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Endothelial-mesenchymal transition and the immune system: an evolving paradigm in muscle fibrosis and heterotopic ossification

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With all its sham, drudgery,
and broken dreams,
it is still a beautiful world.
Be cheerful.
Strive to be happy.

Max Herman, Desiderata

To Elisa

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1. General Introduction

Muscle regeneration is a well-orchestrated process that occurs after injury caused by disease and trauma or, in some cases, drug intake. Complete regeneration leads to full restoration of health, whereas incomplete or defective regeneration can lead to loss of tissue mass or replacement with fibrotic scars, associated to impaired functional recovery. Several evidence supports the emerging concept that inflammation coordinates tissue repair, and it is stated that no regeneration can occurs without inflammation. Neo-angiogenesis is also necessary to establish a new vascular network. Muscle disorders exist in which the irreversible loss of tissue integrity and function is linked to defective angiogenesis with persistence of tissue necrosis and inflammation. How inflammatory, myogenic and vascular components integrate to coordinate skeletal muscle repair and stem cells fate remains still incompletely understood. Based on these considerations, understanding the molecular interplay underlying muscle homeostasis and regeneration is particularly challenging because such knowledge is essential in the development of effective therapies characterized by tissue degeneration.

1.1. MUSCLE DEVELOPMENT, REPAIR AND REGENERATION

1.1.1. Basic Concepts of Skeletal Muscle development

Skeletal muscles (except from head) are derived from mesodermal precursor cells originating from the somites. In the body, mesoderm is divided into four compartments: the axial, paraxial, intermediate and lateral plate mesoderm. Somites are transient segments of paraxial mesoderm that took place progressively on either side of the neural tube, following an anterior-to-posterior developmental gradient[1]. This process is triggered progressively from E8 of embryonic development. During early embryonic development each somite undergoes a programme of maturation and specialization, commonly called differentiation, to produce a ventral sclerotome and a dorsal dermomyotome (Fig.1)[2]. The sclerotome contributes to the cartilage and bone of the vertebral column and ribs; the dermomyotome gives rise to the skeletal muscles of the body and limbs. Myogenesis in the head follows a different route; the majority of these muscles are derived from cranial paraxial mesoderm comprising paraxial head and the more anterior prechordal mesoderm[3] [4].

The determination of skeletal muscle cells is dependent on signaling molecules such as Wnts and Sonic Hedgehog protein, produced by adjacent tissue such as neural tube, the notochord and dorsal ectoderm[2]. A very fine balance of induction (such as molecules from Noggin

pathways) and repression signals (BMPs) permit activation in the somites of the myogenic program genes in the correct spatial-temporal order [5]. The first skeletal muscle is the myotome.

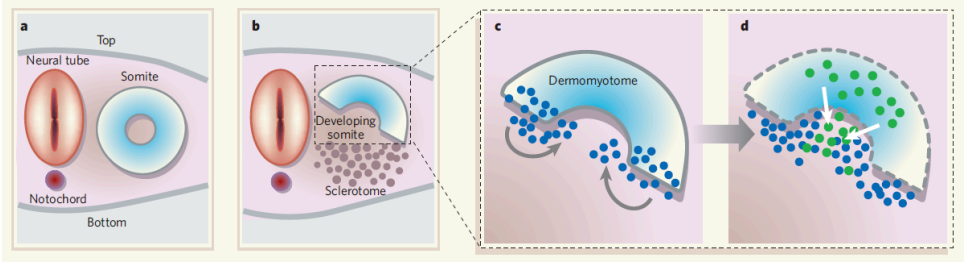

Figure 1. Making of the Myotome. a) Formation of the somites on each side of the developing spinal cord (notochord). Only one side is shown in cross-section. b) Each somite differentiates into a dermomyotome and a sclerotome. c) Proliferating muscle precursor cells (MPCs) move from the lips of the dermomyotome to form a layer of non-proliferating, differentiating myoblasts: the primary myotome. d) Pax3/Pax7 positive cells, originating from the central domain of the dermomyotome, migrate into the primary myotome.

Skeletal muscle is established in successive distinct, though overlapping steps, which involve different types of myoblasts (embryonic and fetal myoblasts, and satellite cells) [6]. At around E11 in the mouse, embryonic myoblasts invade the myotome and fuse into myotubes. At the same stage, myogenic progenitors that have migrated from the dermomyotome to the limb start to differentiate into multinucleated muscle fibers, called primary fibers, during a phase which is usually referred as primary myogenesis. A subsequent "wave" of myogenesis takes place between E14.5 and E17.5. This phase is called secondary myogenesis and involves fusion of fetal myoblasts, either between them to give rise to secondary

fibers (which are originally smaller and surrounding primary fibers) or with primary fibers (Fig.2). At the end of this phase, satellite cells can be morphologically identified as mononucleated cells lying between the fiber membrane and the basal lamina. Satellite cells (SCs) appear at the end of gestation and are responsible for postnatal growth and regeneration. Typical mitotically quiescent (G^0 phase), SCs are present throughout skeletal muscle but show an unequal distribution: at birth, they account for 20-30% of sub-laminar nuclei in mouse skeletal muscle. As mice grow and mature this number rapidly declines to approximately 5% at two months of age and 2% in senile mice. Nevertheless, they have a remarkable proliferative potential[7].

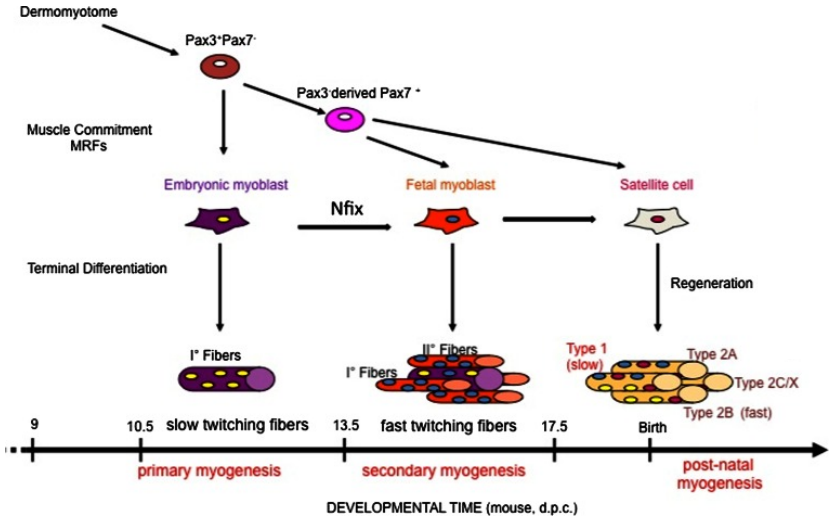

Figure 2. Lineage scheme for skeletal muscle formation. The somitic dermomyotome gives rise to the myotomal cells, which differentiate into the myocytes of the early myotome. During development, from *Pax3*/*Pax7* positive progenitor cells originate muscle precursors (embryonic, fetal and adult myoblasts)

Cell-autonomous activation of myogenesis in different regions of the embryo is controlled by a series of complex transcriptional regulatory networks that, ultimately, result in the expression of members of the basic helix-loop-helix domain-containing myogenic regulatory factors (MRFs). This family includes *Myf5 (Myogenic factor 5)*, *Mrf4* (*Myogenic factor* 6, also known as *Myf6*), *Myod1* (*Myogenic differentiation 1*, also known as MyoD) and *Myog* (*Myogenin*) within nascent and differentiating myoblasts (Fig.3). MRFs are responsible, in collaboration with other cofactors, for directing the expression of genes required to generate the contractile properties of a mature skeletal muscle cell. These genes act downstream of (or in parallel with) the paired domain and homeobox containing transcription factors paired box gene 3 (*Pax3*) and 7 (*Pax7*), in different phases of myogenesis in the embryo and adult (Fig.3) [8].

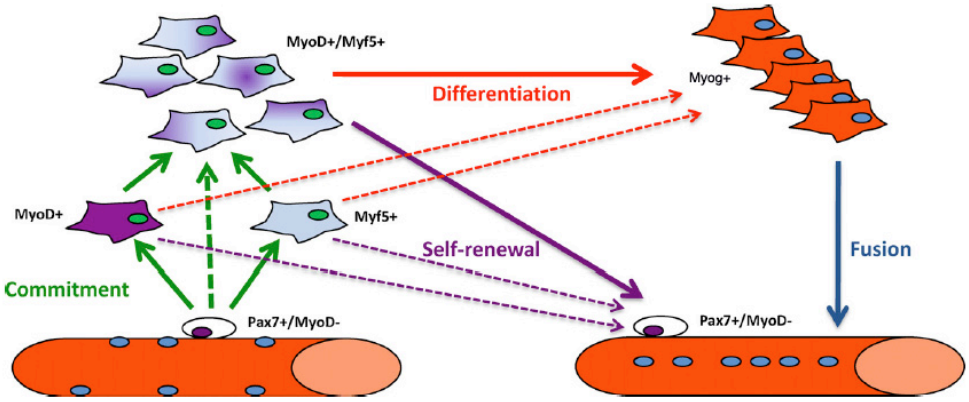

Figure 3 Schematic representation of satellite cell myogenic progression.
SCs, initially Pax7+/MyoD-, can enter the myogenic differentiation program by inducing *Myf5* or *MyoD* expression. Most of the activated SCs carry on their myogenic differentiation progression by expressing both determination genes, and terminally differentiation upregulating Myogenin (Myog+), Dashed lines represent possible, but still not proven connections.

Adult mammalian skeletal muscle is composed by multinucleated postmitotic muscle fibers, which are the basic contractile units [9]. Newly formed multinucleated fibers exhibit central nucleation, and once the nuclei move to a subsarcolemmal position they are called myofibers [10]. The myofibers are individually surrounded by a connective tissue layer and grouped into fascicles (bundles) to form a skeletal muscle (Fig.4).

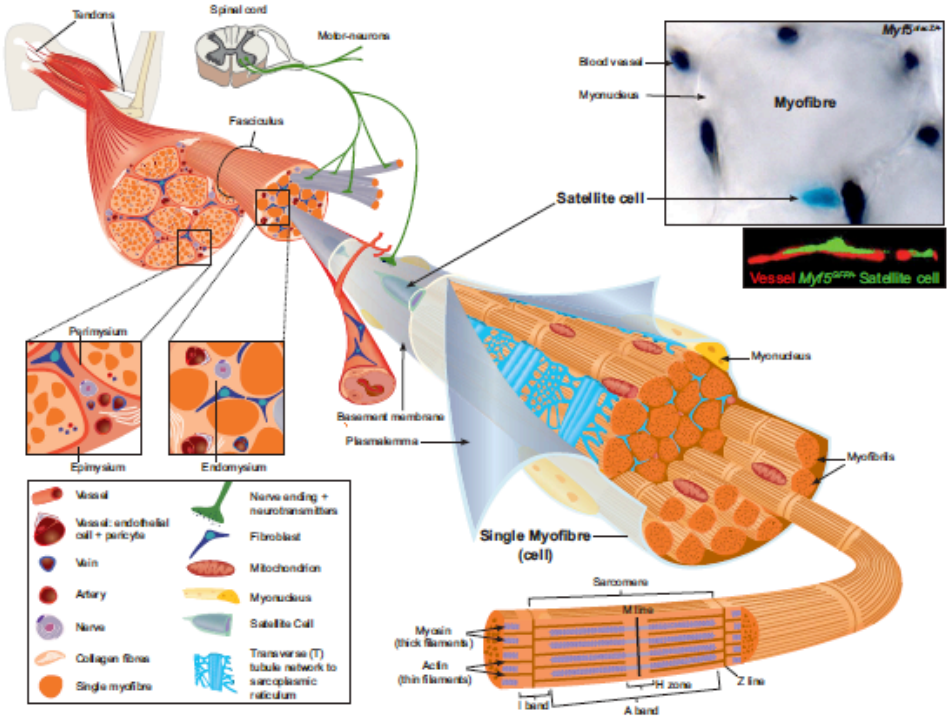

Figure 4. Scheme of skeletal muscle and associated structures. Satellite cells are located between the basement membrane and the plasmalemma of the myofiber. Note the close proximity of the vessel and satellite cell. Three connective tissue layers (epimysium, perimysium, endomysium) can be distinguished in skeletal muscle; these form the network and associated basement membranes in which myofibers regenerate after injury.

The epimysium is the fibrous outer layer that surrounds the complete muscle and is contiguous with the tendons (muscle to bone). The perimysium surrounds the bundles of myofibers, and the endomysium (also called basement membrane) surrounds individual myofibers [10]. Individual skeletal muscles are composed by a mixture of myofibers with different physiological properties ranging from a slow-contracting/fatigue resistant type to a fast-contracting/non fatigue resistant type. The proportion of each fiber type within a muscle defines its overall contractile property.

The interior of a myofiber contains the sarcomeres which are the basic functional units of skeletal muscle. The sarcomere consists basically of thick myosin-rich filaments and thin actin-rich filaments. The basic mechanism of muscle contraction is similar in all myofiber types and it is the result of a "sliding mechanism" of the myosin-rich thick filament over the actin-rich thin filament, after neuronal activation [11]. The contraction of myofibers is transformed into movement by myotendinous junctions at their ends, where myofibers attach to the skeleton by tendons.

The functional properties of a skeletal muscle thus depend on the maintenance of a complex framework of myofibers, motor neurons, blood vessels and extracellular connective tissue matrix.

Characteristic features of mammalian skeletal muscles are the richness in connective tissue and the high degree of vascularization, fundamental to provide essential nutrients for muscle function. During maturation, myofibers are contacted by motor neurons, and express characteristic

molecules for contractile functions, principally different MHC (myosin heavy chain) isoforms and metabolic enzymes [12].

1.1.2. Cellular dynamics in satellite cells niche

The first weeks of postnatal life is a period of intense growth, with body weight increasing 7-8 fold, half of which is accounted for by the increase in skeletal muscle. Muscle growth can be achieved by either an increase in muscle fiber number or an increase in the size of individual myofibers, or a combination of both[13]. Myofiber hypertrophy during growth requires the addition of new myonuclei, and these are supplied by muscle SCs. In adult skeletal muscle is a stable tissue which undergoes a little turnover of nuclei. However, muscle can be injured after direct trauma (for example intense exercise, lacerations, drug intake) or resulting from indirect causes, such as innate genetic defects or neurological dysfunctions. The normal muscular activity can also generate a certain degree of damage to the muscle[14]. Exercise has a profound effect on muscle growth, lead, if not balanced, to hypertrophy and subsequent injuries[15]. Left unrepaired, those injuries may ultimately lead to loss of muscle mass, locomotive deficiencies and eventually lethality. The maintenance of a working skeletal musculature is dependent on its remarkable ability to regenerate.

The cellular dynamics during muscle regeneration are highly complex and occur with distinct temporal and spatial kinetics [16](Fig.5). SCs, the quintessential skeletal muscle stem cells[17], reside in a specialized local

environment whose anatomy changes dynamically during tissue regeneration. After damage, they become activated and some will eventually upregulate transcription factors that trigger the myogenic differentiation programme [18](Fig.3). Once differentiated into myocytes, the cell will align and form new syncytial muscle fibers or fuse to existing fibers. On completion of this regenerative response, the tissue returns to its homeostatic state and the resident cell population re-enter a resting state. Despite there are many differentiating cells, the total number of SCs remains constant through multiple rounds of regeneration[19]. This equilibrium is due to the ability of SCs to self-renew, which provides progeny for differentiation while uncommitted mother cells are retained[20]. In the quiescent niche a few cell types are found in the proximity of SCs, for instance vessel-associated cells and muscle fibers (Fig. 4). This environment remains essentially static and imposes signals that promote the quiescent stem cell state. In contrast, during muscle regeneration the composition of the niche is in a flux that is regulated by a spectrum of cell types. The healing of skeletal muscle in response to trauma depends on the type of injury and on the severity. In general, the healing process consists of three phases: the destruction phase, the repair phase, and the remodeling phase. The destruction phase is characterized by necrosis, formation of hematomas and infiltration of inflammatory cells. Necrosis is generally triggered by disruption of the myofiber sarcolemma, resulting in increasing myofiber permeability. During the repair phase, the necrotic debris is phagocytosed and regeneration of myofibers takes place through the action of muscle stem cells. In the last

phase, remodeling occurs and the regenerated myofibers mature and contract. Once fusion of myogenic cells is completed, newly formed myofibers increase in size and myonuclei move to the periphery of the muscle fibers. The onset, development and resolution of acute damage involve diverse interaction between leukocytes and local cell types. In resting conditions, adult skeletal muscle contains different types of resident leukocyte.

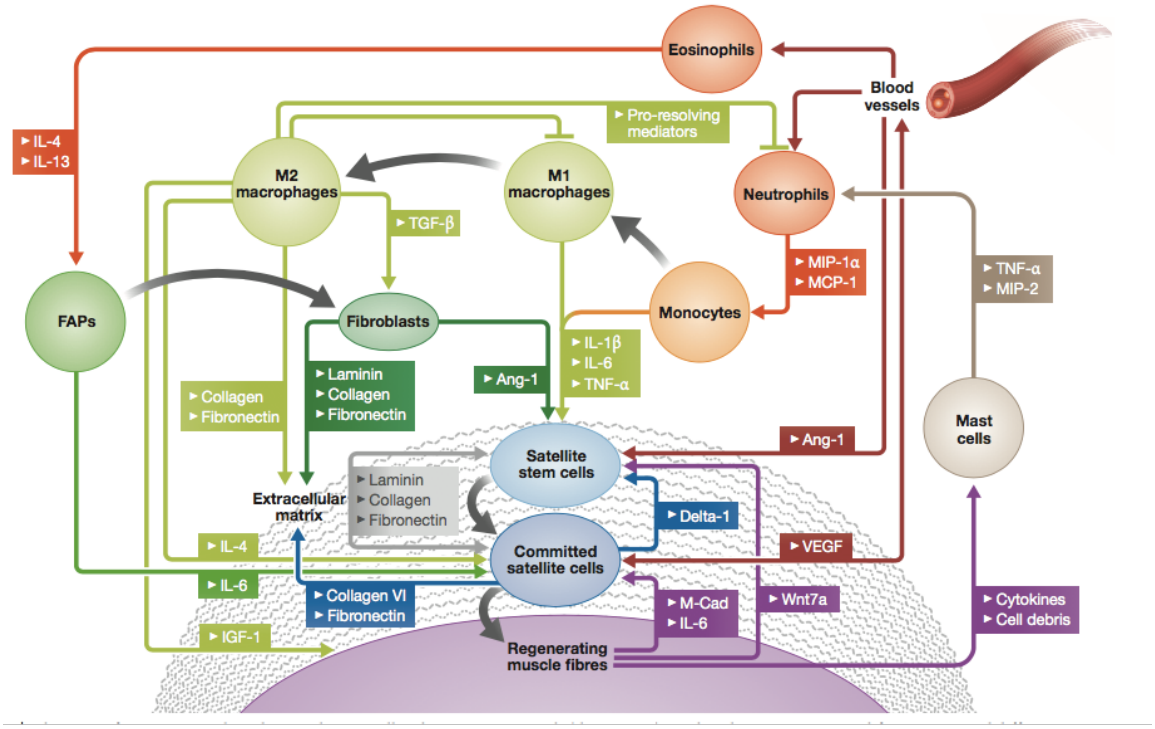

Figure 5. Schematic of extrinsic signals in the muscle stem cell niche. Paracrine signals (thin arrows) regulate the recruitment, proliferation rate and differentiation (bold arrows) of each cell type. (image from Bentzinger et al., 2013 [16])

Mast cells and macrophages are the most abundant. They act as sensors for distress and secrete a number of chemoattractive molecules following muscle injury. In particular, damaged activated mast cells almost instantly begin to secrete TNF-alpha, histamine and tryptase and then initiate the *de novo* synthesis of other cytokines, such as interleukin (IL)-6. At low physiological concentrations, TNF, tryptase and IL-6 promote activation and proliferation of satellite cells[21, 22]. The initial burst of cytokines and chemokines produced by resident leukocytes lead to the rapid attraction of circulating granulocytes [23]. These consist mainly of neutrophils and, to a lesser extent, eosinophils [24]. Neutrophils promote the pro-inflammatory environment that is necessary for the clearance of cellular debris. Under certain conditions, this cell type has been suspected to transiently aggravate tissue damage[25]. Neutrophils also secrete the chemokines MIP-1alpha, MCP-1 and others that favor the recruitment of monocytes. Beyond the first day after injury, monocytes gradually become the predominant leukocytes in the exudate. Once monocytes have invaded the tissue, they begin to differentiate into macrophages. The regulatory function of immune cells for myogenesis is sensitive to perturbation and efficient muscle repair depends on their precise coordination. Thus, even slight imbalances in immune cell population due to sustained and successive inflammatory signals in diseases muscles can disrupt the cellular dynamics in the niche and provide inappropriate environmental cues to SCs. Nonetheless, if appropriately synchronized and controlled, immune cells serve as key effectors in the muscle stem cell niche to guide SCs through regeneration process. Neo-angiogenesis is

also necessary to establish a new vascular network and involves the recruitment of endothelial cells to the site of injury. In addition to the endothelial cells that compose the vasculature, non-hematologic endothelial progenitor cells (EPCs) circulate in the blood and migrate to regions of the circulatory system with injured endothelia. Endothelial cells secrete a variety of mitogenic and/or anti-apoptotic factors, such as VEGF, that influence muscle satellite cells proliferation and differentiation. During muscle injury, the number of capillaries in the tissue initially increases and then returns to baseline about 4 weeks after injury[26].

Finally, quiescent stem cells are found underneath the extracellular matrix (ECM) sheet attached to the muscle fiber plasma membrane[27].

