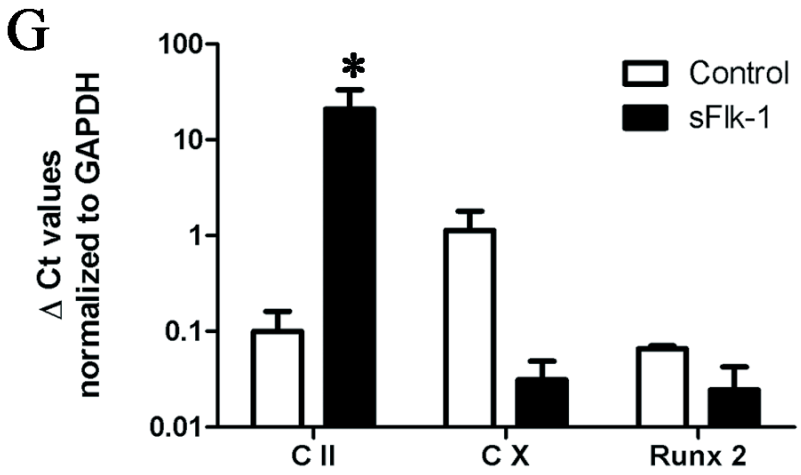

G
Δ Ct values
normalized to GAPDH
100
10
1
0.1
0.01
*
Control
sFlk-1
C II
C X
Runx 2

FIGURE 5

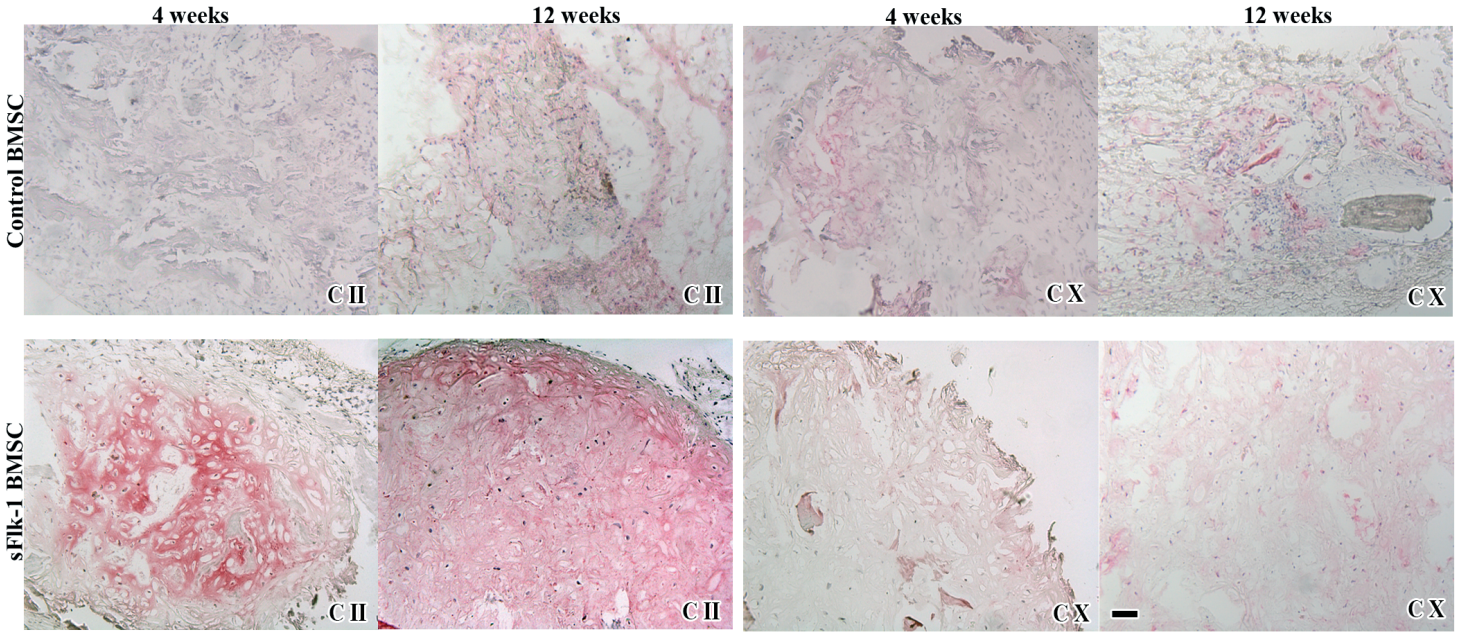
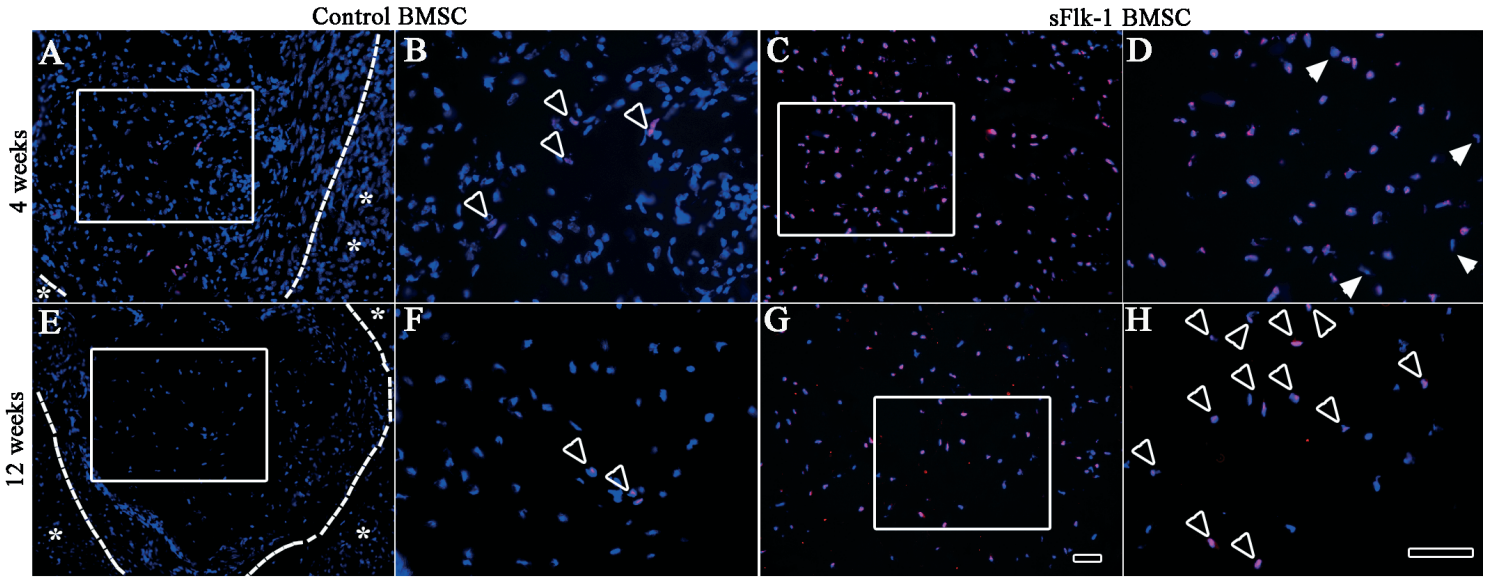
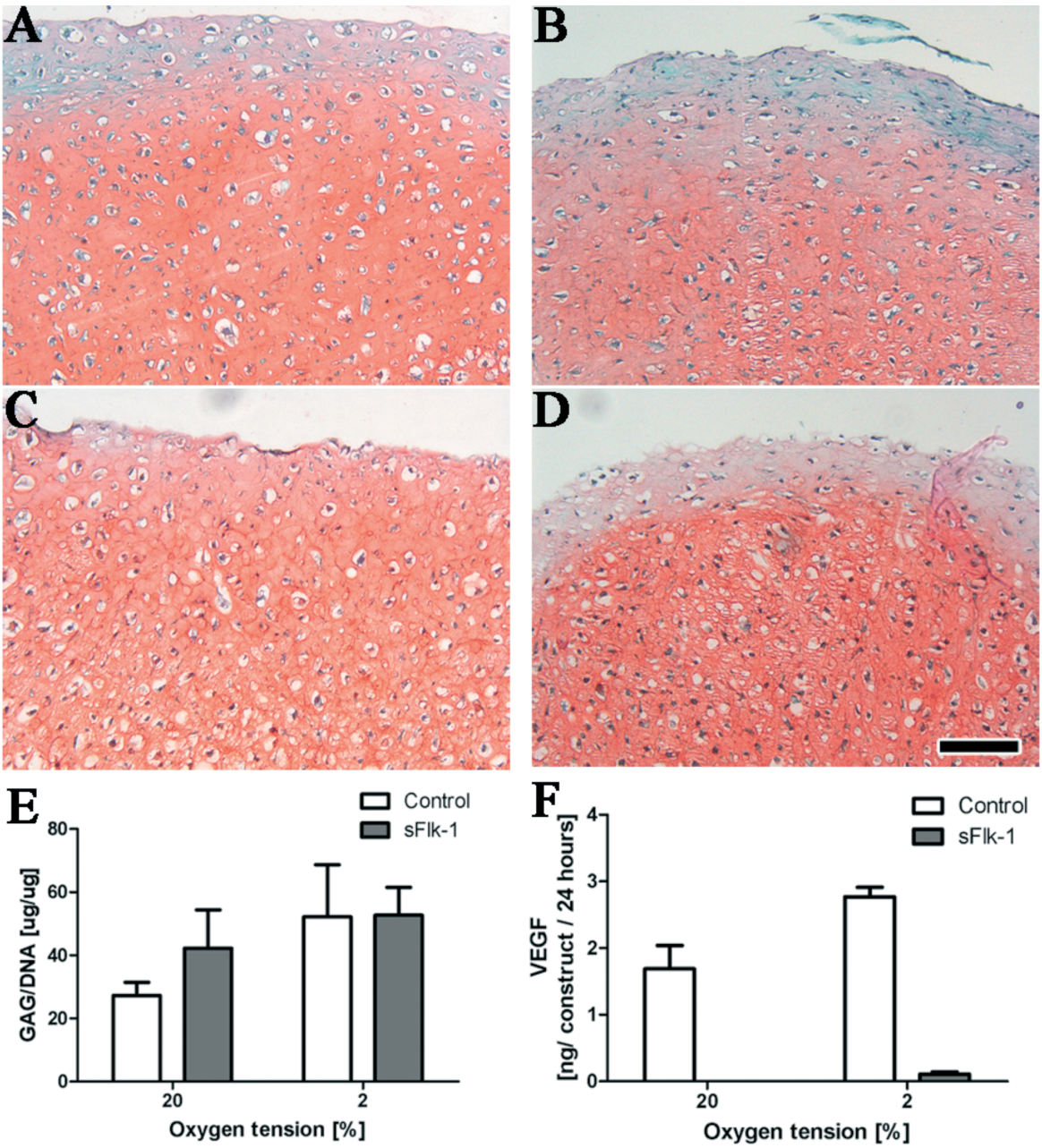