The main source of ECM is fibrogenic mesenchymal stromal cells, such as fibroblasts and fibro/adipogenic precursors (FAPs). During muscle regeneration the extracellular environment in stem cell niche is dynamically rearranged. The function of various ECM components being deposited in regenerating tissue are only beginning to be elucidated. Structurally, this transitional fibrillary ECM serves to preserve the gross integrity of the tissue until degenerated fibers have been cleared and innervated young muscle fibers have been formed in the correct anatomical position[28]. In conclusion, after injury, the stem cell niche in muscle switch from a relatively steady state involving few cell types into an enormously complex environment with spatiotemporally regulated cascades of direct and indirect cellular interactions (Fig.5). With the mouse as a versatile model to study the biology of skeletal muscle, it is

becoming apparent how elaborately fine-turned is the role of the different cell types involved in muscle regeneration, and how detrimental are the consequences of disease-related imbalances in these dynamics. An integrative understanding of the cellular complexity in the niche will allow for the development of therapeutic strategies to normalize or adapt the global behavior of cell population rather than single signaling pathways.

1.1.3. Muscle disease and animal models

Muscle may suffer from a number of disorders, some being fatal to humans and animals. The most common diseases in muscle are the muscular dystrophies, others major include the inflammatory myopathies and neuropathies. Also atrophy and hypertrophy of muscle and the relationship of aging, exercise, and fatigue helps our understanding of the behavior of normal and abnormal muscle. There is actually no curative treatment for muscular dystrophies. Treatment is generally aimed at controlling the onset of symptoms to maximize the quality of life. There are many avenues of research currently under investigation, including gene therapy, stem cell therapy and pharmacological strategies.

The muscular dystrophies are inherited myogenic disorders characterized by progressive muscle wasting and weakness of variable distribution and severity [29], leading to mobility limitations and, in the most severe forms, heart and/or respiratory failure.

Muscular dystrophies are generally characterized by alterations in the muscle histology. Those include variations in fiber size, presence of necrotic and regenerating fibers, inflammatory infiltrate (mainly composed by macrophages) and, ultimately, replacement by fat and connective tissue. Those features do not always show together, except in severe forms like Duchenne Muscular Dystrophy (DMD), which is also the commonest form of the disease. The incidence of DMD is around 1 every 3.500 male births. DMD is caused by mutations in the dystrophin gene, which is the largest known (about 2.5 Mb), which encodes a protein of 427 kDa. In healthy muscle, dystrophin is located at the subsarcolemma and interacts with a number of membrane proteins forming the dystrophin glycoprotein complex (DGC), whose function is to stabilize muscle cell membrane during cycles of contraction and relaxation [30]. Other forms are characterized by variable degrees and distribution of muscle wasting and weakness. Notably, the attenuation of muscle repair in most forms of muscular dystrophy is correlated with a build-up of fibrotic scarring, adipose tissue and immune infiltrations[31]. The increased susceptibility of dystrophic muscle fibers to damage leads to cycles of degeneration and regeneration. In most cases, necrotic and regenerating areas occur concurrently throughout dystrophic muscle. Unlike the beneficial effects of transient ECM protein during normal regeneration, increased inflammation and persistent expression of ECM proteins reduce the differentiation of myoblasts into myofibers. Moreover, the altered elasticity of fibrotic muscle tissue is likely to have a negative influence on the self-renewal of SCs. In agreement with this

idea, anti-fibrotic and anti-inflammatory therapies have reduced the evolution of Duchenne muscular dystrophy in the short term.

Although the degenerative and regenerative phases of muscle regeneration are similar among different muscles and causes of injury, the kinetics and extension of each phase may vary depending on the extent of the injury, the involved muscle and the animal model. Therefore, to study the process of muscle regeneration in a controlled and reproducible way, it has been necessary to develop animal models of muscle injury.

The use of myotoxins such as bupivacaine, notexin (NTX) and cardiotoxin (CTX) is at the moment the most widely used way of inducing muscle regeneration after an acute damage, because of their ease of use and their high reproducibility [32]. CTX is a peptide isolated from snake venoms. It acts as a protein kinase-C specific inhibitor that appears to induce the depolarization and contraction of muscular cells, to disrupt membrane organization and to lyse various cell types. Injection of 25-50 uL of 10 mM CTX in the *tibialis anterior* muscle of an adult mouse induces muscle degeneration leading to a wound coagulum with mononuclear cell infiltration within 1 day of injection. The following 3-4 days see the activation of the inflammatory response and mononuclear cell proliferation. Myogenic cell differentiation and formation of new myofibers starts 5-6 days post injection. After 10 days, the overall architecture of the muscle is restored, although most regenerated myofibers are smaller and display central myonuclei.

It is only after 3-4 weeks after the injection that the muscle returns to a morphologically and histochemically normal state (Fig.6). A potential "caveat" to the use of this damage protocol are the unknown effects of CTX on various muscle cell types, including satellite cells.

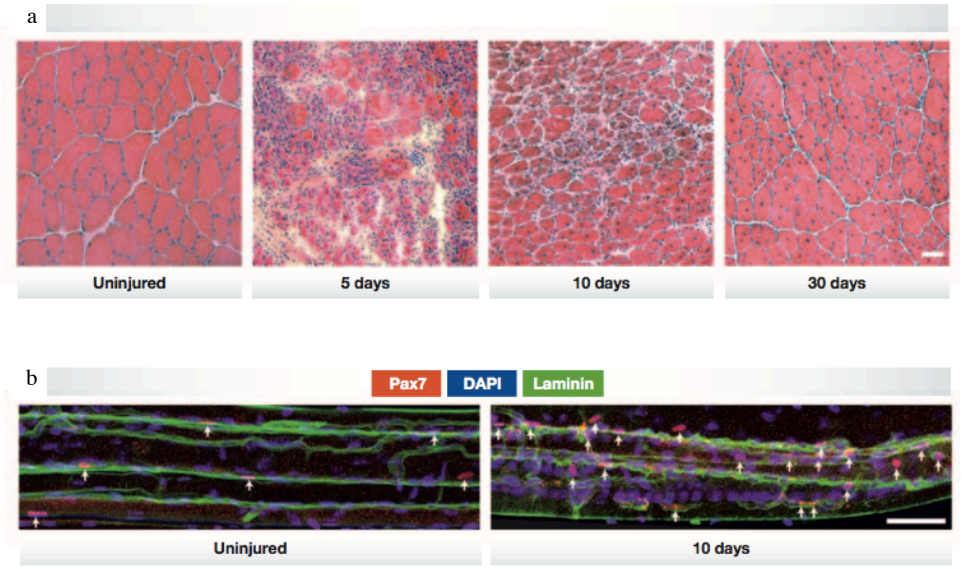

Figure 6. Overview of tissue histology during mouse skeletal muscle regeneration. a) A time course of histological changes in regenerating skeletal muscle(H&E). Scale bar, 50 μm. b) Longitudinal view of whole tissue preparations of uninjured (left) and regenerating (right) skeletal muscle. Immunostaining for the extracellular matrix protein laminin (green) labels the basal lamina surrounding myofibers and capillaries. In regenerating conditions, the proliferation of satellite cells can be observed by the increase in the number of Pax7 (red) expressing cells (arrows). DAPI staining of nuclei (blue) reveals accessory cells in the satellite cell niche. Scale bar, 50 μm..from Betzinger et al. 2013[16]

The most common way of obtaining a model of chronic muscular damage in mammals is to use genetic knockout mice. The *mdx* mouse was first described in 1984 as a naturally occurring dystrophin-deficient mutant. It carries a point mutation in exon 23 of the mouse *dystrophin* gene introducing a premature stop codon, which leads to the absence of full-length dystrophin [33]. Although *mdx mice* mice are normal at birth, skeletal muscles show extensive signs of muscle degeneration by 3 – 5 weeks of age. However, in *mdx* mice, pathology do not fully resemble human DMD, and is thought to be attenuated because dystrophin is partially replaced by its autosomal homologue utrophin [34]. Another widely used murine model for muscular dystrophy is the α-sarcoglycan (αSG*)* knockout mouse, which models a human autosomal recessive disorder caused by a mutation in the same gene, limb-girdle muscular dystrophy 2D (LGMD-2D). Other studies have used canine models for DMD [35]. The best characterized one is the golden retriever dog model (*GRMD*). The disease in *GRMD* dogs is caused by a single base pair change in the dystrophin gene, resulting in a premature stop codon (Fig.7) [36]. Those animals suffer from a rapidly progressing, fatal disease much more severe compared to *mdx* mice, and increasingly similar to human DMD. However, there is a certain degree of variation in disease severity. Moreover, as happens in DMD, *GRMD* dogs display selective muscle involvement although the most affected muscles in dogs (tongue, masticatory and trunk muscles) are different to those of humans [37]. To conclude, despite the less severity of the disease, mouse models have several advantages, namely a large number of papers published on them,

resulting in good understanding of the pathology. In addition, the complexity of regenerating environment and altered systemic milieu in specific disease, however, have made research into this area challenging, and there are many aspects regarding the structural and temporal regulation of muscle repair that remain unknown. Thus, future research into the regulation of molecular mechanism in regeneration and disease holds great potential for the therapeutic enhancement or restoration of muscle homeostasis.

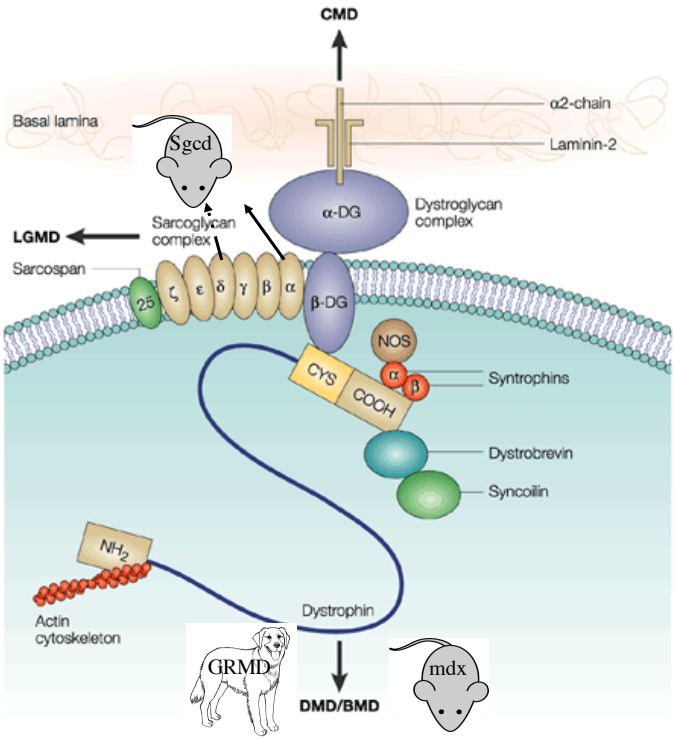

Figure 7. Dystrophin-glycoprotein complex (DGC). Many muscular dystrophies arise from mutations in DGC components. Similar mutations are present in murine and canine models. The most used in preclinical trials are shown.

1.1.4. Myogenic Progenitors Distinct from Satellite Cells

The last 15 years have seen the partial identification and characterization of multi-lineage stem cells derived in culture from various tissues. These findings have challenged the widely held view that tissue-specific stem cells are predetermined to give rise to a specific tissue lineage. In fact, stem cells isolated from various tissues appear to differentiate *in vitro* and *in vivo* upon transplantation into multiple lineages, depending on environmental cues. In particular, progenitor cells isolated from the bone marrow, the neuronal compartment and various mesenchymal tissues can differentiate into the myogenic lineage.

Table 1. Properties of non-SC myogenic progenitors

Cell type	Origin	Proliferation rate	In vitro myogenic differentiation	In vivo Dystrophin expression	Systemic delivery
CD133+	Blood/ Skeletal muscle	Low / High	Induced by muscle cells / spontaneous	Yes	Yes
PICs	Skeletal muscle	High	Spontaneous	Not tested	Not tested
HSCs	Bone marrow	Low	Induced by muscle cells	Yes	Yes
MABs	Vessel wall	High	Induced by muscle cells / spontaneous	Yes	Yes
MADSCs	Adipose tissue	High	Spontaneous	Yes	Not done
MAPCs	Vessel wall	High	Induced by aza-cytidine	Not tested	Not done
MDSCs	Skeletal muscle	High	Induced by muscle cells	Yes	Not done
MECs	Vessel wall	High	Spontaneous	Yes	Not done
MSCs	Vessel wall	High	Induced by aza-cytidine	Yes	Not done

Hematopoietic cells.

The first demonstration that non-muscle cells can contribute *in vivo* to muscle regeneration was reported in 1998 in a study that used transgenic mice expressing the *LacZ* reporter under the control of a muscle-specific promoter (*MLC3f*). Several studies have followed, trying to identify a rare progenitor endowed with high myogenic activity, but those studies have had relatively modest success so far. A subpopulation of circulating cells co-expressing CD133 (a marker of hematopoietic stem cells) and myogenic markers can contribute to muscle repair and to the replenishment of satellite cell pool when transplanted into dystrophic and immunodeficient mice [38, 39]. To date, several subpopulations of hematopoietic cells have been described as possessing myogenic differentiation potency, but none of them seem to be able to exploit this property at a high frequency.

On the other hand, many types of mesoderm-derived stem or progenitor cells (other than hematopoietic cells) have been shown to carry myogenic potential. A list of those cells comprises mesenchymal stem cells (MSCs), muscle-derived stem cells (MDSCs), multipotent adult progenitor cells (MAPCs), $CD133^+$ cells, $PW1^+$ interstitial cells (PICs), mesoangioblasts (MABs), myo-endothelial cells (MECs) and adipose-derived stem cells (MADSCs) [40-42]. A summary of those cells and of their main properties is reported in Table 1. It is important to note that most of those cells have been isolated only retrospectively (and not prospectively) and, in the majority of cases, the precise developmental derivation and physiological function are still unclear. However, the vessel wall appears

to be a common tissue of origin for most of mesoderm-derived myogenic progenitor cells.

Mesoangioblasts.

Among the vessel wall-derived myogenic progenitors reported in Table 1, the mesoangioblast probably is the candidate which has the major therapeutic potential, because it fulfills all the criteria that an ideal stem cell population should have for the treatment of muscle defects. MABs are present in an easily accessible postnatal tissue (skeletal muscle), expandable *in vitro*, able to differentiate into skeletal muscle *in vivo*, and, importantly, being able to cross the vessel wall and home to the site of muscle injury, MABs can reach skeletal muscle through a systemic route [43]. In fact, a phase I clinical trial with MAB allotransplantation in DMD patients is currently ongoing. MABs were first isolated from E9.5 mouse embryonic dorsal aorta as cells expressing early endothelial markers like Flk-1, Sca-1, VE-Cadherin and CD34, but not late ones as von Willebrand factor [44, 45]. They were able to proliferate extensively *in vitro* and to differentiate into several types of solid mesoderm. Mesoangioblast-like cells were later isolated from vessels of postnatal tissues in the mouse, rat, dog and human. Postnatal cells express generally pericyte rather than endothelial cell markers but otherwise are similar to their embryonic counterparts in terms of proliferation and differentiation potency. MABs have been successfully used in preclinical models of cell therapy for muscular dystrophies. Azzoni et al, in 2014[46], identified the putative *in vivo* counterpart of embryonic MABs. They found hemogenic

endothelium-derived cells that contribute to skeletal and smooth muscle *in vivo* and display features of MABs *in vitro*(Fig.8).

Pericytes

Pericytes (also called Rouget cells or mural cells) are contractile connective tissue cells residing beneath the microvascular basement membrane. Pericytes originate from the embryonic sclerotome and are believed to regulate the blood flow in capillaries. As a multipotent stem cell population, they can differentiate into adipocytes[47], chondrocytes, and osteoblasts in vitro and into skeletal muscles both in vitro and in vivo[43, 48].These cells do not express Pax7, Myf5, or MyoD, suggesting that pericyte-mediated myogenesis may follow a myogenic differentiation program distinct from that of SCs. Pericytes display a specific cell surface marker combination, which makes them distinct from other cell types [49-51]. Cells carrying the same surface markers can be isolated from various tissues, including pancreas, adipose tissue and placenta and, regardless of their origins, were able to differentiate into skeletal muscle cells when cultured in vitro.

Myoendothelial cells (MECs)

MECs were identified in human adult skeletal muscle, representing less than 0,5% of the total mononucleated cells [52]. These cells have been prospectively isolated using their molecular signature. In fact, they co-express myogenic and endothelial cell markers (CD56, CD34 and CD144) and are negative for CD45. MECs could regenerate myofibers in the injured skeletal muscle of immunodeficient mice, as well as

exhibiting long term proliferation, retaining a normal karyotype, being not tumorigenic and surviving better under oxidative stress than CD56+ myogenic cells. Clonally derived myoendothelial cells differentiated into myogenic, osteogenic and chondrogenic cells in culture.

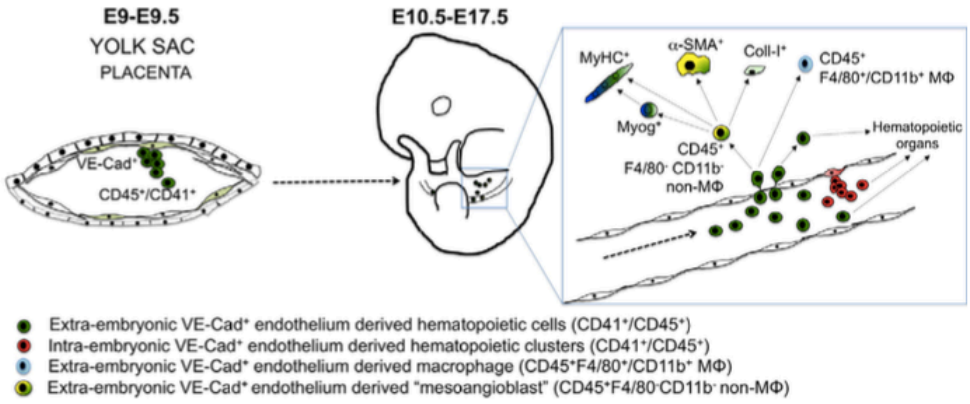

Figure 8. Model of the generation of MABs from extra-embryonic hemogenic endothelium and their contribution to several mesodermal lineages. Extra-embryonic VE-Cad+ hemogenic endothelial cells generate CD41+/CD45+ cells that migrate, probably through circulation, to the embryo proper, where they reach the mesenchyme by extravasation. Here, extra-embryonic-derived CD45+F4/80–CD11b– non-MΦ mesoangioblast cells contribute to several mesodermal lineages, including smooth muscle, skeletal muscle and dermis. Image from Azzoni et al, 2014[46]

1.1.5. Muscle privileged partners

Understanding how stem cell niches are organized in vitro and what interactions their progeny develop with neighboring cell types is a critical issue in stem cell biology.

Macrophages: supportive cells for repair and regeneration

First identified as large phagocytes, macrophages (MPs) play different roles during innate and adaptive immunity. In addition, the last decade has seen the emergence of a multiple properties of MPs, showing that they are more than immune cells [53]. As the presence of MPs is associated with most disease, these cells were firstly thought to be deleterious. However, MPs are also present during the full process of tissue repair and/or regeneration [54]. This led to the identification of the resolution of inflammation and of the restoration of the tissue integrity/function. Those beneficial effects of MPs are mainly due to the trophic factors they release in the environment, and particularly on parenchymal cells.

The term “macrophages” encompasses a variety of cells harboring distinct functional phenotypes. Indeed, depending on the environmental cues they received, MPs may adopt various phenotypes and functions[55]. This versatility makes MPs efficient regulators of tissue homeostasis. MPs have been classified into several subpopulation according to their activation (polarization) state. These populations were defined in vitro, under well-defined stimuli and mainly used human monocyte-derived macrophages. Therefore, these phenotypers likely

don't correspond to what occurs in vivo, were concomitant cues may interfere, leading to a variety of intermediate phenotypes [55, 56]. All macrophages express common markers such as CD11b (Mac1 or CR3), CD68, and CD115 (M-CSF receptor). Classically activated M1 macrophages are induced in vitro by IFN alone or in concert with microbial stimuli (e.g., LPS) or selected cytokines (TNF and GM-CSF). They have proinflammatory functions: they produce effector molecules (reactive oxygen and nitrogen intermediates) and inflammatory cytokines (IL-1β,TNF-α, and IL-6) and participate as inducer and effector cells in polarized Th1 responses. Alternatively activated M2 macrophages comprise cells exposed to low concentrations of M-CSF in the presence of IL-4, IL-13, or IL-10. They participate in polarized Th2 reactions, parasite clearance, damping of inflammation, and promotion of tissue remodeling [57]. This classification of macrophages during muscle regeneration can be simplified into an initial wave of pro-inflammatory, or M1, macrophages that is followed by a second wave of anti-inflammatory, or M2, macrophages. These macrophage subsets, however, are not mutually exclusive, and, at given time point, distinct subtypes can be found in the same regenerating area (Fig.9)[58]. Although the origin of this several phenotypes in macrophages is still debated, distinct functions for both cell types have been established[59]. Depletion models of different types of acute sterile injury have shown that suppression of M1 macrophages leads to persistence of necrotic cells, impaired myoblast proliferation, increased fibrosis and fat accumulation[60]. By contrast, inhibition of the transition from M1 to M2 macrophages in mice negative

for IL-10 resulted in reduced Myogenin expression and fiber growth. These results are supported by the observation that in injured human muscle, M1 macrophages are found close to proliferating myogenic cells and M2 macrophages interact with differentiating myocytes[58]. Treatment of myogenic cells with macrophage-conditioned medium revealed that the effects of M1 and M2 macrophages are also mediated by paracrine signaling[61]. IL1-β, IL-6 and TNF-α secreted by M1 are particularly important to induce proliferative effects on myogenic cells, whereas IL-4 and IGF-1 released by M2 macrophages promote their differentiation[58, 62]. These results are supported by the observation that in injured human muscle, M1 macrophages are found close to proliferating myogenic cells and M2 macrophages interact with differentiating myocytes. Treatment of myogenic cells with macrophage-conditioned medium revealed that the effects of M1 and M2 macrophages are also mediated by paracrine signaling. IL1-b, IL-6 and TNF-α secreted by M1 are particularly important to induce proliferative effects on myogenic cells, whereas IL-4 and IGF-1 released by M2 macrophages promote their differentiation. MPs emerged as "*leading actors*" in various tissue. For instance, MPs play a crucial role in the restoration of skin integrity and homeostasis and exert distinct functions during the multiple phases of skin repair, despite the underlying molecular mechanisms remaining partially unclear [63], Lucas et al. showed that during the early phases of skin repair, infiltrating MPs are alternatively activated and express high levels of growth factors, VEGFα and TGFβ, which

contribute, respectively, to wound angiogenesis and myofibroblasts differentiation[64]. During the midstage of the skin repair response, MPs still express VEGFα and TGFβ but to a lesser extent and they are crucial for vessels stabilization and scar formation. More recently, TGFβ has been described to regulate wound healing through TLR4 receptor. Indeed, $TLR4^{-/-}$ mice display impaired skin wound healing with decreased MPs infiltration and reduced levels of TGFβ.

The plasticity of MPs has been reported to play a role also in parenchymal organ diseases, such as liver or lung fibrosis. Liver fibrosis is a common consequence of chronic liver disease and current evidence suggests that this process is mainly driven by a local inflammatory response[65]. Experimental models of liver fibrosis highlight the importance of hepatic resident macrophages, the Kupffer cells, for sustaining inflammation as well as activating the hepatic stem cells (HSC). However, fibrosis largely depends on recruitment of monocytes into the liver. In a reversible model of liver fibrosis two functionally distinct types of macrophages have been demonstrated to regulate the outcome of the fibrotic response: during the injury phase, infiltrating MPs promote myofibroblasts proliferation and matrix deposition by secreting high amounts of TGFβ and TNF-α, whereas during the recovery phase they sustain matrix degradation, probably by releasing MMP13[66]. Pro-fibrogenic MPs have been shown to derive mainly from circulating Ly-6C high pro-inflammatory monocytes, which massively invade injured liver via the CCR2 receptor both in mice and in humans[67].

Similarly, a critical role of macrophages in regulating lung fibrosis has been recently described. Evidence supports the involvement of alternative activated MPs (M2) in lung fibrotic response via secretion of TGFβ[68, 69]. These results were corroborated by recent observational studies in humans which highlight the presence of M2 macrophages markers in lung diseases: CD163, CCL18, CCL22, and CD206[70].

Returning to skeletal muscle, in chronic diseases several rounds of damage and repair occur: both M1 and M2 polarized macrophages coexist in the tissue, recruited from monocytes. This persistent inflammation leads to fibrosis, fat deposition, and exhaustion of the stem cell pool.

Lemos in 2010 [71] provide new insights into how macrophages influence the fate of FAPs and matrix accumulation in damaged muscle, by tightly regulating the balance of proapoptotic and prosurvival signals driven by TNF and TGFb1, respectively. For successful regeneration, these signals need to appear in two sequential separate waves; in chronic conditions, such as in dystrophic muscle, however, this sequence is lost, leading to timely overlap of TNF and TGFβ1 contradictory signaling. In fact, FAP expansion is preceded by the first wave of infiltrating pro-inflammatory macrophages; in mice deficient in the C–C chemokine receptor type 2, in which macrophage infiltration is reduced after injury, FAPs persisted and accumulated by escaping apoptosis, resulting in fibro/adipogenic tissue accumulation and compromised muscle repair. This finding suggest that inflammatory signals emanating from MPs could regulate survival of FAPs in damaged muscle and, in this way, decide between regeneration and fibrosis in muscle.

Inflammatory myopathies are another class of chronic muscle diseases. They are heterogeneous and classically comprise polymyositis, dermatomyositis (DM), and sporadic inclusion body myositis (IBM)[72, 73]. Interestingly, MPs infiltration is common in all inflammatory myopathies. At present, few data are available concerning the phenotype and the role of MPs in the pathology of inflammatory myopathies. Analyses of muscle biopsies demonstrated that in areas of severe inflammation and necrosis, MPs express both pro-inflammatory and anti-inflammatory markers. Moreover, Reimann et al. demonstrated that the MPs migration inhibitory factor (MIF) is highly expressed in muscle samples of human PM. MIF is a T cell and MPs derived pro-inflammatory cytokine with antiapoptotic, pro-proliferative, and chemotactic effects. In muscle biopsies of PM, MIF has been detected not only in inflammatory cells but also on muscle fiber membrane, thus suggesting a potential role of MIF in the onset of the disease[74].

Finally, Zordan et al. in 2014[75] firstly demonstrated that infiltrating MPs represent a relevant endogenous source of cytokines and angiogenic factors, which support neo-angiogenesis during muscle regeneration by both sustaining the differentiation of endothelial-derived progenitors toward an endothelial fate and by preventing the formation of a fibrotic scar. In pathogenic conditions, when failure of correct MP infiltration occurs, the fate of endothelial-derived progenitors can be skewed to a fibroblastic phenotype, further contributing to an abnormal tissue remodeling. This new finding open our findings open the possibility that ECs in the muscle as well as MPs may represent new targets for

therapeutic intervention for several devastating diseases such dystrophy and other genetic myopathies as well as in aging.

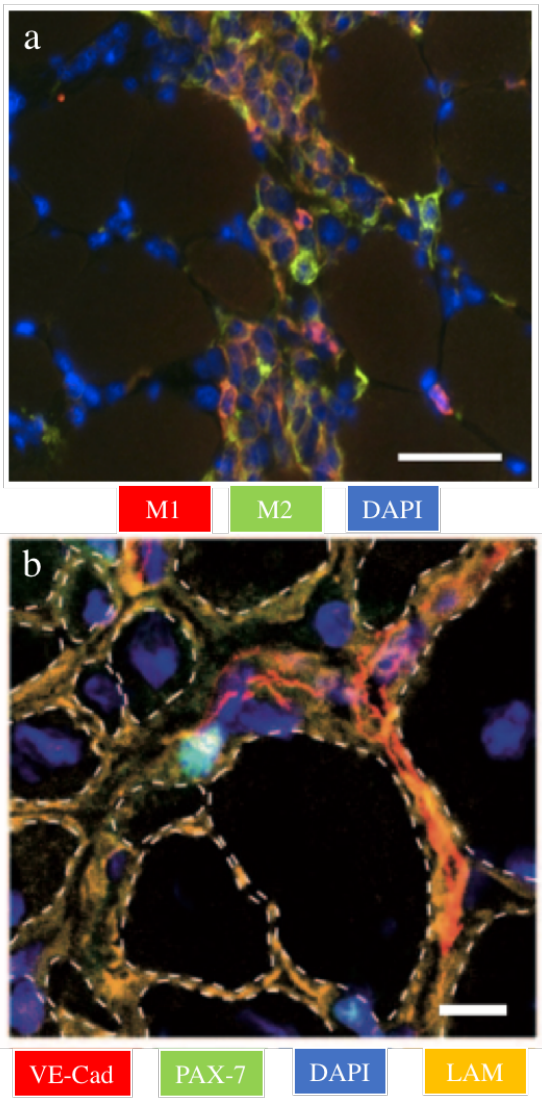

Figure 9. Muscle privileged partners. a) Inflammatory lesion in skeletal muscle shows co-distribution of M1(red) and M2 (green) macrophages. b) VE-Cad (red) positive endothelial cells. ECM is shown in orange and nuclei are labelled with DAPI (blue). SCs are marked with Pax7(green). Scale bar, 10 μm.

Blood vessel: more than a transport network

Blood vessel formation occurs throughout reproductive life as well as during tissue repair or in certain disease conditions, but expansion of the vascular network is essential during development. Most of this vascular growth is mediated by angiogenesis, which involves processes such as endothelial cell (EC) proliferation and sprouting[76, 77]. Subsequent remodeling and blood vessel maturation generate a stable, hierarchically organized, and efficient vascular network devoid of unnecessary connections[76, 78, 79]. The resulting vasculature, which comprises arteries, veins, and interconnecting capillary beds, is optimized to fulfill its conventional and indispensable function in the living organism, namely the transport of gases, nutrients, metabolites, waste products, hormones, and cells

There is now increasing evidence that ECs are not only building blocks of the vascular transport network, but also actively contribute to growth, differentiation, patterning, or repair processes in the surrounding tissue. The paracrine (also termed 'angiocrine') release of signaling molecules by vascular cells, which act on other cell types in proximity of blood vessels, has recently emerged as a fundamental mechanism in many different organ systems. Skeletal muscle is laced with a dense microvasculature, and most quiescent satellite cells are found in close proximity to these vessels [80]. The microvascular bed can be considering a strategic partner of SCs (Fig.9). Endothelial and myogenic cells may derive from common progenitors at development stages, and vascular endothelial progenitor cells are probably essential for muscle

organogenesis[81]. SCs niches are juxtavascular: myogenic cell differentiation is spatiotemporally associated with new vessel formation[82]. Many studies report a high presence and remodeling activity of vascular cells and fibroblasts within 5 to 7 days after acute injury in mice and return to baseline after 28 days. In co-culture experiments, endothelial cells promote the proliferation of satellite cell-derived myoblasts. Reciprocally, differentiating myogenic cells are proangiogenic and increase the formation of capillary-like structures [80]. ECs secrete a variety of mitogenic and/or anti- apoptotic factors, such as VEGF, that influence muscle cells [83, 84]. Intriguingly, differentiating myogenic cells also secrete VEGF and their pro-angiogenic function mainly depends on this factor [83, 84]. This finding suggests an intricate feedforward mechanism through which VEGF in the stem cell niche co-regulates both myogenesis and angiogenesis. In contrast to the predominantly promitotic effects of ECs on myogenic progenitors, cells in the periendothelial position, such as smooth muscle cells and fibrogenic cell types, are crucial for reentry into quiescence on completion of regeneration. SCs transitioning into quiescence increase expression of the Ang1 receptor Tie-2. Forced expression of Ang1 in mouse muscles increases the number of quiescent cells and inhibition of Tie-2 prevents cell-cycle exit on completion of regeneration[85, 86]. Importantly, periendothelial cells seem to be the major source of Ang1 during muscle regeneration. In summary, vessel cells in the stem cell niche coordinate both the acute satellite cell response and the late stages of muscle regeneration, when tissue returns to homeostasis.

1.2. MESENCHYMAL TRANSITION IN DEVELOPMENT AND DESEASE

Endothelial cells (ECs) form an extensive network of blood vessels that has numerous essential functions in the vertebrate body. ECs are emerging as important signalling centers that coordinate regeneration and help to prevent deregulated, disease-promoting processes[87]. The ability of epithelial and ECs to transform into mesenchymal cells is one of the most basic cellular mechanisms in biology. This process, referred to epithelial-mesenchymal transition (EMT) or endothelial-mesenchymal transition (EndMT), regulates various stages of embryonic development and contributes to the progression of a wide array of diseases and in tissue repair[88, 89]. Novel findings regarding the stem cell phenotype generated by EMT and EndMT [90, 91] suggest that they may have therapeutic potential for the treatment of various degenerative diseases.

1.2.1. Endothelial plasticity

ECs can exhibit a wide range of phenotypic variability depending on local physiologic needs throughout the vascular tree (Chi et al, 2003). During embryogenesis, endothelial cells arise from hemangioblasts (a mesodermal progenitor with both endothelial and hematopoietic potential) [92], while smooth muscle cells arise from local mesenchyme and the neural crest [93]. As both vascular cell types originate from different sources, it has long been thought that these cells have distinct

progenitors. However, ten years ago Yamashita et al. described embryonic vascular progenitor cells that differentiate into both endothelial and smooth muscle cells [94]. Later, the results were confirmed by the demonstration that endothelial cells and smooth muscle cells, derived from a single embryonic progenitor, integrate into pre-existing vasculature [95]. A study that used retroviral labelling to analyze somite-derived cells in the chick demonstrated that myogenic and endothelial cells derive from a common somitic progenitor in the limb [81]. A specific subpopulation of cells, the cardiogenic mesoderm, gives rise to the first two types of heart cells, the myocardial and endocardial cells. They latter acquire endothelial markers, like VE-Cadherin, CD31, Tie-1, Tie-2 or VEGFRI/II. Embryonic VE-Cadherin-expressing progenitors (eVE-Cad^{+}), including hemogenic endothelium, have been shown to generate hematopoietic stem cells and a variety of other progenitors, including mesoangioblasts, or MABs (Fig.8, Azzoni et al.)[46] [96].

1.2.2. The endothelial-mesenchymal transition process

Endothelial cells can undergo Mesenchymal transition (EndMT) to gain a different phenotype and acquire invasive and migratory abilities [97]. EndMT is one specific form of epithelial-mesenchymal transition (EMT); similar to EMT, EndMT is a complex biological process in which resident endothelial cells delaminate from the organized layer of cells and invade the underlying tissue. This so-called mesenchymal phenotype can be

characterized by loss of loss cell junctions and endothelial specific markers, such as CD31, and gain of mesenchynmal markers, such as fibroblast specific protein 1 (FSP1, also known as S100A4) or α-smooth muscle actin (α-SMA)[98]. EndMT was first described in embryonic development during the formation of the heart valves. During heart development, cardiogenic mesodermal cells give rise to two types of heart cells, which are the myocardial and endocardial cells. Endocardial cells acquire endothelial markers, such as VE-Cadherin, Tie-1, Tie-2, VEGFRI/II, and PECAM1(CD31). Endocardial/endothelial cells in the atrioventricular (AV) canal were reported to give rise to the mesenchymal heart cushion cells, which form the mesenchymal region of cardiac septa and valves. Similarly, several studies have shown that endocardial–endothelial cells might transdifferentiate into mesenchymal cells during the formation of endocardial cushion tissue in the early embryonic chick heart [99]. Several reports suggest that EndMT may play an important role in embryonic vascular development and formation of intimal thickening [100] and is thought to play a crucial role in stabilizing the neovasculature during vasculogenesis and angiogenesis[101]. More than that, in angiogenesis, EndMT plays an important role in angiogenic sprouting in the early postnatal retina, provides a great number of mesenchymal cells at the tips of the vascular sprouts, and this process can be regulated by vascular endothelial growth factor A (VEGFA)[102]. In the adult, EndMT has been proposed to be an important mechanism in the pathogenesis of many chronic disease, including pulmonary fibrosis, cancer, and in the onset and progression of cerebral cavernous

malformations (CCM)[103-105]. Current evidence indicates that EMT and EndMT are governed by common signaling pathways, including TGF-β and bone morphogenic protein (BMP), Wnt/β-catenin, Notch and Inflammatory cascades (Fig.10). TGF-beta was first shown to induce EndMT in endocardial cells during heart cushion morphogenesis.

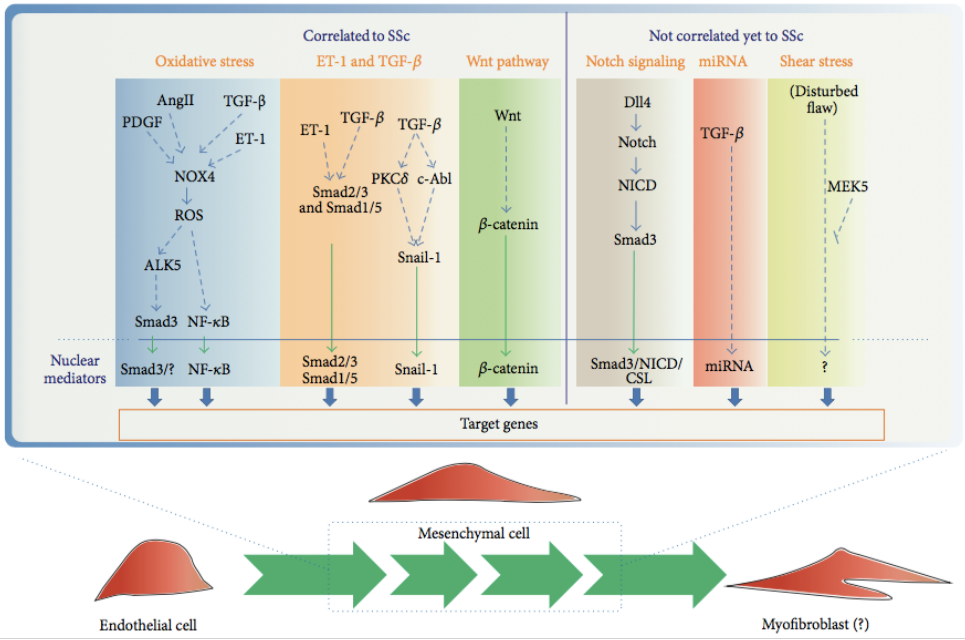

Figure 10. Pathways involved in the EndoMT. The activation of specific nuclear mediator leads to regulation of target genes that are correlated with the increase of mesenchymal markers (such as Col I, α-SMA, and Twist 1) and/or decrease of endothelial markers (CD31, VE-Cad, and Fli-1). The activation of these pathways could lead endothelial cells to acquire initially mesenchymal characteristics and later on to acquire myofibroblastic features. Image from Nicolosi et al, 2016[106]

Genetic ablation of TGF-β, BMP-2, BMP receptor I (Activin receptor type I), ALK2 and b-glycan results in defective heart formation and abolished EndMT in mice. Interestingly, both BMP and TGF-β downstream signaling pathways are up-regulated in a mouse model of CCMs, consisting in the endothelial specific inducible deletion of the Ccm1 or Ccm3 genes. CCM results in malformations of brain blood vessels, which become irregular and leaky, eventually leading to cerebral hemorrhage. Deletion of Ccm3 provokes an increase in b-catenin transcriptional activity, prior to BMP/TGF-β function, that is required for triggering EndMT and acquiring a CCM phenotype. TGF-β/BMP-induced EndMT has been shown to be modulated by Notch signaling: depletion of Ccm1 in ECs results in decreased Notch activity, whereas chemical inhibition of Notch signalling induces BMP-6, thereby promoting EndMT in autocrine manner. Finally, an interplay between TGF-β and Fibroblast Growth Factor (FGF) signaling was described during arterial graft rejection, which is often associated with increased inflammation and reduced FGF receptor (FGFR) expression.

1.2.3. EndMT in human fibrotic disease

Fibrosis represents the final common pathway of many chronic diseases, including systemic sclerosis, IgG4-associated sclerosing, nephrogenic systemic fibrosis, as well as numerous organ-specific syndromes.

Fibrotic disorders collectively affect a very large number of individuals and, due to lack of effective therapeutic approaches, it results in very high

morbidity and mortality rates[107]. Although the etiology of fibrotic disease changes widely, these condition display as a common feature an elevated expression of genes encoding various collagens (type I, III, V and VI) and other extracellular matrix (ECM) proteins. The increased expression of these genes is accompanied by a concomitant reduction in the activity of ECM degrading enzymes, such as Tissue Inhibitors of Metalloproteinases, also known as TIMPS. Recent studies have demonstrated myofibroblast are the cells that ultimately responsible for the severe fibrotic process. Myofibroblast comprise a unique population of mesenchymal cells that express alpha-smooth muscle actin and exhibit a marked pro-fibrotic cellular phenotype. Furthermore, owing to their intrinsic contractile properties and their ability to establish rigid macromolecular stress fiber-like microfilament bundles *in vivo,* myofibroblasts induce changes in the biomechanical properties of the affected tissue causing a progressive increase in tissue stiffness, a newly recognized extremely potent profibrotic stimulus [108-110]. Myofibroblasts originate from various sources including expansion and activation of quiescent resident tissue fibroblasts, migration and tissue accumulation of bone marrow-derived $CD34^+$ fibrocytes, or from the phenotypic transition of epithelial cells. More recent publications, however, have demonstrated that another source of activated myofibroblasts in fibrotic disease are endothelial cells that have acquired a mesenchymal phenotype through EndMT [111]. It is important to emphasize that the transition from ECs into myofibroblast may not need to proceed through complete transdifferentiation and that a partial

transition process may be sufficient for initiation or progression of pathologic fibrogenesis, as discussed by Welch-Reardon[112, 113]. EndMT is a highly dynamic process consisting of multiple steps and end-points, giving rise to a broad range of intermediated phenotypes (including the revers mesenchymal-to endothelial transition, MET) that are therefore difficult to capture and define in human pathological biopsies. Nevertheless, in the current decade lineage tracing experiments developed in mouse models of cancer, cardiac, renal fibrosis, CCMs and more have contributed to highlighting the role of EndMT in such human disease. The results of the numerous studies shown in Table 2 indicate that EndMT plays an important role in the pathogenesis of these common and often fatal disorders. Extensive literature published about the participation of EndMT in fibrotic process suggest that such a role should no longer be considered controversial. Elucidation of the molecular mechanism involved in EndMT may provide novel molecular targets and therapeutic approach to treat different human disease. Additionally, in the future researchers can take advantage of EndMT as part of tissue engineering applications, where ECs might be employed to obtain multipotent mesenchymal cells susceptible of being re-differentiated in a variety of distinct cell types (Fig.11).

Table 2.. Demonstration of EndMT in human fibrotic diseases[105].

Fibrotic Disease	Tissue Source	Method(s)
SSc-associated Pulmonary Fibrosis	Lung Transplants	Immunohistochemistry Immunofluorescence Gene Expression
Radiation-induced Pulmonary Fibrosis	Lung Tissue	Immunofluorescence
SSC-associated Pulmonary Hypertension	Lung Biopsies	Immunofluorescence
Idiopathic Pulmonary Hypertension	Lung transplant	Immunofluorescence Transmission Electron Microscopy Immunoelectron Microscopy
Cardiac Fibrosis	Heart Transplant	Gene Expression
Chronic kidney disease-associated cardiac fibrosis	Heart Tissue (Autopsies)	Immunohistochemistry Gene Expression
Diabetic kidney disease-associated renal fibrosis	Kidney biopsies	Immunohistochemistry
Idiopathic Portal Hypertension Intestinal Fibrosis Radiation-induced Rectal Fibrosis	Liver biopsies Colonic Mucosa Rectal tissues (Surgery)	Immunohistochemistry Immunofluorescence

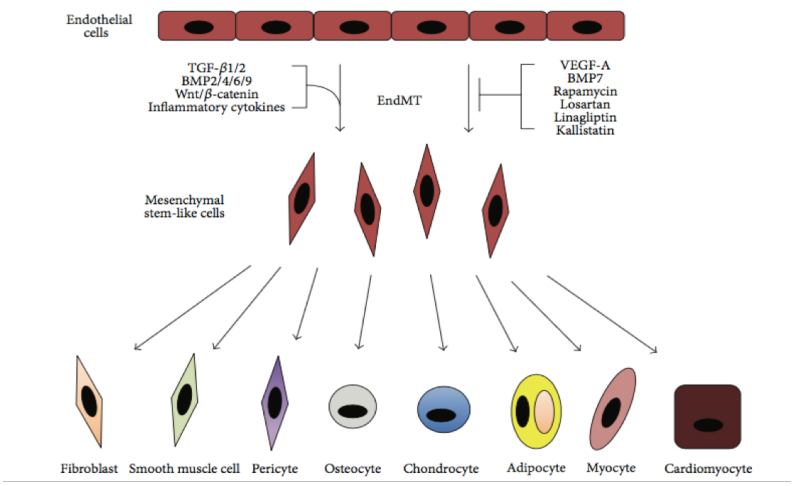

Figure 11: The multipotency of EndMT. Vascular endothelial cells are stimulated to undergo EndMT by various growth factors and inflammatory cytokines such as TGF-β s, BMPs, and Wnt. Endothelial-derived mesenchymal cells take on the properties of multipotent stem cells and can differentiate into fibroblasts, pericytes, smooth muscle, skeletal muscle, cardiac muscle, bone, cartilage, and fat cells. Image from Medici et al, 2016[114]

1.2.4. Bone in muscle: the issue of cellular origin

The multipotency of EndMT supports the idea that, upon appropriate signals and growth factor, endothelial-derived mesenchymal cells can generate various types of connective tissue other than fibroblasts (Fig.11). Vascular cells are also part of stem cell niches and have key role in hematopoiesis, neurogenesis and bone formation[114].