4 weeks
12 weeks
4 weeks
12 weeks
Control BMSC
sFlk-1 BMSC
C II
C II
C X
C X
C II
C II
C X
C X

FIGURE 6



Supplementary FIGURE 1



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Chapter 3

Monocyte Phenotypic Changes Induced by Mesenchymal Stromal Cells Originate Non-Functionalized Cells with Hybrid Mesenchymal-Macrophage Characteristics

Monocyte Phenotypic Changes Induced by Mesenchymal Stromal Cells Originate Non-Functionalized Cells with Hybrid Mesenchymal-Macrophage Characteristics

Introduction

Damaged articular cartilage derived from traumatic lesion has limited potential for repair due to its avascular nature and loss of cellular matrix, and to the self-renewal limitations of chondrocytes, which includes reduced cell migration and proliferation. The currently established method for clinical cartilage repair, autologous chondrocyte transplantation, displays poor binding to adjacent intact articular cartilage and lacks restoration of subchondral bone, commonly leading to the formation of fibrocartilage, which is less rigid when compared to hyaline cartilage. Moreover, host response to damaged tissue, primarily with neutrophil and macrophage infiltration to the damaged site, prevents or impairs complete tissue restoration.

Monocytes are cells that respond to inflammatory cues in the initial phases of inflammation. Monocytes originate in the bone marrow and are later released into the peripheral blood, where they replenish several tissue macrophage populations (Volkman & Gowans, 1965). The differentiation of monocytes into macrophages and other cells (e.g. dendritic cells) is a physiological process that contributes to host defense, tissue remodeling, and repair.

Macrophages have an important role in maintaining tissue homeostasis, for they are competent phagocytic cells responsible for clearance of debris, dead cells, and foreign organisms, as well as remodeling and repairing tissue after the inflammatory process (Gordon, 1998). Among the different classes of macrophages, the classically-activated macrophages (also denominated M1 macrophages) are responsible for immune defense. This response must be thoroughly controlled in order to avoid collateral tissue damage to the host in the advent of severe and unrestrained inflammation. On the other hand,

macrophages are not always pro-inflammatory cells with detrimental effects on tissue regeneration; they also play an important role in inflammation resolution and tissue repair, namely through its "alternatively-activated macrophages", also generally called M2 phenotype.

It has been proposed through several studies that monocytes are capable of differentiating into other cellular types, with the delivery of specific cues in order to alter its default programming into becoming macrophages or dendritic cells. Kuwana and collegues (Kuwana *et al.,* 2003) proposed that circulating CD14+ monocytes could serve as a source for mesenchymal progenitors. Peripheral blood mononuclear cells (PBMC), after being cultured with fetal bovine serum (FBS) and fibronectin, were capable of differentiating into distinct mesenchymal cell lineages. Interestingly, these cells displayed not entirely a monocytic phenotype, also displaying CD34 and type I collagen, indicative of mixed features of monocytes, endothelial, and mesenchymal progenitor cells. More recently, Pufe and colleagues (Pufe *et al.,* 2008) established that cells of monocytic origin could be programmed into becoming type II collagen-producing chondrocyte-like cells when treated concomitantly with IL3 and M-CSF.

Human monocytes were found to be capable of differentiating into endothelial-like cells in vitro in response to a combination of pro-angiogenic factors (Fernandez-Pujol *et al.,* 2000; Schmeisser *et al.,* 2001). Other studies have revealed that multipotent cells of monocytic origin were capable of improving heart function (Dresske *et al.,* 2006), differentiating into cardiomyocyte-like cells (Kodama *et al.,* 2005), hepatocyte-like and pancreatic islet-like cells (Ruhnke *et al.,* 2005), and into keratinocyte-like cells (Medina *et al.,* 2009). In all of the mentioned studies, however, monocytes had to be previously treated with supra-physiological doses of cytokine cocktails (IL3, M-CSF, angiogenic growth factors, etc.). Hence,

it is not currently known if monocytes are capable of differentiating into different lineages without having the supplementation of external cues.

Intriguingly, some studies contest the capability of monocytes to differentiate into cells of other lineages, due to the fact of proper characterization of the cellular phenotype. Rohde and colleagues (Rohde *et al.,* 2006), for instance, claimed that myeloid and monocytic cells share several properties with endothelial cells, which makes it difficult to affirm that monocytes can indeed become endothelial cells (Fernandez-Pujol *et al.,* 2000). Hence, it is imperative to determine whether monocytes can truly differentiate into other cell lineages and exert its functionalities.

In the tissue repair process, monocytes are recruited to the lesion site and directly interact with tissue-competent or progenitor cells (Nucera *et al.,* 2011). Thus, in this study, we aimed at investigating whether MSC that are recruited to the cartilage injury site are capable of instructing monocytes to acquire traits of tissue repairing macrophages and/or stromal cells with a mesenchymal phenotype. For this purpose, we cocultured monocytes with MSC in 3D collagen sponges (direct cell-to-cell contact) or in transwell systems (soluble factor release from MSC to monocytes) for 5 days in under physiological conditions, without the supplementation of cytokines.

Methods

Cell harvest, isolation, and culture

Following informed consent and in accordance to the local ethical committee, bone marrow aspirates were harvested from the iliac crest of healthy donors during orthopaedic surgical procedure (Basel Cantonal Hospital, Switzerland). Bone marrow mesenchymal stromal cells (MSC) were isolated as described by Horn and colleagues (Horn *et al.,* 2008). Fresh bone

marrow were plated at a density of 10^5 nucleated cells/cm^2 and cultured in high glucose DMEM (Gibco, Carlsbad, CA) containing 4.5 mg/ml D-Glucose, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 100 mM HEPES buffer, 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.29 mg/ml L-glutamine (basic medium) supplemented with 10% fetal bovine serum (FBS) and of 5 ng/ml Fibroblast Growth Factor (FGF)-2 (BD Biosciences, Basel, Switzerland) in order to maintain MSC differentiation capacity (Bianchi *et al.,* 2003; Solchaga *et al.,* 2003; Bocelli-Tyndall *et al.,* 2010). Primary MSC were transduced six days after the first plating with previously described retroviral vectors, which consisted of a mouse truncated CD8a gene fused to an Internal Ribosomal Entry Site (IRES) (Misteli *et al.,* 2010). Cells were transduced according to a recently described high-efficiency protocol (Helmrich *et al.,* 2012). Cells were incubated with retroviral vector supernatants supplemented with 8µg/ml polybrene (Sigma-Aldrich, St. Louis, MO) for 5 minutes at 37°C in 60-mm dishes and then centrifuged at 1100g for 30 minutes at room temperature in the dishes, followed by fresh medium replacement. Transductions were repeated twice a day for a total of 6 rounds. Cells were expanded for two passages and re-plated at a density of 10^4 cells/cm^2.

Monocytes were isolated from buffy coats provided by healthy donors (routine blood donations at the Basel Cantonal Hospital) using the traditional Ficoll™ gradient assay. Briefly, the blood was diluted 1:2 in PBS and then 1:3 in Ficoll™ (Sigma-Aldrich). The blood suspension was then centrifuged at 2000 rpm for 30 minutes. After centrifugation, the white blood cell layer was removed and diluted 1:3 in PBS, washed for 15 min at 1500 rpm, resuspended in cold MACS buffer (PBS with 1% EDTA and 1%FBS) and washed again for 10 min at 1200 rpm, then finally placed into a Falcon tube with 5 mL of cold MACS buffer. The white cell population was filtered with a 40uM nylon cell strainer (BD 352340), and then incubated with 300uL of CD14 magnetic beads (Miltenyi Biotec, Auburn, CA). CD14+

monocytes were then isolated according to the manufacturer's instructions and cultured in RPMI-1640, supplemented with 10% FBS, 1% sodium pyruvate, 1% kanamycin, 1% HEPES, 1% non-essential amino acids, 1% Glutamax, and 1:1000 β-mercaptoethanol (Life Technologies) (complete medium). The cells were incubated with the beads on ice for 20 min. The cells were then washed at 1500 rpm for 10 min to obtain a white blood cell pellet. The pellet was resuspended in cold MACS buffer and ran through an LS column (Miltenyi Biotec). After washing the column 3 times with MACS buffer, the final $CD14^+$ flowthrough was harvested.

Culture in transwell system

MSC and monocytes were respectively plated in the upper and lower chambers of transwell plates (polycarbonate membrane with 0.4 mm pore size, Corning, Lowell, MA) and cultured at 37°C for 5 and 14 days in complete RPMI medium. 0.5M cells of each type were plated, keeping a 1:1 proportion. MSC-only and monocyte-only samples were used as controls. Three replicates were performed throughout our studies.

Culture in 3D type I collagen sponges

Monocytes and MSC were cocultured for 5 and 14 days at 37°C in collagen type I scaffolds (Ultrafoam®, Davol, Inc.) of 4mm in diameter in a 1:1 ratio (0.2M cells of each type) in complete RPMI medium. These experiments were performed in duplicate. MSC-only and monocyte-only samples were used as controls.

Digestion of scaffolds

For flow cytometric, proliferation, and gene expression analyses, the samples cultured in collagen type I sponges were digested in order to retrieve the cells. Briefly, the constructs

were incubated with trypsin (0.05%, Life Technologies) for 10 min at 37°C in an orbital shaker. After trypsin removal, the samples were incubated with 1.5% type II collagenase solution (>300 U/mg; Bioconcept Worthington) for 20 min at 37°C in an orbital shaker. The cells were harvested after scaffold digestion and immediately analysed or re-plated.

Analytical assays

FACS sorting

Cells positive for CD8a (BMSC) were sorted by staining with an allophycocyanin-conjugated anti-mouse CD8a (clone 53–6.7; BD Biosciences) as previously described (Helmrich *et al.,* 2012) by using a FACS ARIA sorter (Becton Dickinson, Allschwil, Switzerland) the remaining negative fraction (monocytes) were further analyzed for macrophage and mesenchymal markers by flow cytometry.

Flow Cytometry

Phenotypes of monocytes co-cultured with MSC were analyzed upon staining with the following antibodies: allophycocyanin (APC)-labeled anti-CD90 (clone 5E10, BD559869), anti-CD106 (clone STA, Biolegend 305809), anti-CD14 (clone M5E2, BD561708), anti-CD206 (clone 19.2, BD561763), phycoerythrin (PE)-labeled anti-CD73 (clone AD2, BD561014), anti-CD105 (clone 266, BD560839), anti-CD166 (clone 3A6, Biolegend 343903), anti-CD11b (clone ICRF44, BD555388), anti-CD163 (clone GHI/61, BD556018), anti-nestin (clone 25/NESTIN, BD561230), fluorescein-isothiocyanate (FITC)-labeled anti-CD68 (clone Y1/82A, BD562117), anti-stro-1 (clone STRO-1, Biolegend 340105) , allophycocyanin (APC)-cy7 anti-CD29 (clone TS2/16, Biolegend 303014), and anti-CD44 (clone IM7, Biolegend 103027). The samples were

analyzed by a CyAn™ ADP Analyzer (Beckman Coulter). Analysis was performed using FlowJo software (Tree Star, Ashland, OR).

Gene expression analysis

Total RNA of cells was extracted using Quick RNA Mini prep (ZYMO Research) according to the manufacturer's protocol. cDNA was generated from 3 µg of RNA by using 500 µg/ml random primers (Catalys AG, Wallisellen, Switzerland) and 1 µL of 50 U/ml StratascriptTM reverse transcriptase (Stratagene, Agilent Technologies, Basel, Switzerland), in the presence of dNTPs. cDNA samples were amplified and analyzed by real-time RT-PCR reactions and monitored using the ABI Prism 7700 Sequence Detection System (Perkin- Elmer/Applied Biosystems, Rotkreuz, Switzerland). Taqman Assays on Demand (Applied Biosystems) were used to evaluate the expression of several genes, all purchased from Applied Biosystems: IL1β (Hs01555410_m1), IL6 (Hs00985639_m1), IL10 (Hs00961622_m1), IL12 (Hs01073447_m1), MCAM (Hs00174838_m1), SDF-1 (also known as CXCL12, Hs00171022_m1), TGFβ1 (Hs00998133_m1), TNFα (Hs01113624_g1), and Sox9 (Hs00165814_m1). Type II collagen, on the other hand, had its primers and probe synthesized by Microsynth (reverse primer – GGCAATAGCAGGTTCACGTACA; forward primer – CGATAACAGTCTTGCCCCACTT; probe – CCGGTATGTTTCGTGCAGCCATCCT). Gene expression data for all genes were normalized to human GAPDH (glyceraldehyde-3-phosphate dehydrogenase) as reference gene (Applied Biosystems, Hs02758991_g1).

Chondrogenic Differentiation in pellet culture (performed after proliferation period)

After the period of 5 days coculture, monocytes were re-seeded and cultured at a density of $2.0x10^4$ cells/cm^2 in complete RPMI up to 3 weeks in order for their survival and proliferative

capacities to be assessed. After this culture phase, monocytes were detached and submitted to chondrogenic differentiation.

For the chondrogenic differentiation, serum-free DMEM (Gibco, 4.5% glucose, with NEAA and without L-glutamine and sodium pyruvate) containing 1% sodium pyruvate (Gibco), 1% HEPES (Gibco), 1% penicillin-streptomycin solution (Gibco), 1% ITS + 3 (Sigma-Aldrich, St. Louis, USA) and 1% human serum albumin (HSA 100x, Invitrogen, Carlsbad, USA) was supplemented with 0.1 mM ascorbic acid 2-phosphate (Invitrogen), dexamethasone 10^{-5}M (Invitrogen), and 10ng/ml of TGFβ1 (Invitrogen). In an adaptation from Barbero *et al.,* 2003, Briefly, $2.0x10^{6}$ cells per 0.5mL of medium were placed into conical polypropylene tubes and spun down for 4 min at 1500 rpm to form cell aggregates. The pellets were cultured for 2 weeks. The culture medium was changed twice a week. The monocyte pellets were then analyzed for chondrogenic genes (Sox9 and type II collagen) and glycosaminoglycan content (Safranin-O staining).

Results

Acquisition of a macrophage-like phenotype by monocytes cultured with MSC

Human monocytes isolated from buffy coats were co-cultured with MSC for 5 days either in direct contact (MSC and monocytes cultured in 3D collagen sponge scaffolds) or in indirect contact (transwell system). After the 5 day culture period, monocytes were analyzed for their change of phenotype through FACS and quantitative PCR analyses.

Since the default differentiation of monocytes is for the latter to become macrophages (either M1 (pro-inflammatory), M2 (repair), or a mixed macrophage phenotype), we sought to investigate whether monocytes that have been exposed to MSC cues would still evolve to macrophages when compared to naïve monocytes.