Extraskeletal osteogenesis, often in muscles, is a rare and inexplicable event with serious and clinical consequences. Also defined as Heterotopic Ossification (HO), broadly it describes the formation of endochondral bone in tendons, ligaments and other soft tissue[115]. HO is a debilitating complication of fractures, joint replacement surgery, and neurological trauma, suggesting a process of disordered injury repair, although is unclear why these specific tissue environments foster the formation of ectopic bone (Fig.12). Maybe the most famous cases of HO can be found in patients with fibrodysplasia ossificans progressiva (FOP), a genetic disorder in which patients suffered periodic episodes of acute inflammatory processes that cause tumor-like swellings in muscle tissue (flare-ups), which lead to the formation of cartilage and bone in the inflamed region. Most patients with FOP are confined to a wheelchair by the third decade of life, and require lifelong assistance in performing activities of daily living[116, 117]. The median age of survival is approximately 40 years, and death often results from complications of thoracic insufficiency syndrome. The rarity, variable severity and episodic clinical course of FOP pose substantial uncertainties when

evaluating experimental therapies. Surgical release of joint contractures is generally unsuccessful and risks new, trauma- induced HO. Osteotomy of heterotopic bone or surgical removal of heterotopic bone to mobilize joints is generally counterproductive because additional HO develops at the operative site[118]. FOP arises from gain-of-function mutations in bone morphogenetic proteins (BMP) type I receptor ACVR1 (ALK2), with about 97% of individuals harboring a classic $ACVR1^{R206H}$ variant [119, 120]. This FOP mutation causes an arginine to histidine change in aminoacid 206 (R206H) in the ALK2 protein.

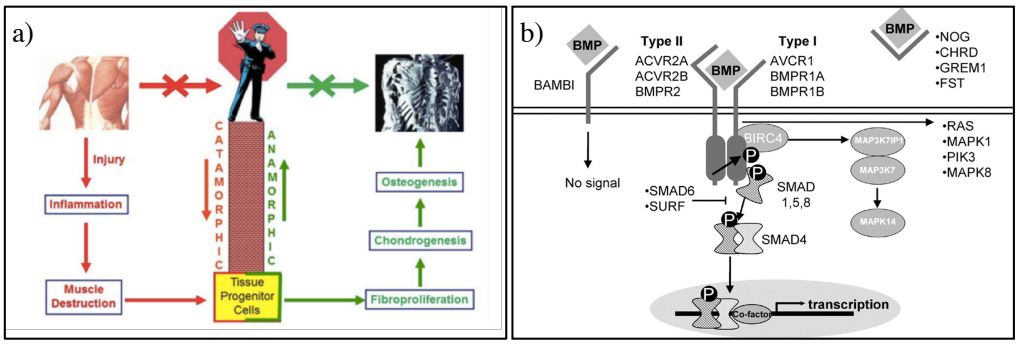

Figure 12.(a) **Schematic Diagram of Skeletal Metamorphosis in FOP and (b) BMP signalling pathway** In FOP, skeletal muscle tissue is transformed into heterotopic bone through a process of skeletal metamorphosis. The process of HEO in FOP involves two major phases - a catamorphic phase (tissue destruction; left column) followed by an anamorphic phase (tissue formation; right column) of transient fibroproliferative and cartilaginous scaffolds, and their replacement with mature heterotopic bone.

Using genetically engineered mice harboring this variant, it is demonstrated that $ACVR1^{R206H}$ drives HO in FOP by conferring aberrant activation of the BMP signaling pathway [121], a signaling defect also observed in mesenchymal stem cells from patient-derived induced stem cells (iPSCs). Because maladaptive BMP/activin/TGF-β family ligand signaling may be a shared property of both genetic and acquired forms of HO [122-124], it has been suggested that FOP and HO might be mediated by common effector and progenitor cells. However, the identity and niche of these progenitors as well as their mechanistic relationship have yet to be determined. The logical candidate would be local tissue mesenchymal stem cells or some other progenitors recruited to the lesions from bone marrow. However, bone marrow transplant studies have been shown to be ineffective in perturbing HO in mice. Other approach sought to identify cell population contributing to HO lesions via immune histology or genetic marking techniques in animal models of HO caused by exogenous BMP ligands. Recently, Dey et al[115] discover two distinct tissue-resident progenitor lineages that drive muscle versus tendon and ligament HO: a $Mx1^{+}$ interstitial lineage in muscle that give rise to injury-dependent intramuscular HO and Scx^{+} lineage participate to apparently spontaneous HO of tendons and ligaments. In contrast to previous approach, they identified cell lineages that are sufficient to initiate HO, resulting from the cell-autonomous effects of ACVR1 mutations. To summarize, different approaches showed candidates with ossification potential: it's uneasy to think that only one-two cell population are recruited to develop a mature and stable extra-skeletal tissue. Probably,

several cell types acquired unusual ability, morphology and contribute the new formation due to altered signals and microenvironments. The endothelial involvement to HO is still unclear: in 2010, Medici suggest that vascular cells emerged as the leading candidate for the cellular origin of heterotopic cartilage and bone[125, 126]. Approximately 50% of the cartilage and bone cells found in lesions appear to be of endothelial origin based on Tie2-Cre lineage tracing and expression of various endothelial markers (Tie1, Tie2, vWF, VE-Cadherin) in these cells. On the other hands, osteoblast and chondrocytes from normal bone or cartilage tissue do not express these endothelial markers[127]. Moreover, Tie-2 marker can be used to identify also a hematopoietic lineage of proangiogenic monocytes and pericyte progenitors. EndMT, until some years ago, was thought to be a process where fibroblast phenotype was the ultimate fate of these cells. However, new studies have shown that EndMT represents a dedifferentiation of endothelial cells to a stem cell phenotype, which can subsequently re-differentiate into bone, cartilage or fat cells (Fig.12). In addition, overexpressing ALK2 carrying the FOP mutation induces EndMT in cultured mature vascular cells [126]. The endothelial cells dramatically changed their morphology from round to elongated, splinde-shaped cells, showed reduced expression of endothelial markers and gained expression of mesenchymal markers. Notably, in the early stages of HO, a mesenchymal condensation forms in the lesions prior to chondrogenesis and endochondral ossification. In future, targeting of molecules responsible for inducing EndMT may prove beneficial for

preventing the formation of ectopic bone in patients with FOP or inflammation-induced HO.

The identification of HO-driving progenitors and pathways has implications for the development of therapy. Current management is focused on early diagnosis, avoidance of injury or iatrogenic harm, symptomatic amelioration of painful flare-ups, and optimization of residual function[128]. In 2016, Cappato et al[129] developed a high-throughtput screening (HTS) assay to identify inhibitors of ACVR1 promoter among drugs already approved for the therapy of other diseases. This drug repositioning strategy has led to the identification of dipirydamole as a possible pharmacological inhibitors of HO (see Chapter IV).

1.3. Scope of the thesis

The final goal of this project is to define the key signals involved in the crosstalk between macrophages and endothelial progenitors and their relevance in muscle development and diseases. We have indeed shown how the perturbation of this signaling triggers the phenomenon of endothelial to mesenchymal transition (EndMT): this can lead to impaired angiogenesis and the exacerbation of fibrosis, which represents a major obstacle for the success of other ongoing preclinical therapies for muscle genetic disease.

Specific aims are:

1) To explore the effects of nitric oxide on endothelial progenitors during skeletal muscle development;

2) To characterize polarized macrophages and endothelial progenitors during muscle regeneration and verify the activation of specific pathways and signaling molecules;

3) To clarify the role of $VeCad^{+}$ cells in a mouse model of heterotopic ossification and define new therapeutic tools for the treatment of related diseases.

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CHAPTER II:

Nitric Oxide Donor Molsidomine Positively Modulates Myogenic Differentiation of Embryonic Endothelial Progenitors.

PLOS ONE

RESEARCH ARTICLE

Nitric Oxide Donor Molsidomine Positively Modulates Myogenic Differentiation of Embryonic Endothelial Progenitors

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Abstract

Embryonic VE-Cadherin-expressing progenitors (eVE-Cad$^+$), including hemogenic endothelium, have been shown to generate hematopoietic stem cells and a variety of other progenitors, including mesoangioblasts, or MABs. MABs are vessel-associated progenitors with multilineage mesodermal differentiation potential that can physiologically contribute to skeletal muscle development and regeneration, and have been used in an *ex vivo* cell therapy setting for the treatment of muscular dystrophy. There is currently a therapeutic need for molecules that could improve the efficacy of cell therapy protocols; one such good candidate is nitric oxide. Several studies in animal models of muscle dystrophy have demonstrated that nitric oxide donors provide several beneficial effects, including modulation of the activity of endogenous cell populations involved in muscle repair and the delay of muscle degeneration. Here we used a genetic lineage tracing approach to investigate whether the therapeutic effect of nitric oxide in muscle repair could derive from an improvement in the myogenic differentiation of eVE-Cad$^+$ progenitors during embryogenesis. We show that early *in vivo* treatment with the nitric oxide donor molsidomine enhances eVE-Cad$^+$ contribution to embryonic and fetal myogenesis, and that this effect could originate from a modulation of the properties of yolk sac hemogenic endothelium.

Introduction

Over the last years, the existence of different stem or progenitor cells with myogenic potential has been widely explored. In addition to the typical skeletal muscle progenitors, the satellite cells, many other multipotent and embryologically unrelated progenitors bearing potential roles in muscle differentiation and tissue repair have been identified [1]. In particular, a population of progenitor cells named mesoangioblasts (MABs) has been identified in the embryonic dorsal aorta [2]. They express markers of hemangioblastic, hematopoietic, endothelial and

mesodermal lineages, and exhibit self-renewal properties and mesodermal differentiation capabilities both *in vitro* and *in vivo* [2, 3].

Using a Cre-loxP based genetic lineage tracing system, we have shown that the hemogenic endothelium in the mouse embryo can undergo mesenchymal transition and is the source of $CD45^+$ progenitor cells. These are distinct from embryonic MΦs and can give rise both to hematopoietic cells and mesenchymal progenitor cells. The latter bear characteristics of embryonic MABs and are able to physiologically contribute to different mesodermal lineages in the embryo, including the skeletal muscle [4].

The ability of MABS to be easily isolated, to differentiate *in vitro* and *in vivo* into skeletal muscle, and to cross the vessel walls when transplanted [2, 5], has prompted their use in exogenous cell therapy approaches for muscle degenerative diseases, in particular in models of muscular dystrophies (MDs). MDs are a heterogeneous group of genetic diseases, characterized by a progressive and irreversible degeneration of skeletal muscle with the most severe cases leading to progressive paralysis and death. MABs have been successful in cell transplantation protocols in dystrophic animals [6–9] thus leading to an ongoing clinical trial for human Duchenne's muscular dystrophy (DMD) patients using the human counterparts of MABs [10]. However, although encouraging, this cell therapy approach is not currently able to fully repair the structural organization and restore the function of the dystrophic muscle. Additional limitations include the high cost and the requirement to tailor the therapy for each patient given the current state-of-the-art. An alternative therapeutical approach to the cell transplantation involves endogenous stem cells which are activated following injury, but in the case of chronic degenerative disease undergo a quick exhaustion. Therefore, an optimal intervention would require the activation of endogenous myogenic stem cells and their expansion and maintenance by molecules acting on specific signaling pathways.

Several growth factors and cytokines have been shown to activate resident mesodermal or circulating stem cells. The observation that pathophysiological features of MDs are associated to an abnormal production of nitric oxide (NO) [11] has prompted studies focusing on the role of NO in muscle development and regeneration and its potential use as a therapeutic agent, either alone [12–16] or in combination with nonsteroidal anti-inflammatory (NSAID) drugs or MAB-based cell therapies [6, 17, 18].

One specific NO donor, molsidomine, was shown to slow disease progression in the absence of NSAIDs and to re-establish the functional capability of the damaged muscle, considerably ameliorating its motor activity [12]. Molsidomine treatment *per se* was able to effectively modulate the features of the inflammatory cells that infiltrate the dystrophic muscles, reducing the fibrotic scar tissue and enhancing its healing function [19] and regulating fibro-adipogenic precursor differentiation [13]. Furthermore, the beneficial effect of molsidomine could be explained by its ability to enhance the self-renewal capacity of satellite cells, thus counteracting the impoverishment of the satellite cells pool [12]. Our group has demonstrated that molsidomine has a favourable impact on embryonic myogenesis in alpha-sarcoglycan (α-SG) null mice by increasing the number of myogenic stem cells [12]. This early effect could be of great importance since it has been shown that in dystrophic muscles, stem cell depletion begins during late embryonic life [20] and experimental treatments in animal models at perinatal stages led to a significant amelioration of the dystrophic phenotype [21]. A better understanding of NO effect on different embryonic progenitors and on the molecular pathways downstream NO signaling in these cells would pave the way to design novel therapies, suitable for treating already in the early stages of the disease and could improve the outcome of other therapeutic strategies at later stages.

Here we investigated whether the positive effect of molsidomine on foetal myogenesis could arise from a modulation of the fate of endogenous embryonic MABs.

Materials and Methods

Animals and Treatment

Mice were housed in the SPF animal facility at our Institute and treated with the approval of San Raffaele Scientific Institutional Animal Care and Use Committee (IACUC 489, 663) in accordance with the Italian law and to the European Community guidelines. Pregnant females were euthanised (CO_2 induced narcosis prior to cervical dislocation) prior to embryos recovery in accordance with the European Community guidelines and with the approval of San Raffaele Scientific Institutional Animal Care and Use Committee (IACUC 489, 663) in accordance with the Italian law.

The transgenic mice that have been used in this study are: Cdh5-CRE^{ERT2} [22]; R26R [23]; R26R-EYFP [24]. Mice were kept as heterozygous and were genotyped as in [4].

Cre recombination was induced by injecting 2mg/25g body weight of Tamoxifen (TAM) (T5648, Sigma-Aldrich, Saint Louis, MO, USA; 10 mg/ml in corn oil) intra-peritoneally (IP) into pregnant females. Staging of early embryos (E9.5) was performed by counting the pairs of somites (E9.5: 18-26sp). For E12.5 and E15.5 embryos we considered that fertilization took place at 6 a.m.

Standard diet (STD) or a diet containing 3 mg/kg of (1-ethoxy-N-(3-morpholino-5-oxadiazol-3-iumyl)-methanimidate (molsidomine) was set based on the daily food intake measured for these animals as in [12]. The experimental groups did not display significant differences in food intake and weight gain.

Embryos and foetuses

For histological analysis, dissected E12.5-E15.5 embryos were fixed for 2–3 hours with a 4% solution of paraformaldehyde (PFA) in PBS at 4°C. After that, embryos were washed in PBS and dehydrated/cryoprotected with passages in PBS solutions with increasing sucrose concentration (10% for 1 hour, 20% for 1 hour, 30% overnight). Embryos were subsequently embedded in OCT and sectioned using a Leica 1850UV cryostat (8μM sections were made).

Immunofluorescence and antibodies

Immunofluorescence on frozen section was carried out as in [4]. The antibodies used are listed in S1 Table. A minimum of 6 embryos were analyzed for each condition. Histological quantifications were done by counting 20 fields (20x and/or 40x) for each data point.

Flow cytometry

eVE-Cad^{+} derived cells were handled as in [4]. Cell sorting and FACS analysis were performed using the MoFLo XDP system (Beckman Coulter, Inc., Brea, CA, US) and LSR Fortessa or FacsCANTO (BD Bioscience, Bedford, MA, USA) respectively. For E9.5 embryos 6–10 embryos and yolk sacs (YS) were pooled for each experiment. For E12.5 embryos 2 embryos were pooled for each experiment. Doublets were excluded by gating on physical parameters; dead cells were gated out based on Hoechst 33258 uptake (H3569, Invitrogen, Carlsbad, CA, USA) or 7-Aminoactinomycin D (A9400, Sigma-Aldrich, Saint Louis, MO, USA). We used fluorescence minus one (FMO) controls and single stain controls to set the position of the gates. Isotype and FMO controls are shown in S1 Fig. Data were analyzed using FlowJo software (TreeStar). The antibodies used are listed in S2 Table.

Quantitative Real-Time PCR

EYFP$^+$ cells sorted from E9.5 or E12.5 embryos were processed using ReliaPrep™ RNA Cell Miniprep System (Z6011, Promega, Milan, Italy). Reverse transcription (RT) was done using the High-Capacity cDNA Reverse Transcription Kit (4368814, Applied Biosystems, Foster, CA, USA). qRT-PCR analysis was carried out using LightCycler 480 (Roche, Basel, Switzerland) or the 7900HT FAST (Applied Biosystems, Foster, CA, USA) Real-Time PCR detection systems. cDNAs were amplified using the GoTaq qPCR Master Mix and the Hot Start Polymerase (A6001, Promega, Milan, Italy). Primer sequences are listed in S3 Table. CT values greater than 35 were considered as negative. Data points were analyzed in triplicate. Quantification was performed using the comparative C_T method. Internal controls: 28S or cyclophilin A.

Gene expression profiling

For gene expression profiling analyses, sorted EYFP$^+$ cells from E9.5-E10.5 Cdh5-CreER^{T2}; R26R-EYFP embryos were processed using ReliaPrep™ RNA Cell Miniprep System (Z6011, Promega, Milan, Italy). cDNA was prepared using RT^2 First Strand Kit (330401 Qiagen, Hilden, Germany). Analyses were done using the Mouse Stem Cell Signaling RT2 Profiler™ PCR Arrays (PAMM-047Z, Qiagen, Hilden, Germany) according to manufacturer's instructions.

Image acquisition and manipulation

Fluorescent images were taken using the following microscopes: Leica TCS SP2 Laser Scanning Confocal or Zeiss LSM 710 Confocal Microscope.

Images were processed using Adobe Photoshop CS6 and Adobe Illustrator CS6.

Statistical analysis

Data were analyzed with Microsoft Excel 14.1.0 and GraphPad Prism 6 and was plotted as mean ± SD or mean ± SEM. To evaluate statistical significance unpaired two-tailed Student's *t*-tests were used assuming equal variance.

Results

Molsidomine treatment affects the specification of VE-Cad$^+$ derived progenitors

To study the effect of nitric oxide on embryonic endothelial derived progenitors, we exploited a transgenic mouse line expressing a tamoxifen inducible (CRE-ER^{T2}) Cre recombinase under the control of VE-Cadherin regulatory sequences, Cdh5-Cre^{ERT2} [4]. These mice were crossed with R26R-EYFP Cre reporter mice in order to obtain double transgenic embryos. We induced Cre recombination by injecting tamoxifen (TAM) IP into the pregnant mother at E8.5, to genetically label VE-Cad expressing cells and their progeny with EYFP. Efficiency and specificity of the recombination were consistent with our previous reports [4].

The NO-releasing drug molsidomine was administered at the beginning of the pregnancy and the treatment continued until the recovery of the embryos/fetuses as in [12]. Control embryos were collected from mice fed with a standard diet.

Firstly, we evaluated by FACS analysis the effect of molsidomine treatment on the total number of embryonic endothelial progenitors (eVE-Cad$^+$). We prepared single cell suspensions from embryos collected at E12.5, a time at which we previously showed that EYFP$^+$ labeled cells start to become heterogeneous and when MAB-like cells can be found abundantly in the mesenchyme [4].

We observed that the percentage of EYFP$^+$ cells on the total cell population was not significantly changed by molsidomine treatment (1.4±0.2% in CTRL embryos vs 1.5±0.3% in molsidomine embryos) (Fig 1A and 1B). This result indicates that the treatment with a nitric oxide donor drug does not induce the expansion of embryonic endothelial progenitors and/or their progeny.

Concomitantly with this analysis, we evaluated the effect of molsidomine treatment on the distribution of EYFP$^+$ cells in the two main subpopulations at this developmental stage: the endothelial (CD31$^+$CD45$^-$) and the hematopoietic (CD45$^+$CD31$^-$).

We observed no significant difference in the fraction of CD31$^+$ CD45$^-$ cells within the EYFP$^+$ population (52±4.8% vs 54.5±5.7% in CTRL and molsidomine embryos, respectively) after molsidomine treatment. The EYFP$^+$ CD45$^+$CD31$^-$ hematopoietic subset was also not significantly changed (16.1±5.1% in treated embryos vs 13.9±1.6% in control embryos) (Fig 1C and 1D). However, in molsidomine treated embryos we detected CD31$^+$ cells also expressing CD45$^+$, a population that we did not observe in control animals (5.4±1.7% in molsidomine embryos vs 0.6±0.3 in control embryos, $p<0.05\%$) (Fig 1C and 1D).

We also determined the proportions of the macrophage (F4/80$^+$) and non-macrophage (non-MΦ F4/80$^-$) subsets in the hematopoietic population, since in our previous work we showed that, amongst the EYFP$^+$ population, mesoangioblast-like cells belonged to the CD31$^-$ CD45$^+$ F4/80$^-$ subpopulation [4].