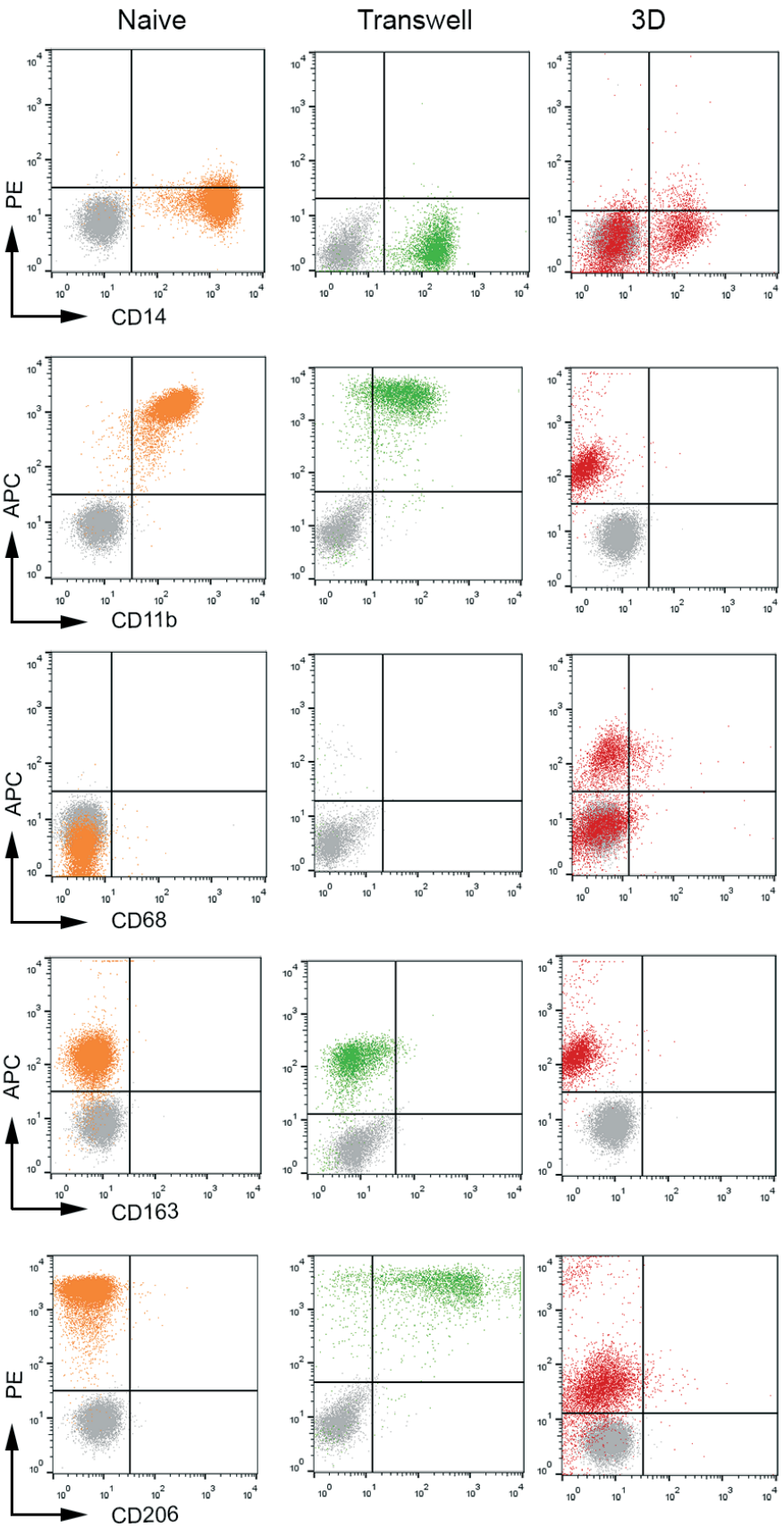

Figure 1. FACS analyses of monocytic/macrophage markers for naïve, transwell, and 3D monocytes. Isotype controls are represented in grey.

In Figure 1, we demonstrate the FACS analyses results for naïve, transwell-cultured, and 3D-cultured monocytes with MSC when analyzed for the acquisition or loss of monocyte/macrophage markers. Naïve monocytes are used as control cells throughout our study, since mono-cultured monocytes did not survive the 5-day culture period. Naïve monocytes were positive for CD14 (99.5%) and CD11b (98%). CD14 is a monocyte-specific marker, which is present in monocyte-derived cells, including macrophages. CD11b is also found in monocyte and macrophage populations. Naïve monocytes were negative for CD68 (an antigen associated with activated macrophage stages), CD163 and CD206 (markers that are used to distinguish M2-related populations).

When monocytes were co-cultured with MSC in transwells, their phenotype were slightly different than naïve monocytes. Transwell monocytes were positive for CD14 (97%), CD11b (88.2%), and negative for CD68. This implies that transwell monocytes had very little differences in macrophage activation-related marker expression. Meanwhile, 1.5% of the transwell monocytes started to express CD163; impressively, 88% of the latter cells were positive for CD206, indicating a clear transition to an M2-like phenotype.

When analyzing 3D-cultured monocytes, only 30% of the cells remained CD14-positive. 95.7% of the cells were positive for CD11b, and only 5% were positive for CD68. Despite the high positivity for CD11b, these monocytes are transitioning away from the monocytic-macrophage lineage; since these cells were negative for CD163 and only 2% of these cells were positive for CD206, we cannot conclude that these cells are acquiring an M2-like phenotype.

In Figure 2, six different macrophage cytokines were analyzed via quantitative PCR. M1-related cytokines (TNFα, IL1β, IL6, and IL12) and M2-related cytokines (TGFβ1 and IL10) had

their expression levels examined in transwell and 3D-cultured monocytes, along with naïve monocytes and MSC used as control cells.

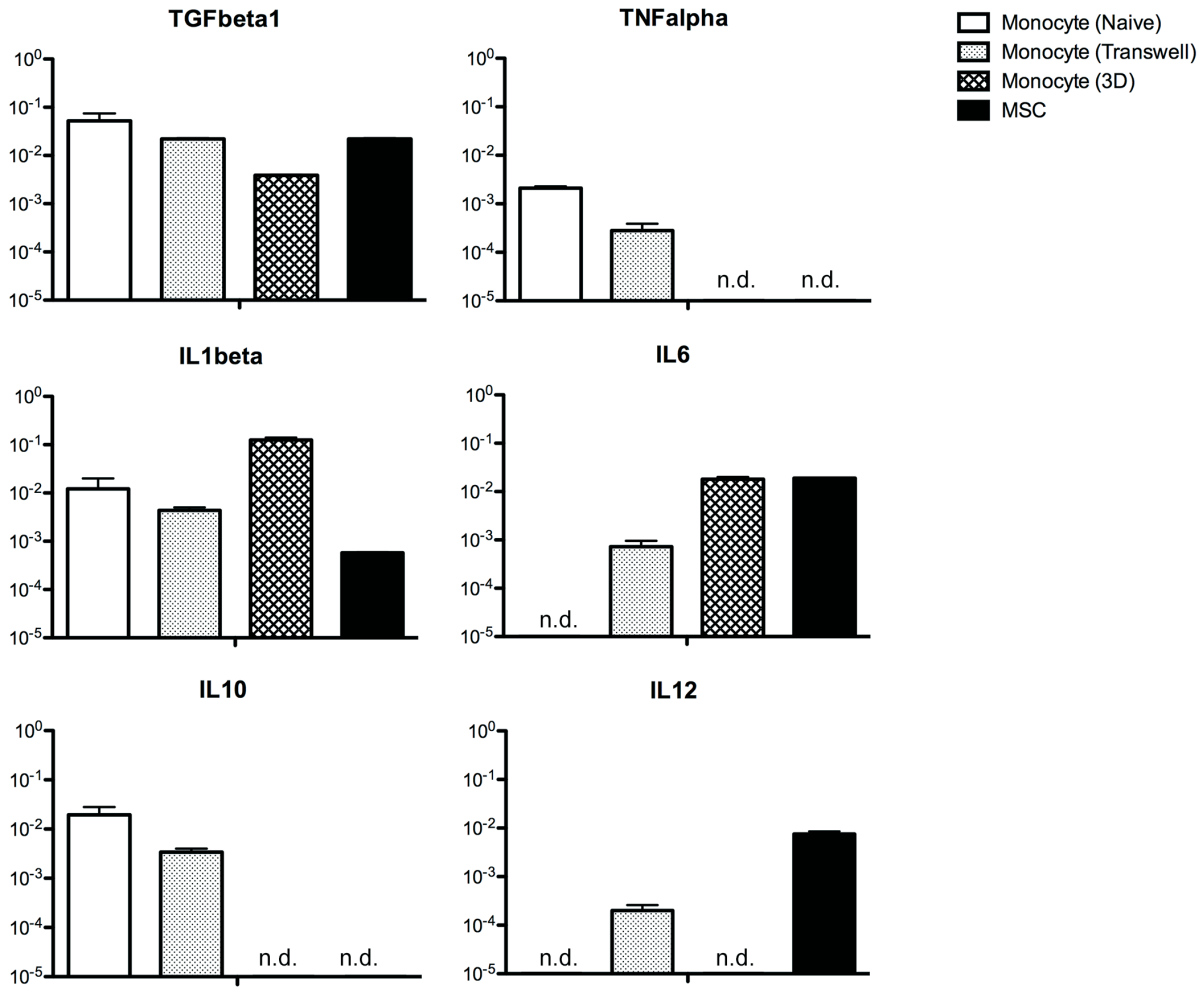

Figure 2. Gene expression analyses of M1-related cytokines (TNFα, IL1β, IL6, and IL12) and M2-related cytokines (TGFβ1 and IL10) for naïve, transwell, and 3D monocytes.

Regarding the M2-related IL10 cytokine, only naïve and transwell monocytes showed some level of expression for this marker. The difference between naïve and transwell monocyte expression was not statistically significant. The fact that 3D monocytes did not express IL10 corroborates the lack of an M2-like phenotype by the latter cells. On the other hand, TGFβ1 was expressed by all cells, including MSC. No significant statistical differences could be observed among the expression of TGFβ1 in the different cell types, although its expression

was slightly diminished in 3D monocytes. Indeed, TGFβ1 plays a crucial role in MSC differentiation (Roelen & Dijke, 2003) and is therefore also expressed in high levels in these cells, along with being an M2 marker.

As for the M1-related cytokines, TNFα is only expressed by naïve and transwell monocytes, although TNFα expression by transwell monocytes is statistically lower than by naïve monocytes (10-fold difference). IL1β is expressed by all groups, but 3D monocytes have a statistically higher expression compared to all other cell types, including naïve monocytes (10-fold difference) and MSC (100-fold difference). IL1β is normally expressed by many cell types (including MSC) and plays and important role in modulating MSC differentiation (Mumme *et al.,* 2012). On the other hand, naïve monocytes do not express IL6 or IL12 unless stimulated. MSC and transwell monocytes express IL6 and IL12, although MSC express much higher amounts of these proinflammatory cytokines when compared to unstimulated transwell monocytes. 3D monocytes, however, only express IL6, and at comparable levels to MSC. Taken together, this data indicates that transwell monocytes display an M2-like phenotype, whereas 3D monocytes show traits comparable to MSC on the cytokine expression level and FACS analyses.

Acquisition of a mesenchymal-like phenotype by monocytes cultured with MSC

After assessing the macrophage phenotype acquired by monocytes cultured in transwells or in 3D collagen scaffolds with MSC, we investigated whether these same monocytes could be capable of acquiring a mesenchymal-like phenotype after direct or indirect contact with MSC.

In Figure 3, we show the FACS analyses results for naïve, transwell-cultured, and 3D-cultured monocytes with MSC when analyzed for the acquisition of mesenchymal markers. For the

FACS analysis, we investigated the expression of CD29, CD44, CD73, CD90, CD105, CD106, CD166, nestin, and stro-1 as MSC markers. In our analyses, CD44, CD90, and CD166 have proven to be unfit markers for proper mesenchymal phenotype characterization, because they are also normally expressed by naïve monocytes and do not give a correct assessment of whether or not monocytes cultured with MSC acquired a mesenchymal phenotype or not. Naïve monocytes were 97.5% positive for CD44, 91% positive for CD90, and 99% positive for CD166. Interestingly, 3D monocytes had a steep reduction in CD44 expression when compared with naïve monocytes (only 66% positive cells) and in CD166 expression (only 51% positive cells). Meanwhile, transwell monocytes also displayed a reduction in CD90 and CD166 expression when compared with naïve monocytes (20% and 48.7%, respectively).

Figure 3 also shows the relative CD105 expression in all three groups. Interestingly, naïve monocytes are 39% positive for CD105; transwell monocytes show a drastic increase in CD105 expression (84.1% positive cells), whereas 3D monocytes actually show a decrease in CD105 expression (3.5% positive cells). Naïve monocytes were also 9.5% positive for CD29. Transwell monocytes show a reduction in CD29 expression (only 1.5% positive for CD29), while 3D monocytes have almost double the CD29 expression when compared with naïve monocytes (22% positive cells).

In our experimental setup, the most reliable MSC phenotypic markers were CD73, CD106, nestin, and stro-1. Naïve monocytes were virtually negative for these markers (being only 0.05% for nestin and 5% positive for CD106). Nestin expression, however, remained negative for all experimental groups. On the other hand, stro-1 was only slightly positive for 3D monocytes (6.5% positive cells) and negative for transwell monocytes.

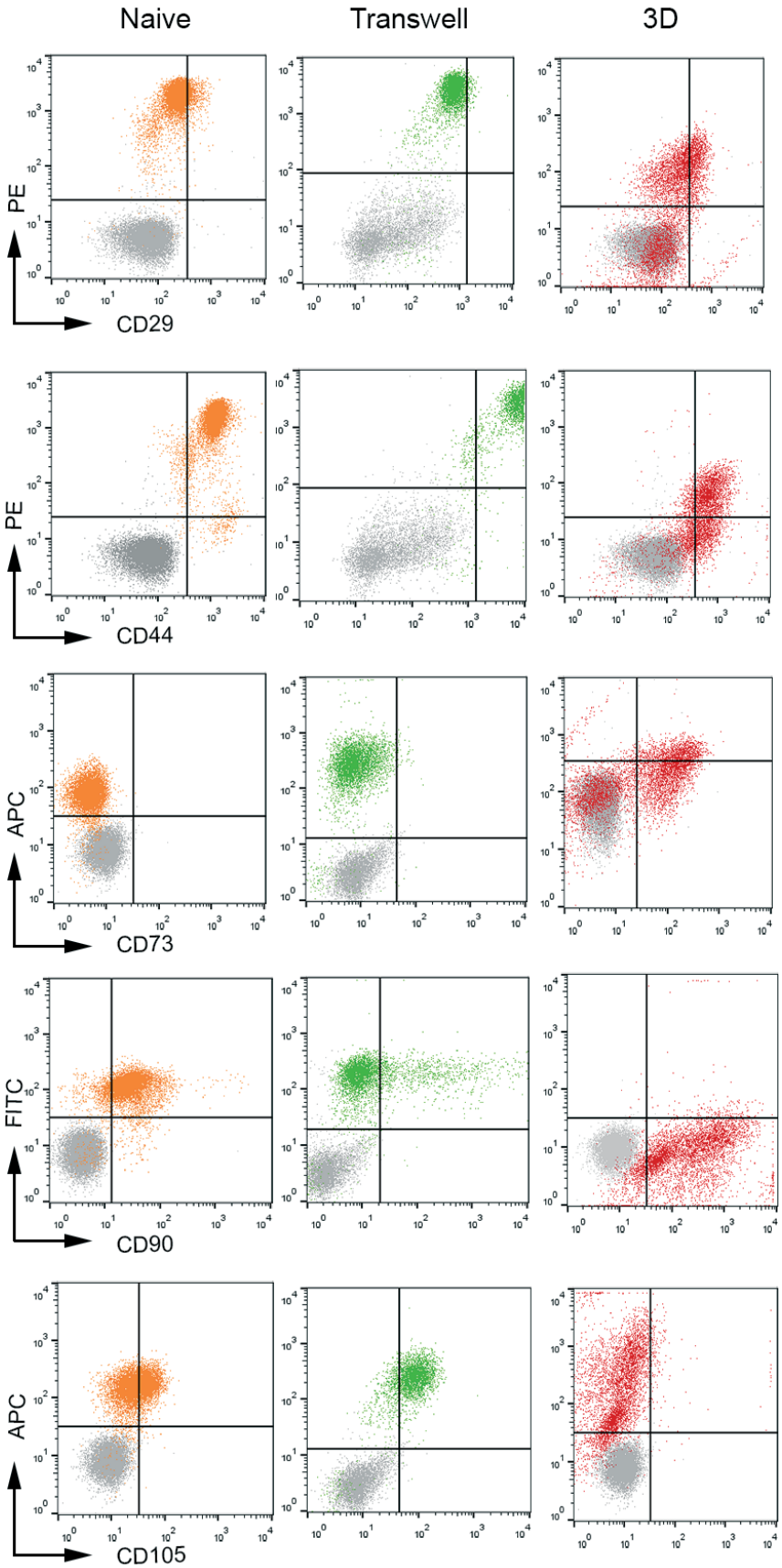

Figure 3. FACS analyses of MSC markers for naïve, transwell, and 3D monocytes. Isotype controls are represented in grey.