In molsidomine treated embryos we observed an increased trend in the number of CD45$^+$F4/80$^-$ cells within the EYFP$^+$ population (14.7±5.6% in treated embyos vs 8±1.9% in control embryos) and a corresponding significant decrease in the number of MΦ CD45$^+$F4/80$^+$ (12.4±1.8% in control embryos vs 3.3±0.7% in molsidomine treated embryos, $p<0.01$) (Fig 1E). This increase was even more evident by analyzing the percentage of the non-MΦ F4/80$^-$ inside the CD45$^+$ population, higher in molsidomine treated embryos (77.7±7.3% vs 38.9 ±3.4% in control embryos, $p<0.01$) (Fig 1F).

Molsidomine treatment increases the myogenic differentiation of eVE-Cad$^+$ progenitor cells

We have previously demonstrated that cells expressing VE-Cad at E8.5 and/or their progeny physiologically contribute to the myogenic lineage without differentiating through a somitic intermediate [4].

We therefore examined how molsidomine influences the contribution of eVE-Cad$^+$ derived progenitors to the development of the skeletal muscle at two different timepoints, E12.5 and E15.5, corresponding respectively to the times of establishment of embryonic and foetal myogenesis [25].

We first evaluated by immunofluorescence analysis the distribution of endothelial EYFP$^+$ CD31$^+$ cells and the extension of the endothelial network in control and molsidomine treated embryos. At E12.5 we observed no changes in vascularity and the distribution of EYFP$^+$ CD31$^+$ cells was comparable in the control and treated embryos (Fig 2A and 2B). This was also the case at E15.5 (Fig 2A and 2B). To further verify this, we performed a transcriptional analysis of eVE-Cad$^+$ derived cells at E12.5, to evaluate how and to what extent the treatment with molsidomine alters endothelial gene expression.

We extracted RNA of EYFP$^+$ cells sorted from E12.5 molsidomine-treated and control embryos (using the same gating strategy depicted in Fig 1A and in [4] and we performed qRT-PCR analysis evaluating the expression of a panel of endothelial genes (Fig 2C). Consistently with the immunofluorescence analysis, all of these genes (CD31, CD34, VE-Cadherin) were expressed at similar levels in control and treated embryos. FACS analysis using antibodies

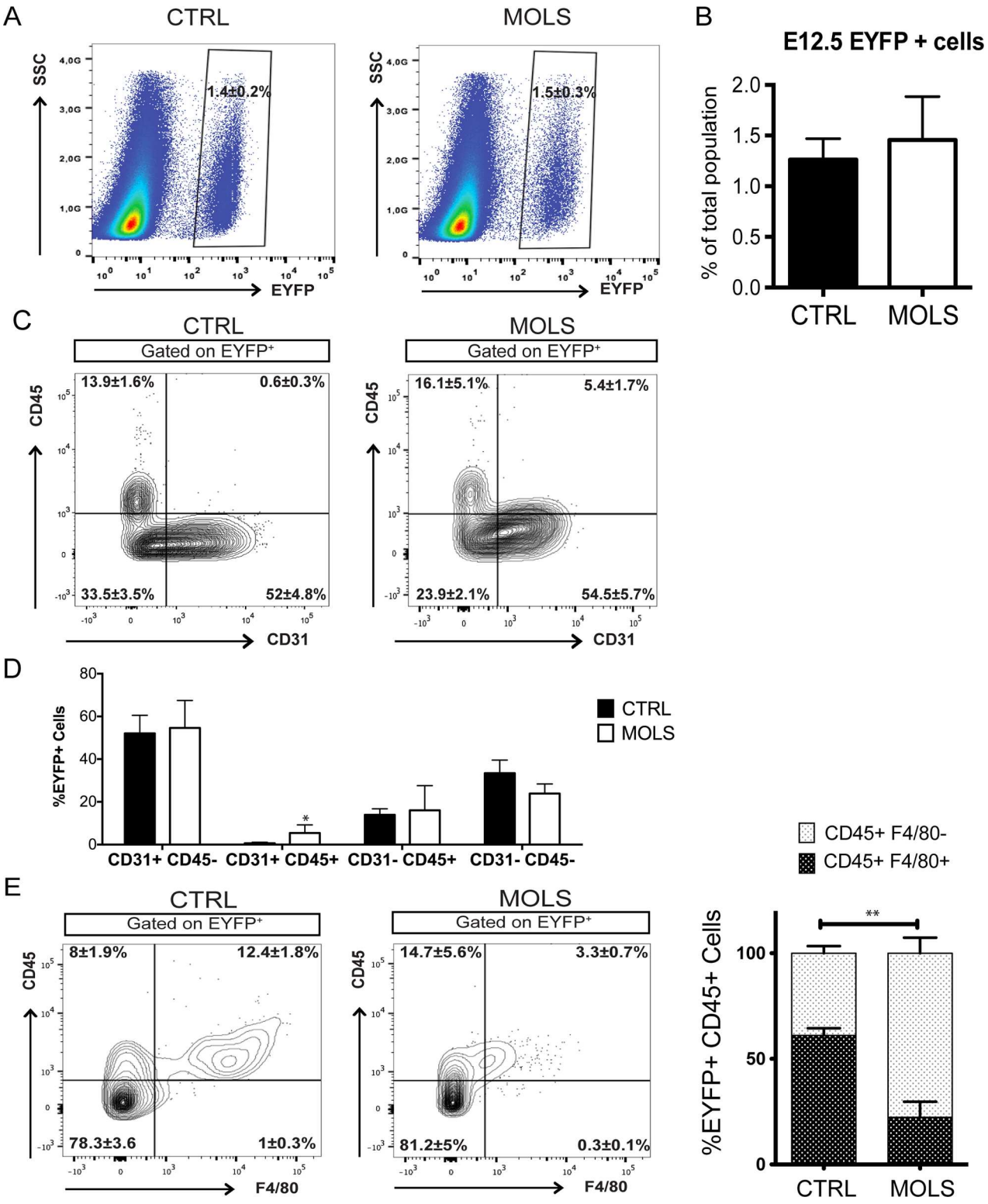

Fig 1. Effect of molsidomine treatment during embryogenesis on eVE-Cad$^+$ derived cells. A) EYFP$^+$ gating strategy used for FACS analysis of single cells suspensions from E12.5 Cdh5-CREERT2; R26R-EYFP embryos (minus the head and fetal liver) B) Graph representing the percentage of EYFP$^+$ cells obtained by FACS analysis of single cell suspensions from E12.5 embryos (At least 5 embryos per group, n = 3 independent experiments). Data are represented as mean ± S.E.M. C) FACS analysis of E12.5 embryos cells showing the expression of CD45 and CD31 within the EYFP$^+$ subset. D) Graph summarizing the percentage of the different populations

shown in (C), obtained with at least 4 control and 5 molsidomine-treated embryos, n = 3 independent experiments. Data are expressed as mean ± S.E.M; *p<0.05 (MOLS vs CTRL). E) FACS analysis on E12.5 embryos showing the expression of CD45 and F4/80 within the EYFP$^+$ subset. F) Graph summarizing the percentage of F4/80$^+$ cells within the EYFP$^+$CD45$^+$ subset obtained with 4 control and 5 molsidomine-treated embryos, n = 3 independent experiments. Data are expressed as mean ± S.E.M; **p<0.01 (MOLS vs CTRL).

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specific for CD31, CD34, VE-Cadherin (Fig 2D and S2 Fig) confirmed these results, suggesting that NO does not significantly alter endothelial differentiation of eVE-Cad$^+$-derived progenitors.

We next examined the contribution of eVE-Cad$^+$ progenitors to embryonic and foetal myogenesis. We first evaluated the distribution of EYFP$^+$ cells expressing the myogenic determination marker MyoD (Fig 3A). At E12.5 we could detect an increase in the number of EYFP$^+$ myoblasts (EYFP$^+$ MyoD$^+$ cells) in sections of molsidomine treated embryos compared to control ones (Fig 3A and 3B). This was in agreement with the qRT-PCR analysis of EYFP$^+$ cells sorted from E12.5 molsidomine-treated and control embryos showing that in EYFP$^+$ cells from molsidomine-treated embryos MyoD expression was upregulated (Fig 3C). Moreover, Pax3, Desmin and eMHC, other markers of embryonic and foetal myogenesis, were also upregulated in EYFP$^+$ cells derived from molsidomine treated embryos.

We then evaluated by immunofluorescence the expression of MyHC, a marker of differentiated myogenic cells (using the antibody MF20). At both E12.5 and E15.5, we could detect an increase in the number of EYFP$^+$ myoblasts (MyHC$^+$ cells) and myotubes in sections of molsidomine treated embryos compared to control ones (Fig 4A–4C). At E12.5, the number of labeled myoblasts/myotubes was approximately 1.5 times higher in molsidomine treated embryos compared to control, while at E15.5 this increase reached almost 4-fold in treated embryos (Fig 4B and 4C).

Therefore, we could conclude that molsidomine treatment increases eVE-Cad$^+$ derived contribution to skeletal muscle development.

Molsidomine treatment modulates the properties of VE-Cad$^+$ hemogenic endothelium

We have previously demonstrated that embryonic MABs originate from a population of cells with mesodermal potency co-expressing hematopoietic and mesodermal markers and arising from extraembryonic VE-Cad$^+$ hemogenic endothelial cells in both YS and placenta [4]. The emergence of these cells from extraembryonic hemogenic endothelium occurs in a very limited temporal window, indicating that extra-embryonic and embryonic hemogenic endothelia have a different timing of activity and possibly distinct biological characteristics, implying diverse responsiveness to distinct molecules and signaling pathways.

To investigate differences between EYFP$^+$ populations labeled in different endothelial compartments, which include hemogenic endothelium, we first profiled eVE-Cad$^+$ derived cells from YS and embryo proper in basal conditions by gene expression analysis, focusing on a panel of genes mainly involved in stem cell-related signaling pathways.

We sorted EYFP$^+$ cells from E9.5 embryo proper and YS with Cre induction at E8.5. From previous data [4] the majority of these cells are endothelial cells (ECs) (Fig 5A). By comparing the gene expression profile of eVE-Cad$^+$ derived cells from embryos or YS at E9.5 we found that groups of genes involved in the FGF, Wnt, Hedgehog and Notch signaling pathways had a higher expression in embryonic compared to YS eVE-Cad$^+$derived cells (Fig 5B). In particular, Fzd3 and Notch3 were significantly increased in the embryo proper. Of those supergroups, only one gene (*Fzd8*) was more expressed in YS cells. Amongst TGF-β related genes, only Acvr2b was more expressed in the embryo proper, while the others, in particular Tgfbrap1,

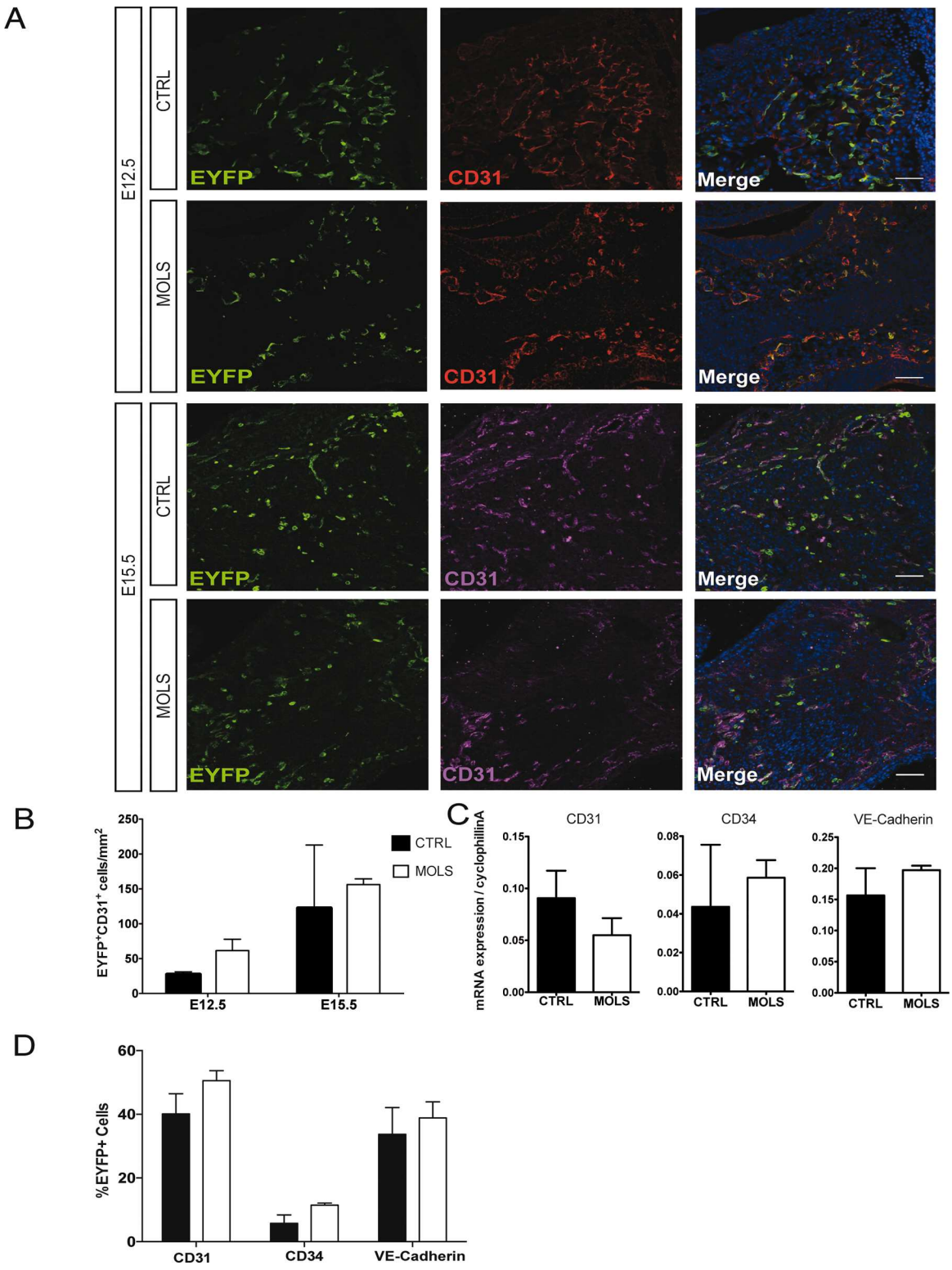

Fig 2. Molsidomine treatment during embryonic development does not affect eve-cad^{+} derived cells contribution to angiogenesis. A) Immunofluorescence (IF) using anti-EYFP and anti-CD31-specific antibodies on transverse sections of Cdh5-CREERT2;R26R-EYFP E12.5 embryos and E15.5 fetuses, untreated (CTRL) and treated with molsidomine (MOLS). Nuclei are stained with Hoechst. Scale bars: 50 um. B) Graph represents the quantification of the number of EYFP^{+}/CD31^{+} cells in the embryo, performed by counting cells in at least 20 fields in different areas (20x and/or 40x) for each data point.

Data are expressed as mean± S.E.M. (n = 6 embryos). C) qRT-PCR analysis on EYFP$^+$ cells freshly sorted at E12.5. Gene expression data are relative to cyclophilin A. D) Graph summarizing the percentage of CD31$^+$, CD34$^+$, VE-Cad$^+$ cells within the EYFP$^+$ populations obtained with 6 control and 5 molsidomine-treated embryos, n = 3 independent experiments. Data are expressed as mean± S.E.M. (n = 4 embryos per group).

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were upregulated in the YS at E9.5. These data indicate that E9.5 YS ECs are distinct from the endothelium in the embryo proper, possibly reflecting functional differences.

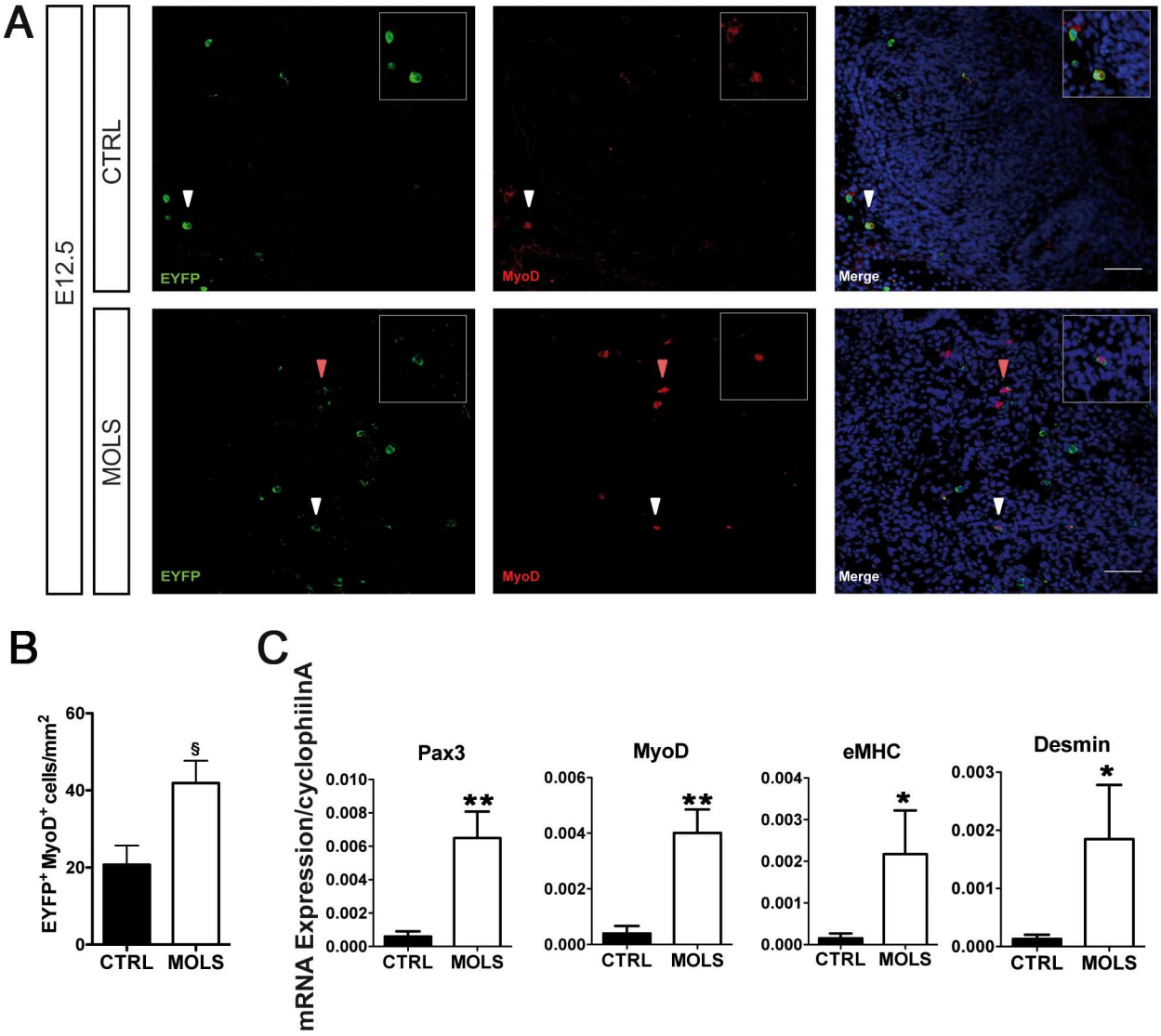

Fig 3. Molsidomine treatment during embryonic development increases eve-cad$^+$ derived cells contribution to early myogenesis. A) Immunofluorescence (IF) using anti-EYFP and anti-MyoD-specific antibodies on transverse sections of Cdh5-CREERT2;R26R-EYFP E12.5 embryos, untreated (CTRL) and treated with molsidomine (MOLS). Nuclei were stained with Hoechst. Arrowheads indicate EYFP$^+$ MyoD$^+$ single-nucleated myoblasts. Inset in panels represent a 5x magnification of the cells indicated by the white arrowhead. Scale bars: 50 um; B) Graph representing the quantification of the number of EYFP$^+$ MyoD$^+$, cells in the embryo, performed by counting cells in at least 20 fields in different areas (20x and/or 40x) for each data point. Data are expressed as mean± S.E.M. §p = 0,06; MOLS vs CTRL (n = 4 embryos per group). C) qRT-PCR analysis of EYFP$^+$ cells freshly sorted at E12.5 using primers specific for Pax3, MyoD, Desmin and MyHC. Gene expression data are relative to cyclophilin A. Data are expressed as mean± S.E.M. **p≤0,01;*p≤0,05, MOLS vs CTRL (At least 6 embryos per group, n = 3 independent sorting experiments)

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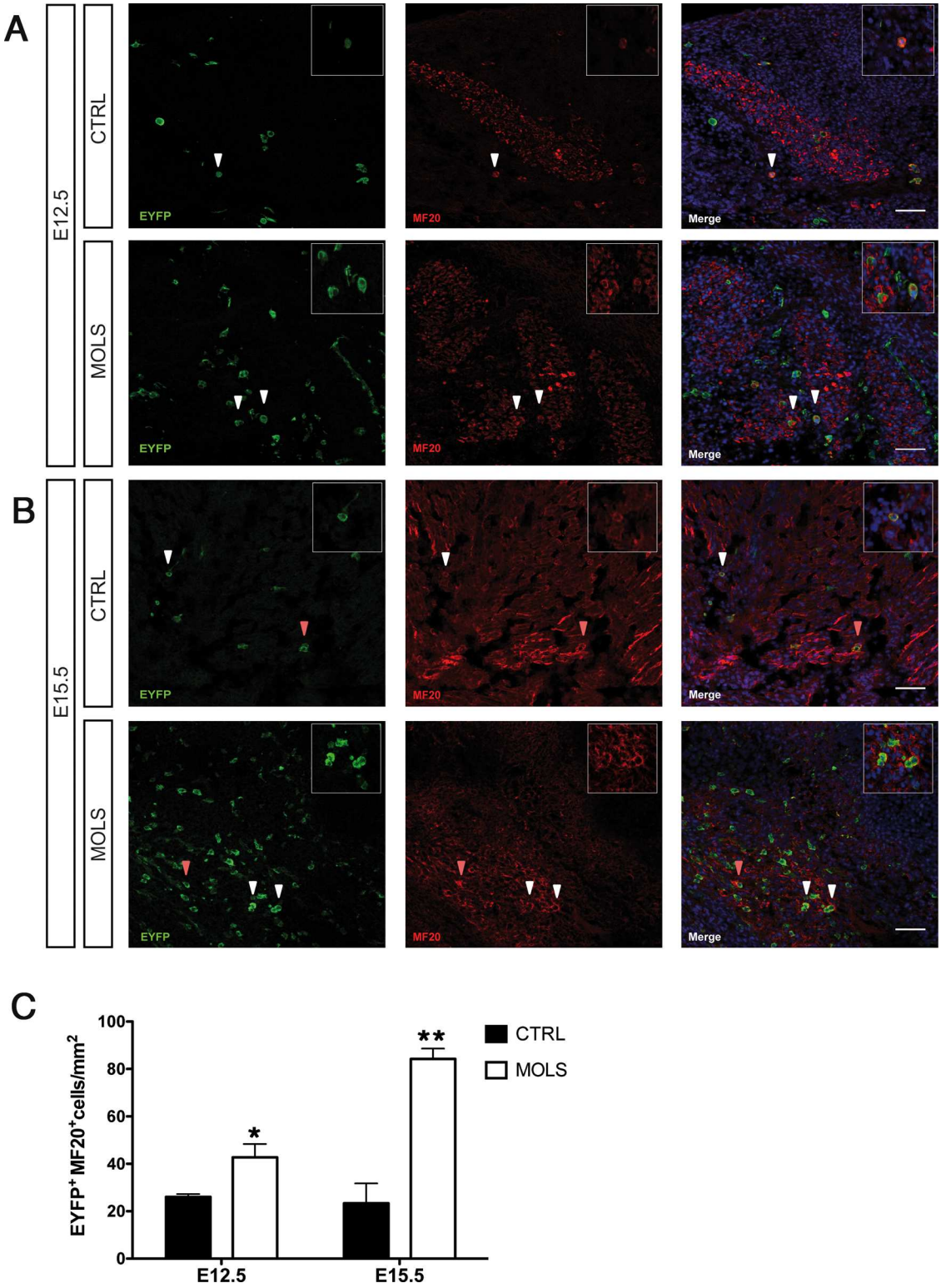

Fig 4. Molsidomine treatment during embryonic development increases eVE-Cad$^+$ derived cells contribution to MF20$^+$ myotubes. A) Immunofluorescence (IF) using anti-EYFP and MF20 specific antibodies on transverse sections of Cdh5-CREERT2;R26R-EYFP E12.5 embryos untreated (CTRL) and treated with Molsidomine (MOLS). Nuclei were stained with Hoechst. Arrowheads indicate EYFP$^+$MF20$^+$ single-nucleated myocytes. Inset in panels represent a 5x magnification of the cells indicated by the white arrowhead. Scale bars: 50 um; B) Immunofluorescence (IF) using anti-

EYFP and anti-MF20-specific antibodies on transverse sections of Cdh5-CREERT2;R26R-EYFP E15.5 fetuses untreated (CTRL) and treated with molsidomine (MOLS). Nuclei were stained with Hoechst. Arrowheads indicate $EYFP^+MF20^+$ single-nucleated myocytes/myofibers. Inset in panels represent a 5x magnification of the cells indicated by the white arrowhead. Scale bars: 50 um. C) Graph representing the quantification of the number of $EYFP^+/MF20^+$ cells per mm^2 in E12.5 and E15.5 embryos, performed by counting cells in at least 20 fields in different areas (20x and/or 40x) for each data point. Data are expressed as mean± S.E.M. *p≤0,05; **p≤0,005 MOLS vs CTRL (n = 4 embryos per group).

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We have shown that molsidomine treatment could affect the myogenic differentiation of MABs/eVE-Cad^+ cells while not changing the number or distribution of endothelial cells. We therefore reasoned that nitric oxide could directly modulate properties of different hemogenic endothelia, thereby affecting the nature of specific embryonic endothelial or hematopoietic populations, some of which physiologically contribute to myogenesis. To evaluate whether molsidomine differentially acted on yolk sac and embryonic eVE-Cad^+ cells, we next isolated $EYFP^+$ cells from control and molsidomine treated YS and embryo proper at E9.5. The

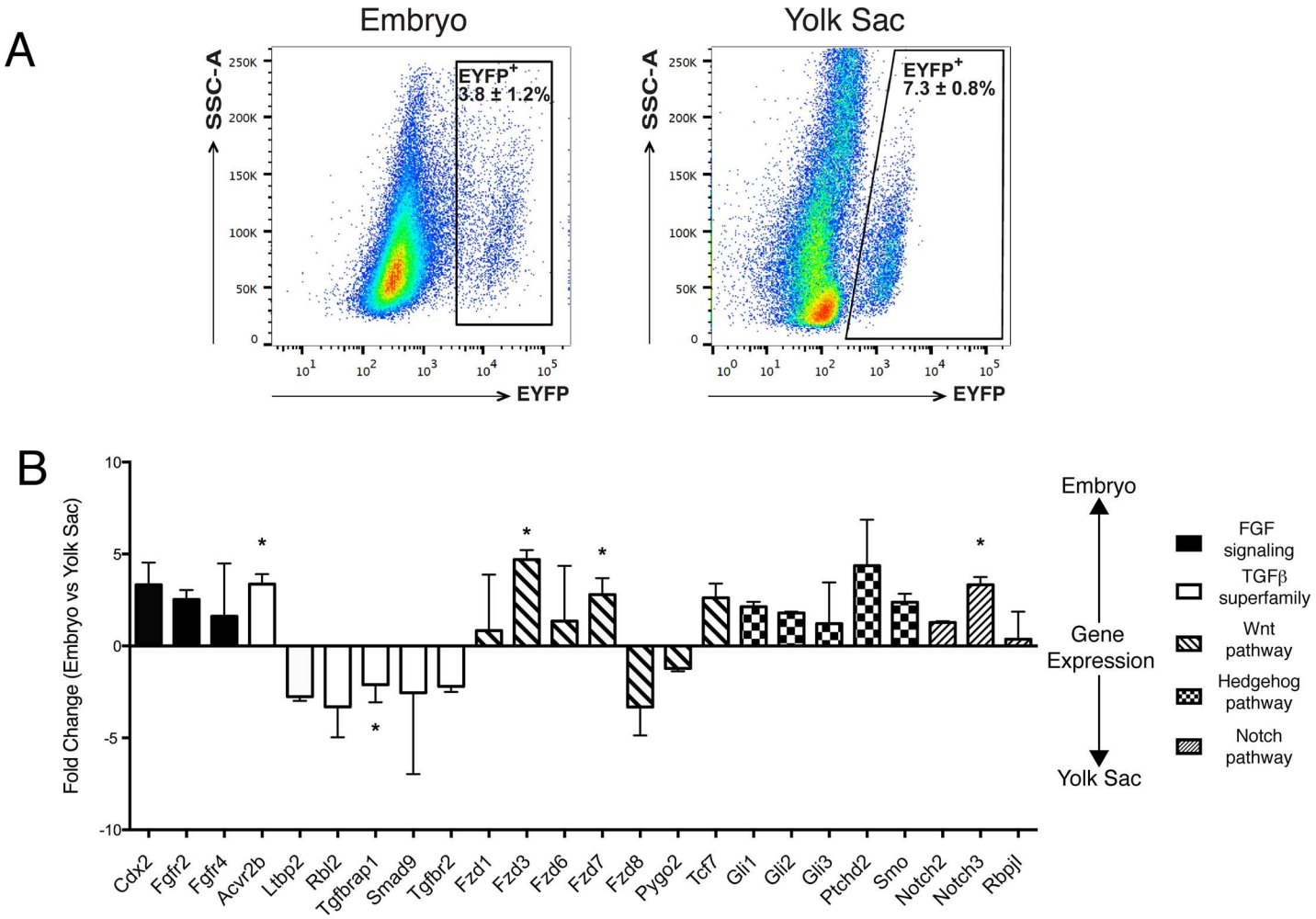

Fig 5. E9.5 eVE-Cad^+ cells in the embryo and YS express a non-.overlapping set of genes. A) Gating strategy employed to isolate $EYFP^+$ cells from E9.5 Cdh5-$CREER^{T2}$;R26R-EYFP embryo proper and yolk sac by fluorescence activated cell sorting (FACS). Cre recombination was induced with TAM 24 hours before collection. Values are expressed as mean ± S.D. n≥3 independent experiments. B) $EYFP^+$ cells were FACS sorted from E9.5 Cdh5-$CREER^{T2}$;R26R-EYFP embryo and yolk sac. Cre recombination was induced with TAM 24 hours before collection. A Real-Time PCR-based array analysis was performed. Positive fold changes represent genes more expressed in the embryo respect to the YS; negative fold changes represent the opposite. At least 10 embryos and yolk sacs were pooled. Values are expressed as mean± S.E.M. *p≤0,05. n = 3 independent sorting experiments.

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percentage of EYFP$^+$ cells was comparable between molsidomine treated and control embryos (Fig 6A). We then compared expression levels of the above mentioned genes in the YS and embryo proper of molsidomine-treated and control embryos (Fig 6B and 6C). In general, molsidomine treatment led to an upregulation of all these genes in the embryo proper and a downregulation in the YS, with few exceptions. In particular, the expressions of Gli2, and to a lesser extent Gli1, were higher in molsidomine- treated YS, while *Eng* and *Tfgbr3* were more expressed in the control embryo proper.

These data suggest that molsidomine treatment differentially modulates the functional properties of yolk sac and embryonic eVE-Cad$^+$ cells in early embryogenesis, thus potentially influencing the fate of their progeny.

Discussion

Several studies have clearly shown that NO administration in animal models, both alone or in combination with NSAIDs, significantly improves muscle regeneration and ameliorates the dystrophic phenotype by slowing down disease progression [6, 18, 26]. Based on this, several Phase I clinical trials have been launched and resulted in successful outcome [27, 28].

The mechanism by which NO exerts its positive effect on muscle regeneration is likely to be due to activity at multiple levels. In particular, several studies suggest that NO can influence the function of endogenous myogenic progenitors and of other cells involved in muscle development and regeneration [29].

Here we show that the NO donor molsidomine is able to modulate the differentiation, and possibly the commitment, of embryonic hemogenic endothelium-derived eVE-Cad$^+$ cells, including the *in vivo* counterparts of embryonic MABs.

MABs are progenitors with physiological myogenic potential, found in embryonic and adult vessels. They have been successfully used in preclinical MD models of systemic cell therapy, leading to a significant structural and functional recovery of the skeletal muscle tissue [1, 6, 8, 9]. Despite their recent use in clinical trials, in-depth studies on their developmental origin, physiological role and biological characteristics are still missing. In particular, the fine understanding of mechanisms guiding the fate choice of these endogenous progenitors could help to identify novel ways for their *in vivo* manipulation, not only in adult life, but during embryonic and fetal development. These findings will bear great therapeutic relevance as stem cell loss in dystrophic muscles begins as early as late embryogenesis in affected individuals [20].

To date the role of NO in early embryogenesis and myogenesis has been poorly investigated. It has been shown that molsidomine treatment of α-SG null pregnant mice has beneficial effects during embryonic myogenesis, with recovery of Pax7$^+$ cells in treated embryos compared to untreated dystrophic ones [12]. Additionally, recent studies have demonstrated that NO promotes embryonic myogenesis in the chicken embryo through upregulation of genes involved in myogenic differentiation [30]. However, no effect on myogenic determination genes, such as MyoD and Myf5, was found in mouse models, highlighting possible differences between mouse and chicken development. NO stimulates the proliferation of satellite cells via signaling pathways that require Vangl2 and cGMP, thus favoring muscle regeneration by counteracting the exhaustion of the satellite cells pool observed during repetitive acute and chronic damage [12]. Moreover, through a cGMP-independent pathway, NO leads to downregulation of the peroxisome proliferator-activated receptors gamma (Pparγ1) expression in fibro-adipogenic precursors (FAPs) in the skeletal muscle, therefore inhibiting their differentiation and reducing adipose tissue deposition and fibrotic scar formation [13]. The effect of NO on MABs has been investigated only on transplanted cells in the adult muscle [6, 31] but never on the endogenous pool of progenitors cells.

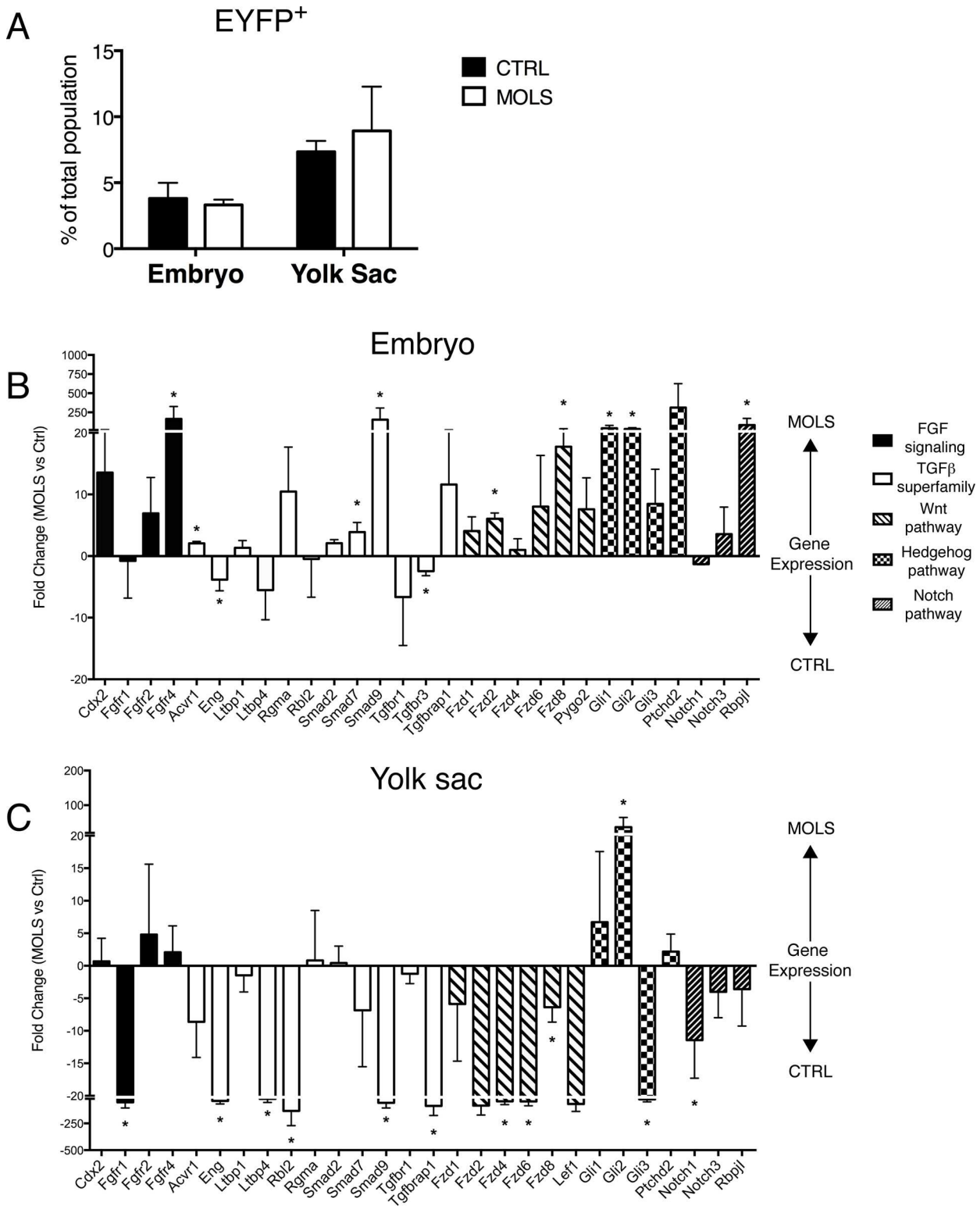

Fig 6. Molsidomine treatment modulates the gene expression signature of eVE-Cad^{+} cells. A) Graph summarizing the percentage of EYFP^{+} cells in molsidomine-treated (MOLS) and control (CTRL) E9.5 embryos and yolk sacs with Tamoxifen induction at E8.5. Values are expressed as mean ± S.D. n≥2 independent experiments. B-C) EYFP+ cells were FACS sorted from E9.5 Cdh5-CREER^{T2};R26R-EYFP molsidomine-treated and control embryos and yolk sacs. Cre recombination was induced with TAM 24 hours before collection. A Real-Time PCR-based array analysis was performed. Positive fold changes represent genes more expressed in molsidomine-treated (MOLS)

embryos (B) and yolk sacs (C); negative fold changes represent genes more expressed in control (CTRL) embryos (B) and yolk sacs (C). At least 10 embryos and yolk sacs were pooled. Values are expressed as mean± S.E.M, *p≤0,05. n = 3 independent sorting experiments.

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Here we demonstrated that NO treatment during embryogenesis results in a greater contribution of eVE-Cad$^+$-derived cells (embryonic MABs) to both embryonic and foetal myogenesis. Immunofluorescence analysis showed a higher percentage of EYFP-labeled myoblasts and myotubes in molsidomine treated embryos and gene expression profiling revealed higher expression levels of myogenic genes in eVE-Cad$^+$-derived cells compared to treated embryos. Importantly, molsidomine effect on myogenic differentiation of embryonic MABs appears to be specific, since we did not detect any significant difference in the number or distribution of eVE-Cad$^+$-derived endothelial cells and in the expression levels of endothelial specific genes. This is in agreement with *in vitro* data showing that nitric oxide is not required for endothelial differentiation of embryonic stem cells until late stages [32].

Embryonic MABs originate from eVE-Cad$^+$-derived CD45$^+$ cells, that are distinct from F4/80$^+$ macrophages and have the ability to differentiate into both hematopoietic cells and mesenchymal progenitor cells [4]. Here we show that molsidomine-treated embryos contain an increased number of CD45$^+$ non-MΦ eVE-Cad$^+$ derived cells relative to the controls, as well as an increased number of eVE-Cad$^+$ CD31$^+$ CD45$^+$ cells, the latter possibly representing a more transitional population [4]. These results suggest that NO may act not only on myogenic differentiation of MABs, but during the early specification of MAB progenitor cells. The increase in CD45$^+$ non-MΦ eVE-Cad$^+$ derived cells could result from a modulation of the activity of hemogenic endothelium. Accordingly, nitric oxide has been shown to positively modulate hematopoiesis by upregulating Runx1 in the hemogenic endothelium [33, 34]. However, recent evidence has suggested that hemogenic endothelial cells are not an homogenous population [35, 36]. We have previously shown that the timing of activity of extra-embryonic and embryonic hemogenic endothelia is different, possibly due to distinct biological characteristics [4]. In particular, the YS is the source of multiple progenitors emerging independently of the embryo proper. These include erythro-myeloid progenitors (EMPs) later generating tissue-resident macrophages which persist until adult life [37], and the first immune-restricted cells with lymphoid and myeloid potential, appearing before hematopoietic stem cells (HSCs) [38]. At least some of these progenitors, including embryonic MABs [4], originate from yolk sac hemogenic endothelium [36]. This exclusive potency of extra-embryonic hemogenic endothelium implies a specific sensitivity to different signaling pathways. Interestingly, very little is known about the nature and function of different endothelial populations in the early embryo. We have focused on a specific panel of genes that has allowed us to highlight significant differences between ECs of YS and embryo proper, and to point out how NO can modulate these differences.

Definitive hemopoiesis in the AGM region, but not in the YS is Notch-dependent [39–41]. Indeed, we found that genes belonging to the Notch pathway are upregulated in the embryonic endothelium, suggesting that it is intrinsically responsive to these signals. Molsidomine treatment appears to downregulate Notch1 in the YS while Rbpj1 is upregulated in the embryo proper. The effect of molsidomine on the other pathways that we investigated shows a similar trend, with genes in the embryo proper being upregulated while their expression is reduced in the YS.

Hedgehog has been described to play a role in AGM hemopoiesis in zebrafish [42, 43]. In particular, reciprocal BMP-hedgehog gradients in the dorsal aorta appear to be critical for HSC emergence [43], while the involvement of Shh signalling in mouse hemopoiesis is less clear [44–47]. Here we show that several components of the Shh pathway are more abundantly expressed in the embryo proper in respect to the YS, while some members of the BMP/TGF

pathway are more expressed in the YS. The latter are indeed known to play important roles also in the extraembryonic and early gastrulating embryo [48, 49]. Interestingly, molsidomine treatment decreases TGF/BMP activation mainly in the YS, while increasing Shh signalling in both embryo proper and YS, with the exception of the Gli3 gene.

BMP pathway also crosstalks with FGF signaling in early hemopoiesis and endothelial differentiation. It has been shown in the zebrafish model that FGF is required for the specification of aortic hemogenic endothelium via repression of BMP signaling [50]. In our control embryos, FGF signaling components are more expressed in the embryo proper. Interestingly, molsidomine treatment induces upregulation of FGF genes in the embryo, while at least Fgfr1 is highly reduced in the YS, while, as mentioned above BMP genes are downregulated mainly in the yolk sac. Moreover it has recently been reported in zebrafish that TGF/BMP pathway also interacts with the Notch pathway to orchestrate the specification of hemogenic endothelium [51]. This suggests a complex signaling network through which molsidomine may influence eVE-Cad$^+$ cell fate.

Wnt signaling appears to be critical for both primitive and definitive hemopoiesis [36, 52]. *Fzd8*, the only Wnt related gene more expressed in the YS, is known to play important roles in the extraembryonic tissue and early gastrulating embryo. Molsidomine treatment differentially affects the expression of Frizzled receptors in YS and embryo proper. Again, molsidomine upregulated genes involved in this pathway in the embryo proper, while downregulating them in the YS.