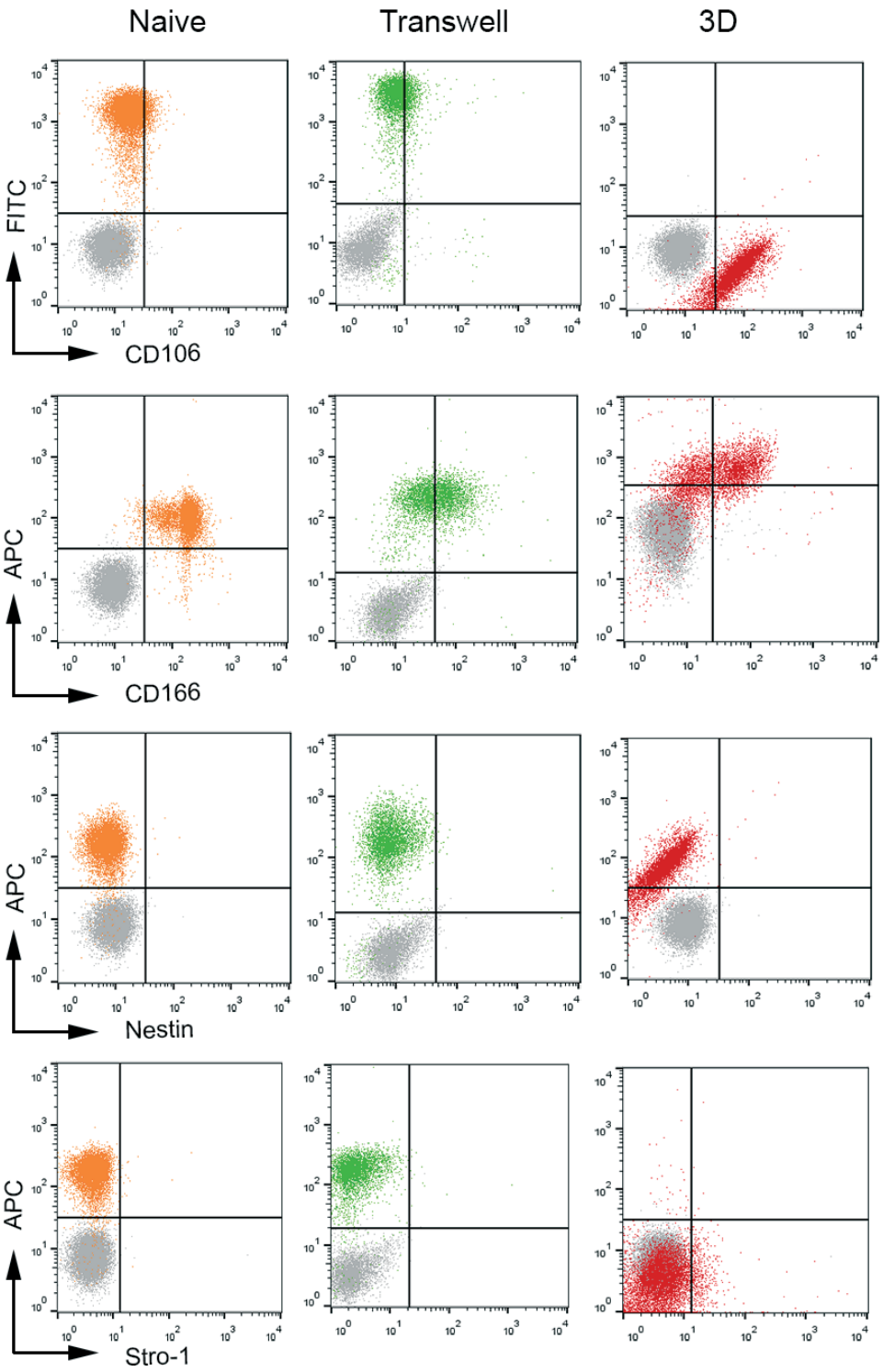

Figure 3 *continued*. FACS analyses of MSC markers for naïve, transwell, and 3D monocytes. Isotype controls are represented in grey.

CD73 was little expressed by transwell monocytes (only 1.25%), and highly expressed by 3D monocytes when compared with naïve monocytes (56% positivity).

As for CD106, transwell monocytes had a significant increase in its expression when compared with naïve monocytes (30% positive cells), and a whopping 82% positivity for 3D monocytes.

In Figure 4, we analyzed the gene expression levels of MCAM (melanoma cell adhesion molecule, CD146, or cell surface glycoprotein MUC18) and SDF-1 (stromal cell-derived factor 1, also known as C-X-C motif chemokine 12 or CXCL12), which are mesenchymal markers.

SDF-1 was expressed by naïve monocytes, but it is expressed 100 times more by MSC. Transwell monocytes displayed very little expression of SDF-1 (even lower than naïve monocytes). 3D monocytes, on the other hand, expressed highly significant levels if SDF-1, even higher than MSC expression. Importantly, only MSC and 3D monocytes expressed MCAM. Taken together, the FACS and quantitative data analyses indicate that 3D monocytes display several MSC traits and may acquire a mesenchymal phenotype through direct cell-to-cell contact with MSC, whereas transwell monocytes, which are only indirectly in contact with MSC through soluble factor interaction, display little mesenchymal traits.

Chondrogenic differentiation of monocytes cultured with MSC in 3D collagen scaffolds

Since the prerogative of functional MSC is their ability to differentiate into cells of the mesenchymal lineage (e.g. chondrocytes), we investigated whether transwell-cultured and 3D-cultured monocytes could undergo chondrogenic differentiation *in vitro* in a similar manner as MSC.

Figure 5 shows naïve, transwell, and 3D monocytes after their 3-week post-coculture recovery period. We observed that naïve monocytes died after a few days in culture. Transwell monocytes retained their round-like morphology (similar to naïve monocytes) and

survived. 3D monocytes, on the other hand, developed a spindle-like morphology similar to mesenchymal cells.

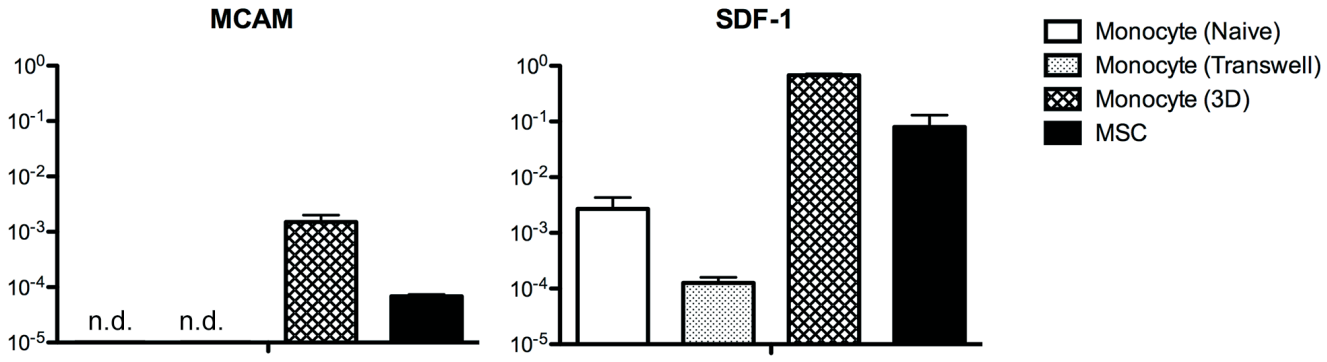

Figure 4. Gene expression analyses of MSC-related cytokines (MCAM and SDF-1) for naïve, transwell, and 3D monocytes.

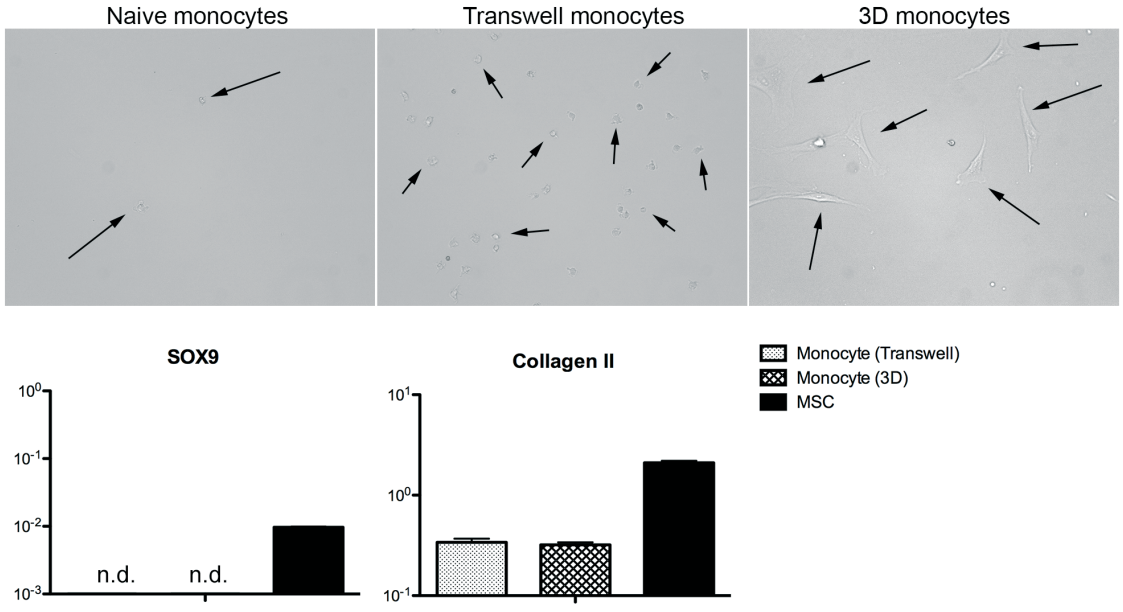

Figure 5. Phase-contrast images of naïve, transwell, and 3D monocytes in culture for a week after the 5-day coculture period with MSC. Sox9 and type II collagen expression of transwell and 3D monocytes in chondrogenic pellet culture after MSC coculture.

During the chondrogenic differentiation period, no pellet formation could be observed, which hindered further possible histological analyses of the monocytes. When compared

with the original pellet culture described by Barbero and colleagues (Barbero *et al.,* 2003), 0.5M monocytes in 500uL of chondrogenic medium was not enough for a visible pellet to be observed. Quantitative PCR analysis, however, was performed.

Figure 5 also shows the quantitative PCR data for SOX9 and type II collagen for transwell and 3D monocytes after they underwent *in vitro* chondrogenic differentiation. SOX9 is not expressed by either transwell or 3D monocytes; it is only expressed by control MSC. On the other hand, the expression of type II collagen by transwell and 3D monocytes are very similar to each other, about 10 times lower when compared with MSC. Hence, monocytes that have been either in direct or indirect contact with MSC do not seem to display the same chondrogenic capability as do MSC.

Discussion

The objective of this study was to investigate whether mesenchymal stromal cells (MSC) could instruct monocytes into acquiring an M2-like macrophage phenotype and/or mesenchymal traits, with the intent to benefit from monocytes that infiltrate cartilage lesions and could eventually help MSC in the regeneration of the tissue. Here, we demonstrate that monocytes are capable of expressing some M2/MSC characteristics upon direct or indirect coculture with MSC, which could further benefit MSC in their intrinsic tissue-repairing function. Monocytes were not capable of differentiating into chondrocytes in our experimental setup; however, though not being directly capable of replacing damaged tissue, monocytes may still play a role in aiding MSC in tissue regeneration.

MSC have various immunomodulatory effects of different immune cells, varying from suppressing the proliferation of T cells (Le Blanc *et al.*, 2003; Di Nicola *et al.,* 2002). Mainly, MSC mediate immunosuppression by negatively modulating the function of B cells (Corcione

et al., 2006), natural killer cells (NK) (Spaggiari *et al.,* 2006), and dendritic cells (Nauta *et al.,* 2006; Jiang *et al.,* 2005). MSC also provide cytokine and growth factor support for expansion of hematopoietic and embryonic stem cells (Majumdar *et al.,* 2000; Cheng *et al.,* 2003).

The mechanism in which MSC mediate immunosuppressive actions upon different immune cells remains elusive, however, several soluble factors released by MSC are thought to play a role in their immunosuppressive effects, such as hepatocyte growth factor (HGF), prostaglandin E2 (PGE2), TGFβ1, indoleamine 2,3-dioxygenase (IDO), and IL10 (Aggarwal & Pittenger, 2005, Beyth *et al.,* 2005). A few other studies have suggested that contact-dependent mechanisms may also be implicated in the immunosuppressive effects of MSC (Krampera *et al.,* 2003; Augello *et al.,* 2005).

Due to their intrinsic organic functions, immune cells are capable of adapting to different environments upon different immune response mechanisms. Not only do immune cells execute various inflammatory and host defense mechanisms, they are active participants in tissue repair and in resolving inflammation after its onset. Various subpopulations of adaptive and innate immune cells have been found to actively replace tissue, or directly help in its recovery.

Gattinoni and colleagues, for instance, uncovered a human T cell subpopulation with a high proliferative rate and self-renewal properties. Moreover, these cells were capable of deriving central and effector memory, and effector T cells, displaying a multipotent phenotype (Gattinoni *et al.,* 2011). The stem cell-like properties of these cells may be crucial for immunological memory, in which stem-like cells are able to self-renew lymphocytic cell populations which can differentiate into effector cells upon antigen re-exposure.

Several reports have claimed that monocytes ($CD14^+$ cells) were capable of differentiating into progenitor/stem cell-like cells or mature cells of other lineages. Zhao and colleagues

have identified adult pluripotent stem cells that originated from human monocytes. These cells displayed fibroblastic appearance, were capable of fast expansion when exposed to macrophage colony-stimulating factor (MCSF) and, being $CD14^{+}CD34^{+}CD45^{+}$, also displayed hematopoietic traits. Strikingly, these cells were capable of acquiring a macrophage-like phenotype upon induction with lipopolysaccharide (LPS) and T lymphocyte phenotype after exposure to IL12. Moreover, Zhao also achieved to obtain epithelial cells, endothelial cells, neuronal cells, and hepatocytes after inducing the monocyte-derived pluripotent stem cells with epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), nerve growth factor (NGF), and hepatocyte growth factor (HGF), respectively (Zhao *et al.,* 2003).

Kuwana and colleagues (Kuwana *et al.,* 2003) have performed seminal and important work assessing whether $CD14^{+}$ monocytes could represent a source of mesenchymal progenitors. These cells, termed MOMPs (monocyte-derived mesenchymal progenitor), were obtained once peripheral blood mononuclear cells (PBMC) were cultured *in vitro* on fibronectin in the presence of FBS. MOMPs displayed a fibroblastic morphology, along with a unique $CD14^{+}CD34^{+}CD45^{+}$type I collagen^{+} molecular signature. These cells showed mixed molecular characteristics of monocytes, mesenchymal, and endothelial cells. MOMPs were found to derive from CD14+ monocytes; the latter required fibronectin binding and exposure to soluble factors from peripheral blood $CD14^{-}$ cells in order to give rise to MOMPs.

In our studies, human MSC were cocultured with monocytes in both transwell and 3D culture system for 5 consecutive days. We hypothesized that, through the release of soluble factors or through cell-to-cell contact, MSC would be able to instruct monocytes to sustain a M2-like macrophage phenotype and/or instruct monocytes to acquire a mesenchymal phenotype in order for monocytes to actively aid the functions of MSC in tissue replacement.

Our results indicate that, while transwell monocytes (monocytes cocultured with MSC in a transwell system) displayed a mixed M1-M2 phenotype, 3D monocytes (monocytes cocultured with MSC in 3D collagen sponges) started to transition away from the monocytic lineage, though still presenting a few macrophage characteristics. Transwell monocytes exhibited a very high CD206 expression (M2) associated with high TNFα, IL6, and IL12 expression (M1). The cytokine expression, however, is unspecific, since monocytes and/or MSC may also express the latter. This unspecific macrophage phenotype may relate to the default differentiation pathway of monocytes, despite the fact that MSC have been reported to induce M2-like macrophages (Kim & Hematti, 2009), and that cytokines released by M2 macrophages support the growth of MSC, such as IL10, TGFβ1, and VEGF (Freytes *et al.,* 2013). Moreover, when human MSC were cocultured with purified subpopulations of immune cells, they altered the cytokine secretion profile of dendritic cells, NK cells, and T_H1 and T_H2 cells, inducing a more anti-inflammatory phenotype of these cells (Aggarwal & Pittenger, 2005); this could be an indicator that M2 phenotype can indeed be sustained by monocytes in direct or indirect contact with MSC.

3D monocytes, on the other hand, suffered substantial loss of CD14 and lack of specific macrophage cytokine expression. These cells, however, did have high IL1β expression, even when compared with the expression of IL1β by MSC. IL1β is expressed in high levels by MSC, where it plays a role in MSC recruitment and in modulating the differentiation capacity of these cells (Mohanty *et al.,* 2010).

When the capacity of monocytes to acquire a mesenchymal phenotype was tested, several markers that are known to be expressed by MSC were tested. Several difficulties were encountered in this process, namely finding ideal markers that could provide an indication of monocyte-to-mesenchymal transition. No single marker is specific to MSC; indeed, MSC are

defined by the co-expression of multiple markers. In our experiments, many markers that are commonly expressed by MSC were also expressed by monocytes, proving to be unreliable for mesenchymal phenotype testing. For our experimental purposes, CD73, CD106, nestin, and stro-1 were the most reliable mesenchymal markers, since they were not expressed by monocytes. Whereas nestin remained negative in all conditions, CD73, CD106, and stro-1 were significantly expressed by 3D monocytes. For transwell monocytes, however, only CD106 had a significant expression, but still much lower than its 3D counterpart. On the cytokine level, only 3D monocytes expressed significant levels of mesenchymal cytokines, namely MCAM and SDF-1. This indicates that 3D monocytes are more prone to acquiring a mesenchymal phenotype, and could therefore directly participate in tissue regeneration, directly contributing to the repair functions of MSC. As shown with the chondrogenic differentiation analysis, monocytes are not capable of differentiating themselves into fully mature and functional mesenchymal cells, but they could play a role in aiding MSC in tissue repair, either by being directly recruited by MSC in a cell-to-cell contact – and thus acquiring a mesenchymal-like state - or by responding to soluble factor released by MSC and transforming themselves into M2 macrophages, which could further the potential of MSC in repairing damaged tissue.