We speculate that the distinct potency of yolk sac and embryonic hemogenic endothelia could be at least in part derived from differential responsiveness to extrinsic signals, such as nitric oxide. Interestingly, yolk sac EMPs still emerge in absence of circulation [36] suggesting that hemogenic endothelium in the yolk sac might have different requirements for nitric oxide signaling, as well as YS derived multipotent progenitors. This would explain the different behavior that we observed in the embryo and yolk sac ECs upon molsidomine treatment and why the endothelial differentiation of eVE-Cad$^+$ derived cells was not largely changed.

The crosstalk of NO with other signaling pathways in the embryo is mostly unexplored and this is the first evidence of its broad effect on the physiology of embryonic endothelial progenitor derived cells. More specific studies will be required to get additional insights into the role of NO in the fate commitment of endothelial progenitors and to unravel the role of its downstream pathways and their crosstalk in the differential nature and developmental fate of YS and embryonic ECs. These studies may ultimately pave the way on new approaches to manipulate cell fate in the embryo and at the same time develop new therapeutic approaches.

Conclusions

In this study, using a mouse model we demonstrate that treatment with the NO donor molsidomine during pregnancy modulates the fate of embryonic endothelial-derived progenitors. Notably, while not affecting endothelial network formation, molsidomine treatment enhances the contribution of endothelial-derived progenitors to embryonic and fetal myogenesis. We also provide evidence that molsidomine treatment affects several important signaling pathways in the embryonic endothelium, possibly resulting in the expansion of a CD45$^+$ non-MΦ eVE-Cad$^+$ population, that we have previously shown to include mesoangioblast-like cells. These findings may help explain the biology behind the beneficial effect of NO in embryonic myogenesis and could in the future have therapeutic relevance for the early *in vivo* and *ex vivo* treatment of muscular dystrophy.

Supporting Information

S1 Fig. Isotype and Fluorescence Minus One (FMO) controls for FACS staining. A) Anti-IgG2b,k- PE and Anti-IgG2a,k-APC isotype controls for FACS analysis of E12.5 embryos. B) Representative density plots showing FMO controls for FACS analysis of E12.5 cells. Plots

show gating of CD31 and CD45 within the EYFP$^+$ population. C) Representative density plots showing FMO controls for FACS analysis of E12.5 cells. Plots show gating of CD31 and F4/80 within the EYFP$^+$ population.
(TIF)

S2 Fig. Effect of molsidomine treatment on eVE-Cad$^+$ cells during vascular embryogenesis. A-C) Representative FACS plots of E12.5 embryos showing the percentage of (A) CD31, (B) CD34 and (C) VE-Cadherin within the EYFP$^+$ subset in control and molsidomine treated embryos. Data are expressed as mean ± S.E.M. (At least n = 3 embryos per group).
(TIF)

S1 Table. List of antibodies used for Immunofluorescence.
(PDF)

S2 Table. List of antibodies used for FACS analysis and FACS sorting.
(PDF)

S3 Table. List of primers used for quantitative real-time PCR.
(PDF)

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CHAPTER III:

EndMT and the immune system in muscle repair
(*Data from manuscript in preparation).*

EndMT and the immune system in muscle repair

(Data from manuscript in preparation)

The homeostatic response of skeletal muscle to injury involves the regulated interaction among various cell populations. After acute injury the initial phase of muscle repair is characterized by necrosis and by the activation of an inflammatory response.

We showed that macrophages (MPs) are necessary for efficient vascular remodeling in the injured muscle[1]. In particular macrophages sustain the differentiation of endothelial-derived progenitors to contribute to neo-capillary formation, by secreting pro-angiogenic growth factors. When we perturbed this process, by depleting circulating monocytes and infiltrating MPs, we observed delayed angiogenesis and myogenesis. In addition, endothelial-derived progenitors appeared to contribute markedly less to capillary formation and neo-angiogenesis. They down-regulate the expression of endothelial markers, whereas up-regulating mesenchymal markers, including collagen I, suggesting that EndMT (see Chapter 1.2.2) is occurring. To better understand the interplay between macrophages and vascular cells, we set an in vitro co-culture system (Fig.1). Following the expression changes of genes regulator of the mesenchymal transition such as CD31 and Twist, we have demonstrated that polarized macrophages directly modulate EndoMT in endothelial progenitors. Indeed, M2a and M2c macrophages appear to directly counteract TGF-β-induced EndMT. In particular, we observed a decrease of endothelial marker CD31 when we treated endothelial progenitor with TGF-β and upregulation of mesenchymal-related genes. When we added M2 macrophages, we detected a

restoration of CD31 expression levels and down-regulation of COL-I and TWIST (Fig. 1b).

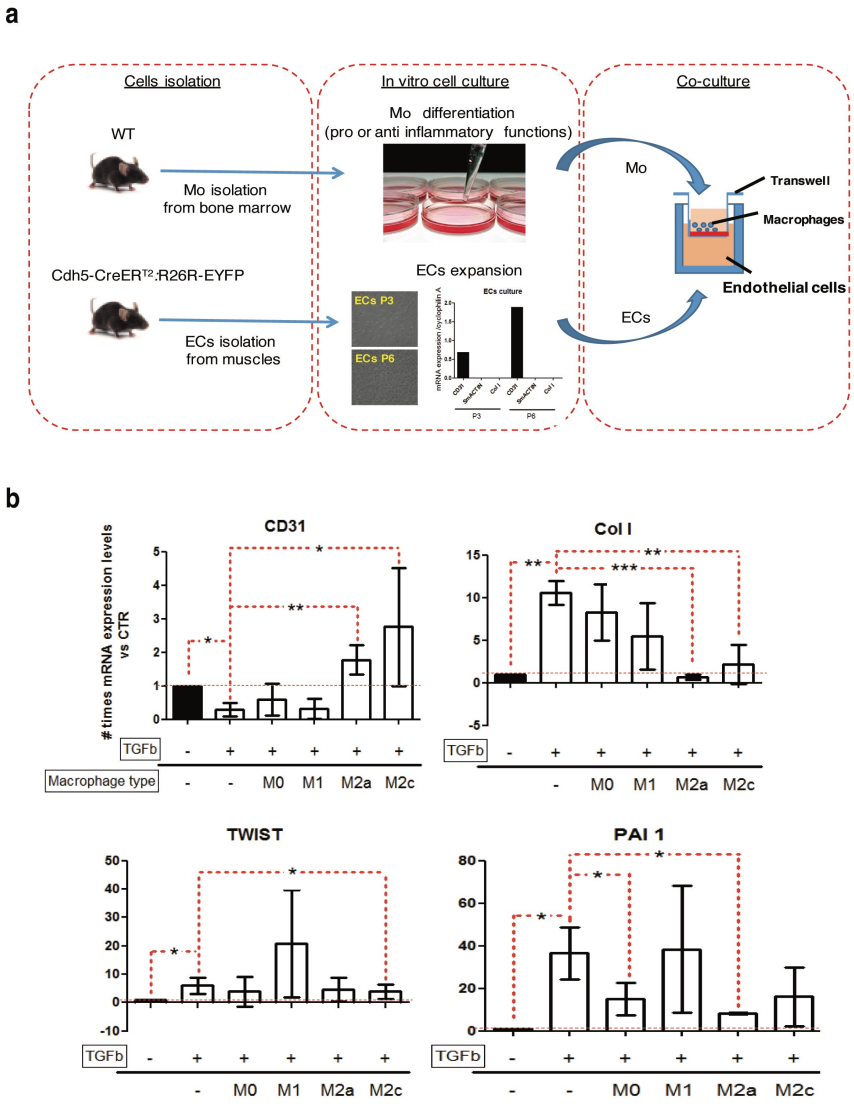

Figure 1. EndMT in vitro system. a) Schematic diagram of co-culture method. Freshly isolated endothelial progenitors [2] were cultured for 4 days with TGF-® , whereas Bone Marrow-derived monocytes were polarized with IFN©, IL-4 to obtain different macrophages phenotypes. After 4 days, macrophages were put in indirect contact with ECs for O/N. b) Expression levels of marker genes. pvalue<0.05*;<0.01**; <0.005***. Data ± SD (n=5).

The action appears to be specific, since M0 and M1 do not influence these parameters. The subdivision of macrophages in M1 and M2 subtypes is a convenient simplification of the reality but M1 and M2 are unfortunately the extremes of a continuum of intermediate cells, which becomes quite clear when looking in vivo instead of in cells cultured in vitro with IFNγ vs IL-4. It is hard to find "real" M1 or M2 cells in mice; but we should rather focus on the question whether macrophages are more or less "skewed towards the M1 or M2 direction".

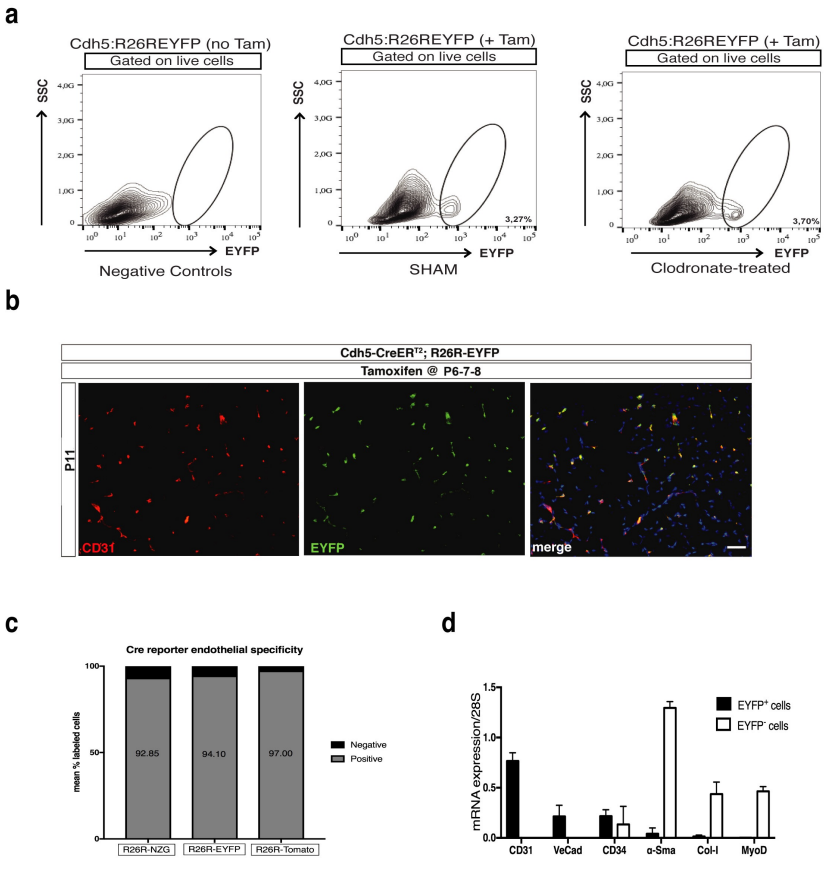

Figure 2. Phenotype of freshly sorted endothelial cells. a) Gating strategy for endothelial cells isolation. b) IF performed on TA of Cdh5-CreERT2;

R26R-EYFP mice activated with TAM injection at postnatal days (P) 6–8, at P11. c)Cre endothelial specificity of different reporter mice. d) Gene expression of VE-Cad^{+} derived cells after postnatal Cre activation.

So *in vivo* we decided to deplete all Cd11b^{+} cells with the help of clodronate encapsulated in liposomes that induces an apoptosis of phagocytes cells [3, 4]. We followed the fate of Endothelial cells (ECs) during muscle regeneration after acute damage and we obtain a depletion of about 50-60% of macrophages (Fig.3a). To elucidate the molecular mechanism that underlie EndMT in the skeletal muscle, we took advantage of in vivo lineage tracing of vascular associated cells, using transgenic mice in which an endothelial specific promoter (Cdh5) guides the expression of a Tamoxifen inducible Cre recombinase (Fig.2) (see Chapter II)[5, 6]. Crossing our transgene line with appropriated reporter, we have been able to obtain a EYFP, Tomato or nLacZ irreversible labeling of VE-cad^{+} cells (Fig.2b)[7, 8]. We performed a RNA-Sequencing on freshly sorted ECs (Fig.3b) isolated from clodronate-treated mice (CLL) in comparison with control mice (SHAM), which received empty liposomes. Validation occurred with Real-Time PCR (Fig. 3d.). From this analysis, we have generated a "gene expression signature", a list of about 100 candidate genes associated with EndMT in muscle (Fig. 3b). Enrichment analysis revealed that ECs from CLL mice up-regulated genes related to Extracellular Matrix and Extracellular region (Fig. 3c). Notably, ECs appeared to be involved also in process of "ossification" and "bone development" (Fig.3c)

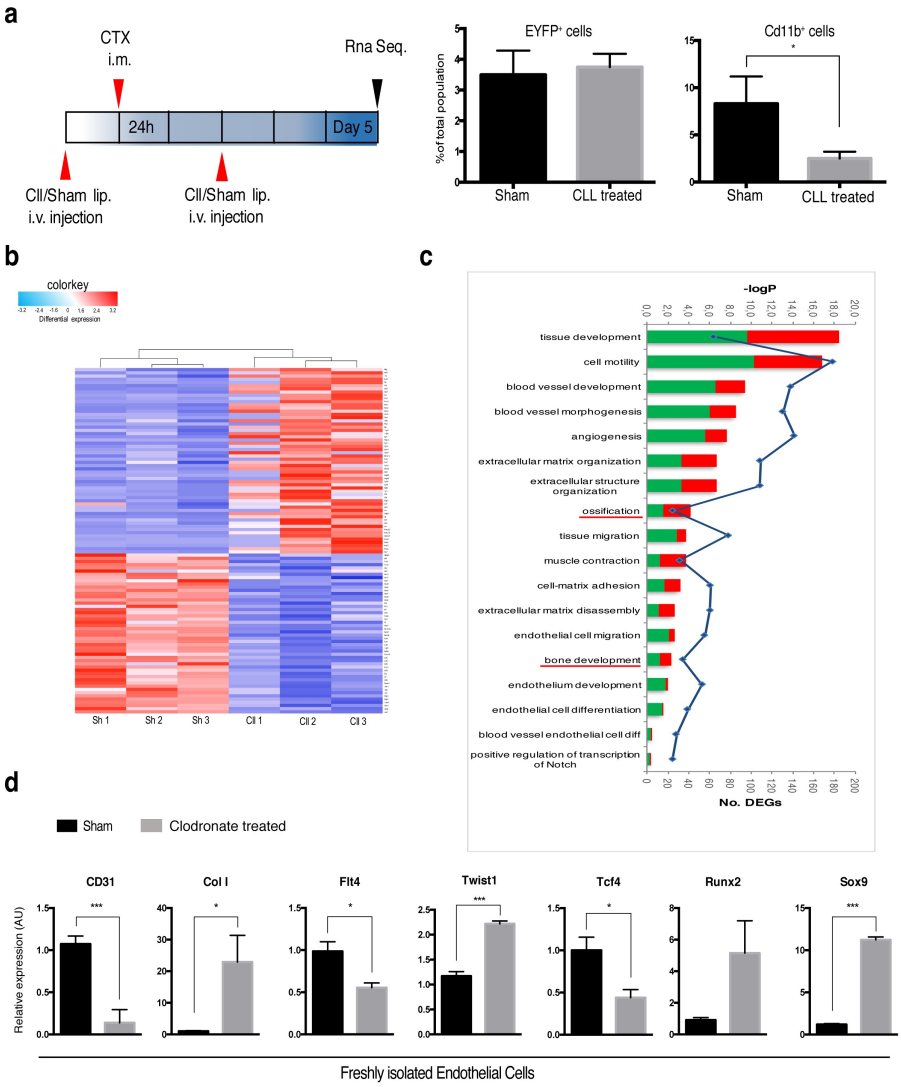

Figure 3. NGS analysis on eVE-Cad⁺ cells (Eyfp⁺) and *Gene Ontology enrichment.* a) Time course of experiment. Graphic showing percentage of Eyfp⁺ sorted cells and Cd11b⁺ cells. b) Heatmap of genes. c) Gene Ontology (GO) Biological Process terms enriched in CLL versus control samples (SHAM) are represented. Number of up- and down-regulated differentially expressed genes (DEGs) associated to each term are shown as red and green bars, respectively. All the enrichments shown are statistically significant (blue line indicates significance expressed as -log Pvalue). d) mRNA expression of selected genes. pvalue *<0.05; **<0.01; ***<0.005. Data ± SD (n=5).

With the aim to provide new insights on molecules that could mediate EndMT in vivo, we focused our attention on the Flt4 protein, also known as VEGFR3. Vascular endothelial growth factor (VEGF) potently promotes angiogenesis, and is indispensable for vascular development[9, 10]. Angiogenesis, the growth of new blood vessels, involves specification of endothelial cells to tip and stalk cells, which is controlled by Notch signalling, whereas vascular endothelial growth factor receptors VEGFR-2 and VEGFR-3 are implicated in angiogenic sprouting. Endothelial deletion of VEGFR3 leads to excessive angiogenic sprouting, and decrease the level of Notch signalling, indicating that VEGFR-3 possesses passive and active signalling modalities[10]. VEGFR-3 is activated by the VEGF homologues VEGF-C that is expressed by macrophages. With EndMT in vitro system, we evaluated if macrophages had the ability to modulate the expression of Flt4 genes and protein on endothelial precursor cells. Preliminary analysis (Fig.4) support the idea that the ligand VEGF-c binds Flt4 restoring the Notch signaling pathway (in particular Notch4-Dll4), which leads to decreased sensitivity to other growth factors (for example TGF-β) and supports neoangiogenesis. In particular, Flt4 was localized in nuclear compartment on ECs. When we added TGF-β, ECs changed morphology and VEGFR3 was translocated from the nucleus to the cytoplasm (Fig.4). M2a macrophages (derived from culturing bone marrow derived macrophages in specific polarizing conditions) seemed to counteract this translocation and reestablished ECs morphology in *in vitro* assays (Fig.4a, Fig.4b). No dramatic changes were observed in mRNA expression of Notch4 and FLt4. VEGF-C mainly signals through

VEGFR3. VEGFR3-signalling is likely not be not only involved in lymphatic, but also in cardiovascular development. It has been shown that heterozygous mice carrying a VEGF-C loss of function demonstrate delay in retinal vascularization and decrease vessel branching density. In contrast, they exhibit increased vessel sprouting and decreased level of Notch target gene expression [10]. However, further studies are needed to get insights in the role of Flt4 in the nucleus and to associate the function of this protein with the mesenchymal transition of ECs.

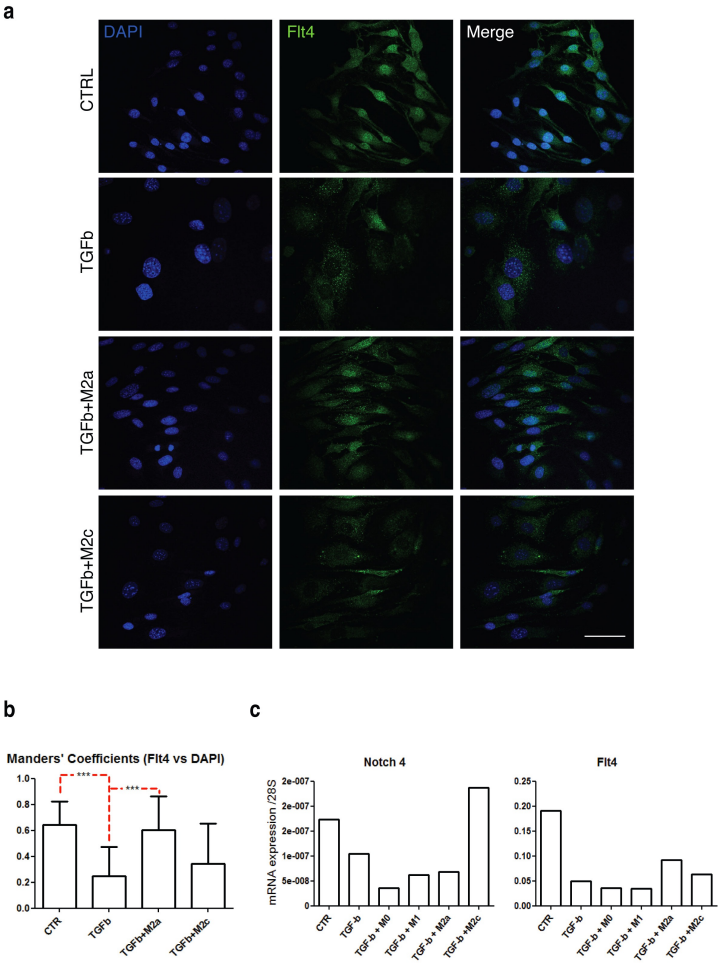

Figure 4. Macrophages induce translocation of Flt4 protein (preliminary). a) Representative image of ECs after co-culture: Immunofluorescence staining showing Flt4(green) and DAPI(blue). Scale bar: 20⌈m. b) Manders overlap Coefficients (Flt4 vs DAPI). c) mRNA expression of Notch4 and Flt4. pvalue*<0.05;<**0.01;<***0.005.

RNA-Seq analysis has suggested that fibroblasts are not the ultimate fate of ECs after EndMT. Our data support the hypothesis that, upon appropriate signals and growth factor, endothelial-derived mesenchymal cells can generate various types of connective tissue other than fibroblasts [11]. In particular, vascular cells have key role in hematopoiesis (see Chapter II) and bone formation, as suggested by gene enrichment analysis (Fig.3c). The development of an endochondral bone in skeletal muscle, or in general soft tissue, is defined as Heterotopic Ossification (HO). An example of HO can be found in patients with Fibrodysplasia Ossificans Progressiva (FOP, OMIM 135100). FOP arises from gain-of-function mutations in bone morphogenetic proteins (BMP) type I receptor ACVR1 (ALK2). This mutation causes an arginine to histidine change in aminoacid 206 (R206H) in the ALK2 protein and confers aberrant activation of the BMP signaling pathway (See Chapter 1.2.4) [12-16]. Different approaches during these years showed candidates with osteogenic potential [17-19]. The endothelial involvement to HO is still debated [19, 20]. It is possible that acute inflammatory processes lead to a disordered injury repair, several cell types acquire unusual ability, morphology and contribute to the new bone formation due to altered signals and microenvironments.