Conclusion

In this study, we investigated the ability of MSC in instructing monocytes to acquire a macrophage and/or mesenchymal phenotype. Our results indicate that, while monocytes do not actively contribute to cartilage tissue formation as do MSC, they could actively participate in supporting and assisting MSC with their functions, either by acquiring a mesenchymal phenotype or by maintaining a M2-like phenotype throughout the regeneration process. Future assessments are needed to investigate the precise mechanism

in which MSC could benefit the presence of monocytes at the lesion site, and how this process could be further explored *in vivo*.

Acknowledgements

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Final Conclusions and Future Perspectives

Results Summary and Final Conclusions

This thesis aimed at investigating the effects of angiogenesis regulation in cartilage formation by two cell sources that are currently used for articular cartilage repair, namely nasal chondrocytes (NC) and mesenchymal stromal cells (MSC). Moreover, we analyzed if MSC, while being recruited to repair a cartilaginous lesion and detaining immunomodulatory properties, could induce monocytes (inflammatory cells infiltrating the cartilage lesion site) to acquire traits of tissue repair cells. Hence, MSC could significantly benefit from their ability to control angiogenesis and inflammatory events during cartilage repair processes.

Chapter 1 showed that two distinct methods for blocking VEGF, namely sFlk-1 overexpression and an anti-angiogenic peptide (WHLPFKC) were both capable of greatly inhibiting angiogenesis and promoting chondrogenesis *in vivo* by non-precultured-NC primaries. High and low primaries were tested; the terms “high” and “low” refer to the chondrogenic capacity of these NC. Both types of primaries were capable of undergoing chondrogenesis *in vivo* when expressing sFlk-1 or when associated with the anti-angiogenic peptide. Importantly, whereas control “high” chondrogenic NC could undergo chondrogenesis *in vivo*, both anti-angiogenic approaches greatly enhanced cartilage formation in these donors. Importantly, for the “low” chondrogenic NC primaries, only the ones overexpressing sFlk-1 were capable of generating cartilage *in vivo* in an ectopic mouse model. The cartilage formed was stable up to 8 weeks *in vivo*, and did not show signs of hypertrophy or endochondral ossification.

Chapter 2 showed that VEGF signaling blockade by sFlk-1 effectively inhibited angiogenesis and induced *in vivo* chondrogenesis by MSC, without the supplementation of additional chondrocytic morphogens. It has also been shown that hypoxia is effectively a trigger for MSC chondrogenesis to take place, and that VEGF blockade by sFlk-1 *in vivo* promotes the necessary hypoxic environment for chondrogenesis to occur in an ectopic mouse model. sFlk-1 overexpression also enhanced MSC cell survival *in vivo* up to 12 weeks. Furthermore, the cartilage formed by sFlk-1-overexpressing MSC preserved a stable chondrogenic phenotype, as it did not exhibit traits of cartilage remodeling and endochondral ossification.

Chapter 3 showed that monocytes that were cocultured with MSC in transwells acquired some M2 traits, and monocytes cocultured with MSC in 3D collagen scaffolds were more prone to acquiring a mesenchymal phenotype. This could imply that monocytes infiltrating into the cartilage lesion site could be instructed by the resident MSC to become tissue repair cells thus actively participating in the healing of the cartilage defect. This phenotype change induction could be done either by direct MSC-to-monocyte contact or by the release of soluble MSC factors. It could also mean that, by directly regulating monocyte phenotype, MSC could also hinder pro-inflammatory macrophages from further damaging the tissue.

Relevance of the Study and Future Perspectives

The self-repair capacity of articular chondrocytes is very limited. Being an aneural and avascular tissue, cartilage regeneration is a complex process with currently restricted success. Problems concerning tissue engraftment, natural fibrocartilage

scar tissue formation, and *in vitro* expansion time of chondrocytes are among some of the challenges faced in current clinical cartilage repair approaches.

Autologous Chondrocyte Implantation (ACI, a technique that involves the injection of cultured chondrocytes into a chondral defect) and Matrix-Induced Autologous Chondrocyte Implantation (MACI, a porcine type I/type III collagen bilayer seeded with chondrocytes) have become the established treatment for symptomatic osteochondral defects. These procedures are designed to heal cartilage damage by filling the cartilage defect with cells capable of repairing the cartilaginous tissue.

The studies performed in chapters 1 and 2 show a remarkable advantage for potential therapeutic MSC and NC cartilage engineering. Both studies focus solely on angiogenesis as a key regulator of cartilage formation, without the need of additional growth factors to trigger chondrogenesis or *in vitro* preculture. These clear advantages make these cartilage repair approaches highly attractive for a clinical application setting. With these approaches, the cartilage formed was stable, did not undergo hypertrophy, and did not require pre-commitment of neither cell source in order for chondrogenesis to take place.

Before placing the anti-angiogenic approach into practice, studies in an orthotopic environment, where cartilage is exposed to several physiological stimuli such as mechanical loading, are imperative to determine the long-term stability of the newly formed cartilage. Moreover, due to safety issues regarding the oncogenic potential of retroviral vectors, the use of self-inactivating lentiviral vectors for stable and sustained transgene expression could be used as an alternative. Other approaches, along with the dendronized anti-angiogenic peptide, could be implemented by a scaffold-based delivery of clinically used anti-angiogenic agents, such as humanized

monoclonal VEGF antibodies. Moreover, biodegradable scaffolds could be used with an anti-angiogenic approach in order to trigger the initial chondrogenic response, being degraded as soon as the newly formed cartilage is in place and angiogenic regulation of the cartilage milieu is no longer required. Finally, once the interdependence between chondrogenesis and angiogenesis is defined, the development of "off-the-shelf" products, such as biomaterials capable of controlling angiogenesis at different stages, could trigger chondrogenesis at various developmental stages depending on the extension of the chondral damage.

Several additional factors can contribute for a successful cartilage repair process. Monocyte and macrophage infiltration at the cartilage lesion site can be detrimental, if the inflammatory process that initiates with these cells is left unresolved, but it can also be highly beneficial for the new tissue being formed. Monocytes, when acquiring an M2 macrophage phenotype, can assist tissue-competent cells into regenerating the damaged tissue. Moreover, due to their high plasticity, monocytes are capable of acquiring several traits of differentiated cells of non-myeloid lineages, which can further contribute to aiding repairing cells in their biological functions. In chapter 3, by looking into the phenotype that monocytes acquired after being directly or indirectly cultured with MSC, a hybrid M2-MSC-like phenotype was identified; though monocytes under our experimental conditions do not become tissue-competent cells, as shown by their lack of chondrocyte differentiation capacity, they may actively assist MSC in cartilage tissue repair by different mechanisms, such as the release of soluble factors that may recruit MSC to the lesion site, or by hampering the inflammatory process in order for tissue repair by MSC to take place. Other environmental factors may be added to the culture in the

future to better mimic the cartilage repair milieu in which MSC and inflammatory cells are interacting.

Thus, understanding how MSC-controlled inflammation, mainly derived from monocytes, can regulate cartilage formation and repair may shed light into new therapeutic approaches in cartilage regeneration. The generation of biomaterials capable of polarizing macrophages into a desired phenotype (M2-like or MSC-like) can overcome the detrimental effects of innate immunity and aid MSC in cartilage repair.

Carolina Cunha

Ahornstrasse 29
4055 Basel
+41 76 7078310
carol_medeiros@yahoo.com

Personal

Born on May 16th, 1983 in Recife, Brazil, *Brazilian and American Citizenship*.

Education

04/2011 – 07/2014 **University of Basel**, *PhD student in Tissue Engineering*, Basel, Switzerland.

09/2008 – 03/2010 **University of Göttingen**, *International Master Program in Neuroscience-International Max Planck Research School*, Göttingen, Germany.

06/2002 – 01/2008 **Federal University of Pernambuco**, *PharmD*, Recife, Brazil.

Research Projects

University of Basel **Microenvironmental VEGF sequestration enhances in vivo cartilage formation**.

University of Basel **Spontaneous in vivo chondrogenesis of bone marrow-derived mesenchymal progenitor cells by blocking VEGF signalling**.

University of Basel **Monocyte phenotypic changes induced by mesenchymal stromal cells originate non-functionalized cells with hybrid mesenchymal-macrophage characteristics**.

University of Göttingen **Identification of Neuroligin-2 protein interactors**.

Federal University of Pernambuco **Schizophrenia: current therapies and future perspectives**.

Scholarships and Awards

09/2008 – 03/2010 **Stipend of the Excellence Foundation for the Promotion of the Max Planck Society**, *Göttingen, Germany*.

09/2012 – 09/2012 **Travel Award recipient – TERMIS World Congress**, *Vienna, Austria*.

Skills

Languages

Portuguese **Native Speaker**

English **Native Speaker**

German **Advanced**

French, Spanish **Basic**

Italian	**Beginner**

Software

Office	**Word, Excel, Powerpoint, Outlook (all advanced)**
References	**Endnote (basic)**
Photo & Imaging	**Adobe Illustrator and Adobe Photoshop (basic)**
Data Analysis	**GraphPad Prism (basic)**