In the present study we have demonstrated that VE-cad^{+} cells contribute to BMP-induced heterotopic bone. In vivo micro-

computerized tomography (μCT) scans were carried out to assess progression of ossification and any effect on the normal skeletal structure. In vivo μCT imaging was performed using the IVIS SpectrumCT, and that allowed us to obtain three dimensional fluorescence images using a transillumination excitation source placed below animal. We analyzed the growth of endochondral bone after 10 and 21 days from hBMP2 injection in clodronate-treated mice (CLL) in comparison with control mice (Sham: treated with hBMP2 injection and empty liposomes) (Fig.5). We detected an increase of fluorescence signals in CLL-mice in the area of new ossification at 10 days, and this is confirmed 21 days from BMP-induction (Fig.5b). Additionally, mineralized volume and bone density were significant increase in CLL-mice at 21days (Fig.5c, Fig.5d). We also performed immunofluorescence analysis during early phase of HO (Fig.6). Our work has also shown that under normal condition few percentage of endothelial precursor contribute to all stages of ossification; depletion of macrophages, however, significantly improved their contribution to the population of chondrocytes (Fig. 6b, β-gal^{+}Sox9^{+}) and osteoblast (Fig.6c, β-gal^{+}Osx^{+}). These cells belong to the mesenchymal lineage and this result could be due to the de-differentiation of ECs (EndMT). Next step will be to repeat this kind of analysis crossing our lineage tracing model with a murine model of FOP, in order to better reproduce the altered microenvironment of FOP patients and confirmed EndMT as a potential new therapeutic target. In future, inhibiting endothelial trans-differentiation may significantly delay disease progression and allow patients to maintain adequate organ function for a longer period.

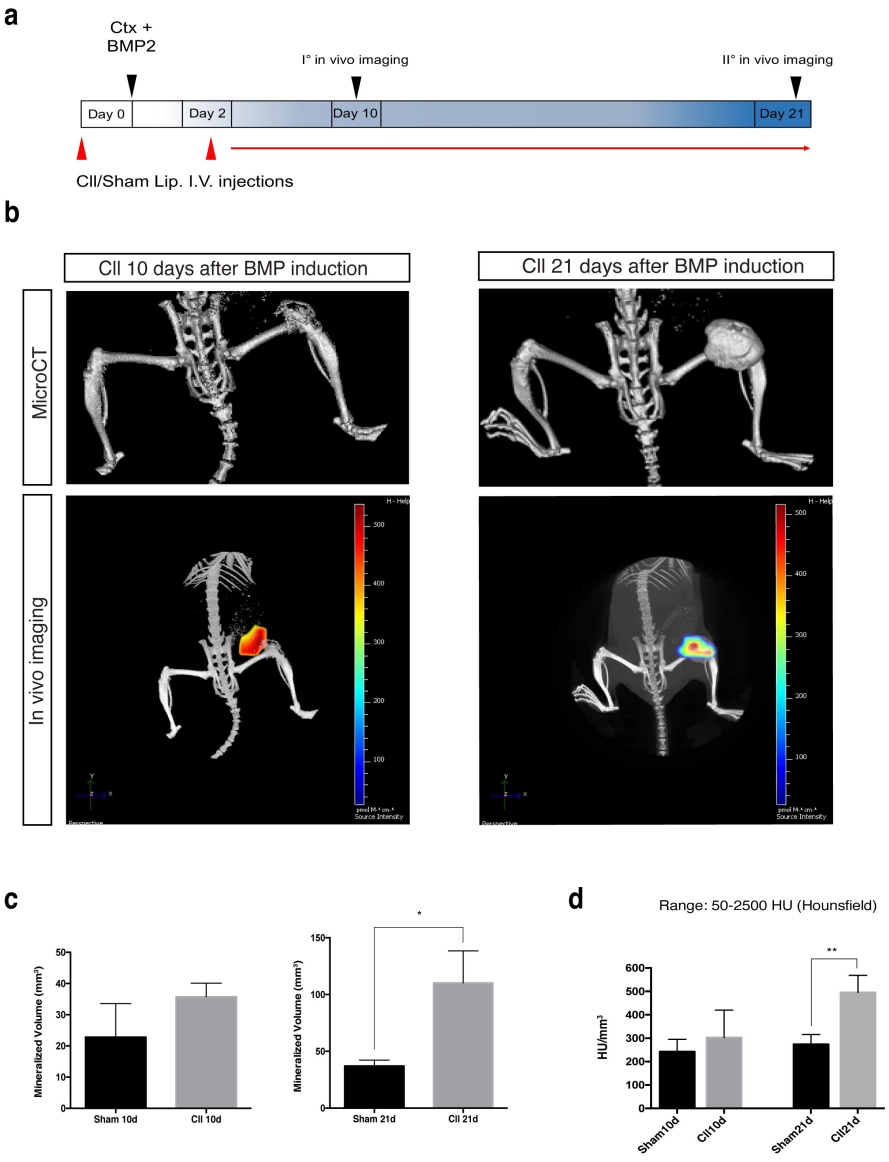

Figure 5. In vivo imaging of HO. a) Time course of treatments. b) ⌈CT and In vivo Imaging of CLL-treated mice 10 and 21days after BMP injections. c) Where ectopic mineralization was detected the total mineralized volume (mm^3) and bone density were quantified. *pvalue<0.05; **<0.01. Data ± SD (n=5)

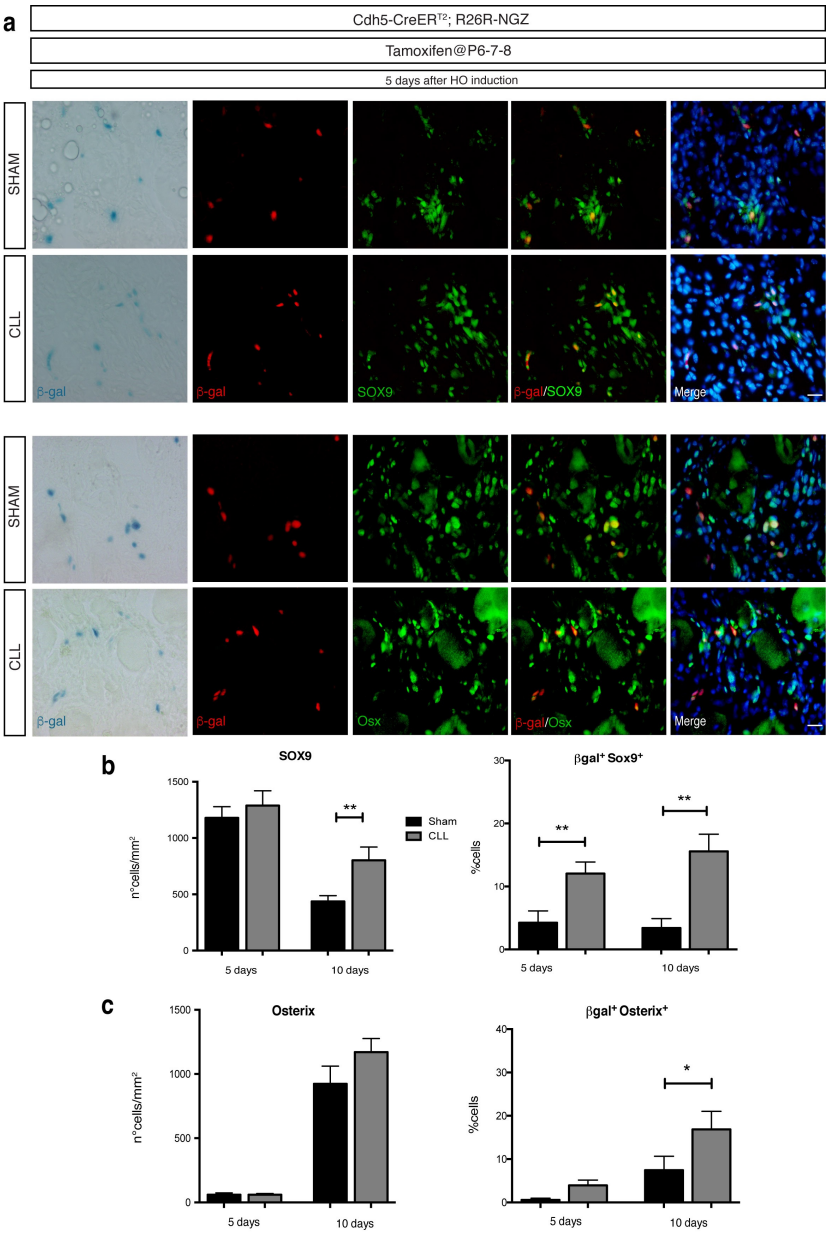

Figure 6. CLL treatment during HO development increases eVE-cad$^+$ derived cells contribution to ectopic bone. a) Immunofluorescence (IF) using anti-Sox9, anti-Osterix (green) specific antibodies and ®-gal on transverse sections of Cdh5-CREERT2;R26R-NGZ mice, untreated (SHAM) and treated with clodronate liposomes (CLL). Nuclei are stained with Hoechst (Blue). Scale bars: 50⌠m. b-c) Graph summarizing the

quantification of the number of ®-gal$^+$,Sox9$^+$,Osx$^+$ and percentage of co-localization performed by counting cells in at least 20 fields in different areas (20x) for each data point. Data are expressed as mean± S.E.M. (n=5) pvalue<0.05*;<0.01**.

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CHAPTER IV:

High-throughput screening for modulators of ACVR1 transcription: discovery of potential therapeutics for fibrodysplasia ossificans progressiva.

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CORRECTION

Correction: High-throughput screening for modulators of *ACVR1* transcription: discovery of potential therapeutics for fibrodysplasia ossificans progressiva

Serena Cappato, Laura Tonachini, Francesca Giacopelli, Mario Tirone, Luis J. V. Galietta, Martina Sormani, Anna Giovenzana, Antonello E. Spinelli, Barbara Canciani, Silvia Brunelli, Roberto Ravazzolo and Renata Bocciardi

There was an error concerning Fig. 4 in *Dis. Model. Mech.* **9**, 685-696.

The wrong figure was used for Fig. 4. The correct figure is shown in this Correction with its legend. The PDF and full-text version of this article have been corrected.

The authors and DMM office apologise to the readers for this error.

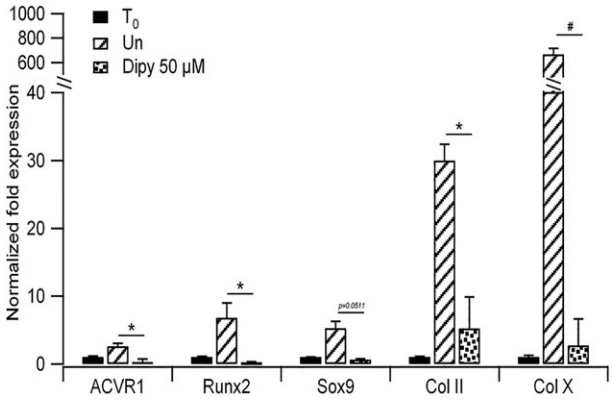

Fig. 4. Effect of Dipy on the expression of chondrogenic markers. RT-qPCR on RNA extracted from ATDC5 cells cultured as alginate spheres for 14 days in differentiation medium. Bars show mean and s.d. of three independent experiments. Expression levels were normalized on *GAPDH* and *18S* and compared to that of cells at T_0 (cells harvested at the beginning of the differentiation protocol). Un, untreated cells. ns, non-significant; $^{*}P<0.05$, $^{\S}P<0.001$.

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RESEARCH ARTICLE

High-throughput screening for modulators of *ACVR1* transcription: discovery of potential therapeutics for fibrodysplasia ossificans progressiva

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ABSTRACT

The *ACVR1* gene encodes a type I receptor of bone morphogenetic proteins (BMPs). Activating mutations in *ACVR1* are responsible for fibrodysplasia ossificans progressiva (FOP), a rare disease characterized by congenital toe malformation and progressive heterotopic endochondral ossification leading to severe and cumulative disability. Until now, no therapy has been available to prevent soft-tissue swelling (flare-ups) that trigger the ossification process. With the aim of finding a new therapeutic strategy for FOP, we developed a high-throughput screening (HTS) assay to identify inhibitors of *ACVR1* gene expression among drugs already approved for the therapy of other diseases. The screening, based on an *ACVR1* promoter assay, was followed by an *in vitro* and *in vivo* test to validate and characterize candidate molecules. Among compounds that modulate the *ACVR1* promoter activity, we selected the one showing the highest inhibitory effect, dipyridamole, a drug that is currently used as a platelet anti-aggregant. The inhibitory effect was detectable on *ACVR1* gene expression, on the whole Smad-dependent BMP signaling pathway, and on chondrogenic and osteogenic differentiation processes by *in vitro* cellular assays. Moreover, dipyridamole reduced the process of heterotopic bone formation *in vivo*. Our drug repositioning strategy has led to the identification of dipyridamole as a possible therapeutic tool for the treatment of FOP. Furthermore, our study has also defined a pipeline of assays that will be useful for the evaluation of other pharmacological inhibitors of heterotopic ossification.

KEY WORDS: *ACVR1*, Transcriptional regulation, BMP signaling pathway, FOP, Dipyridamole, High-throughput screening, Drug repositioning

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INTRODUCTION

Fibrodysplasia ossificans progressiva (FOP; OMIM 135100) is a rare genetic disease with a prevalence of about one per 2-million people. The inheritance is autosomal dominant, although most cases are due to sporadic new mutations (Shore et al., 2005). Individuals with FOP are characterized by a peculiar congenital toe malformation and, usually starting within the first decade of life, by a progressive heterotopic ossification (HO) that takes place following some types of injury (such as trauma, medical surgery, intramuscular immunization, infections) or spontaneously. Inflammatory soft-tissue swellings, commonly called flare-ups, progressively transform skeletal muscles, tendons, ligaments, fascia and aponeuroses into a second skeleton of heterotopic bone (Kaplan et al., 2008).

The FOP gene (*ACVR1*) encodes a type I receptor of bone morphogenetic proteins (BMPs), ACVR1 (also known as ALK-2). The most recurrent *ACVR1* mutation is in the glycine-serine (GS) domain (c.617G>A, p.R206H) (Shore et al., 2006). Additional mutations have been identified in the GS and in the kinase domain of the protein in 3% of all known individuals with FOP (for a review, see Kaplan et al., 2009; Bocciardi et al., 2009). The consequence of *ACVR1* mutations is an alteration of inter-intramolecular interaction of the mutant receptor that causes a deregulation of the downstream BMP signaling (Shore et al., 2006; Bocciardi et al., 2009; van Dinther et al., 2010; Song et al., 2010; Groppe et al., 2011; Chaikuad et al., 2012).

At present, no established medical treatment is available for FOP. Early diagnosis prevents unnecessary interventions, such as biopsies or surgical operations that can exacerbate the progression of the disease, and high-dose glucocorticoids are used in the management of inflammatory flare-ups (Kaplan et al., 2013).

In recent years, much effort has been devoted to designing new therapeutic approaches to FOP treatment and to identify new, potentially useful, drugs (Kaplan et al., 2013; Sanvitale et al., 2013; Yu et al., 2008a; Kitoh et al., 2013). A promising alternative to the discovery of new drugs is the drug repositioning strategy, in which a drug already developed for a specific disease can be used to treat a different condition. Drug repositioning reduces costs and accelerates the drug development process. Moreover, this approach might contribute to clarify the mechanism of action of a given compound by establishing a relationship between the molecular basis of the disease and the ability of the compound to intervene at a certain step of the disease process (Shameer et al., 2015).

A possible strategy to find drugs for the treatment of a genetic disease could rely on a sensitive, specific and fast cell-based assay. In this way, a large number of small molecules can be screened [high-throughput screening (HTS)] to find agents that correct the

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basic defect. The recent identification and characterization of the promoter region of *ACVR1* (Giacopelli et al., 2013) inspired us to develop an HTS assay by generating cells stably expressing the luciferase reporter gene controlled by a 2.9-kb region of the gene promoter. We expected that this type of assay would allow the identification of molecules that, by inhibiting the *ACVR1* promoter, would also negatively regulate the downstream signaling that is upregulated and hyper-responsive to BMPs because of the mutation in the receptor.

In this work, we describe the screening of a library of 1280 US Food and Drug Administration (FDA)-approved compounds, in order to identify modulators of *ACVR1* gene expression. Characterization of hit molecules included a series of second-level assays to evaluate the effect of compounds on chondrogenic and osteogenic differentiation models *in vitro* and *in vivo*.

We found that dipyridamole, commonly used as an antithrombotic and vasodilator drug, has an inhibitory effect on *ACVR1* expression, as well as on the whole BMP signaling pathway, and is able to affect chondrogenesis and osteogenesis, both in cellular assays and in a BMP-induced HO mouse model.

RESULTS

Screening of the Prestwick Chemical Library

Our primary screening was designed to find drugs that downregulate BMP signaling by targeting the expression of the *ACVR1* gene at the transcriptional level. Accordingly, we developed a quantitative assay based on expression of a reporter gene under the control of the *ACVR1* promoter. To this end, we generated clones of the ATDC5 cell line (mouse chondrogenic cell line derived from teratocarcinoma) stably expressing the luciferase coding sequence under the control of the 2.9-kb promoter of the gene, previously characterized by our group (Giacopelli et al., 2013). We obtained several clones that were expanded and selected for the level and stability of reporter gene expression over time. The availability of different clones, with putative different integration sites of the reporter construct in the genome of ATDC5 cells, allowed us to verify that the effect measured for a given compound was not related to a 'position effect' operated by the genomic region surrounding the reporter construct itself.

The generated cell system and the compound analysis procedure were tested by screening a small library of 43 molecules with chromatin-modifier properties. This allowed us to validate the protocol for the primary screening and provided us with a positive control because we identified resveratrol as a transcriptional activator of *ACVR1* gene expression (Fig. S1).

We used these cells to screen the Prestwick Chemical Library, which includes 1280 FDA-approved compounds, with the idea that 'repositioning' of an already approved drug could have the great advantage to overcome several steps of the drug discovery process. The screening detailed in Table 1 (see also Fig. S2) was performed in duplicate: compounds were added to cells seeded in 96-well plates for 24 h at the concentrations of 20 and 2 µM, respectively. We included in each plate DMSO, the vehicle in which compounds are dissolved, and resveratrol (20 µM) as a transcriptional activator of the *ACVR1* promoter and positive control. When we started this work, no transcriptional inhibitors of the *ACVR1* expression were known. However, during the screening of the second plate of the Prestwick Chemical Library, we detected dipyridamole as an inhibitor of *ACVR1* expression. Therefore, this compound was subsequently included in all the remaining plates as an additional control. To monitor the performance of the screening, we used the Z′-factor statistical parameter (Zhang et al., 1999). The calculated

Table 1. HTS assay protocol

Step	Parameter	Description
1	Cell plating (Pr2.9-Luc ATDC5)	3×10^5 cells/well
2	Controls	10 µM resveratrol, positive control; 1% DMSO, neutral control; 20 µM dipyridamole, negative control*
3	Prestwick Chemical Library	1200 compounds diluted to 20 and 2 µM
4	Incubation	24 h at 37°C and 5% CO_2
5	Viability assay	Addition of 20 µl CellTiter-Fluor reagent to living cells
6	Viability assay (incubation)	1 h at 37°C and 5% CO_2
7	Viability assay (detection)	Detection of the fluorescent signal (GLOMAX Automated Plate Reader $380\text{-}400_{Ex}/505_{Em}$ nm)
8	Reporter gene assay	Addition of the ONE-Glo reagent (100 µl), cell lysis
9	Assay readout	Detection of the luminescence signal (GLOMAX Automated Plate Reader)
10	Evaluation of HTS performance	Calculation of the Z'-factor statistical parameter according to Zhang et al. (1999)
11	Data analysis	Evaluation of cell viability (Vi) and effect of compounds (E) on the reporter gene activity. Selection of compounds for further characterization

Step	Notes
1	96-well format culture plates
2,3	80 compounds per plate (columns 2 to 11); columns 1 and 12 were used for positive, negative and neutral controls (8 wells for DMSO, 4 wells for each control)
5-7	12-tip dispense of the CellTiter-Fluor reagent; automated fluorescence detection
8-9	These steps are fully automated; the ONE-Glo reagent is added *in situ* by injection followed by luminescence detection
10	$0\geq Z'\leq 1$, with $Z'\geq 0.5$ for a good HTS (Zhang et al., 1999) $Z'_{(+Resv)}=0.63\pm0.1$ and $Z'_{(-Dipy)}=0.65\pm0.1$
11	Evaluation of cell viability, $Vi=(fluoC_x/\text{average } fluo_{DMSO})\times100$ Normalization, $N_x=LumC_x/fluoC_x$ and $N_{DMSO}=LumC_x/fluoC_x$ Effect of compounds, $E=(N_x/\text{average}N_{DMSO})\times100$

*Identified in the second experimental plate and introduced as negative control from plate 5 to 15.

Z′-factor was 0.63±0.1 and 0.65±0.1 when considering resveratrol and dipyridamole, respectively. These values are considered optimal for an HTS assay (Zhang et al., 1999). During the primary screening, we also evaluated the toxicity of all tested compounds by an *in situ* fluorescence-based assay (Table 1 and see Materials and Methods for details). We therefore normalized the activity of the luciferase reporter gene driven by the *ACVR1* promoter with a fluorescence signal proportional to the number of viable cells at the end of the treatment. This allowed us to select molecules not affecting cell viability, inducing a reduction in the luciferase activity of at least 0.4- or an upregulation of at least 2.4-fold compared to cells treated with DMSO (Table S1).

Validation assays of dipyridamole

According to our inclusion criteria, the primary screening provided a list of compounds putatively working as activators (4 hits) or inhibitors (18 hits) of *ACVR1* transcriptional activity (listed in Table S1). Among these latter molecules, we found that dipyridamole (abbreviated henceforth as Dipy), was the compound that, during the retesting of primary hits, generated the

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most reproducible and significant results. Therefore, Dipy was selected for further experimental confirmations.

Dipy showed a dose-dependent suppression of the luciferase activity driven by the *ACVR1* promoter, with the strongest effect at 50 µM (Fig. 1A). The inhibition was detectable after 6 h of treatment for the highest dose, further increasing at 24 h (Fig. 1B).

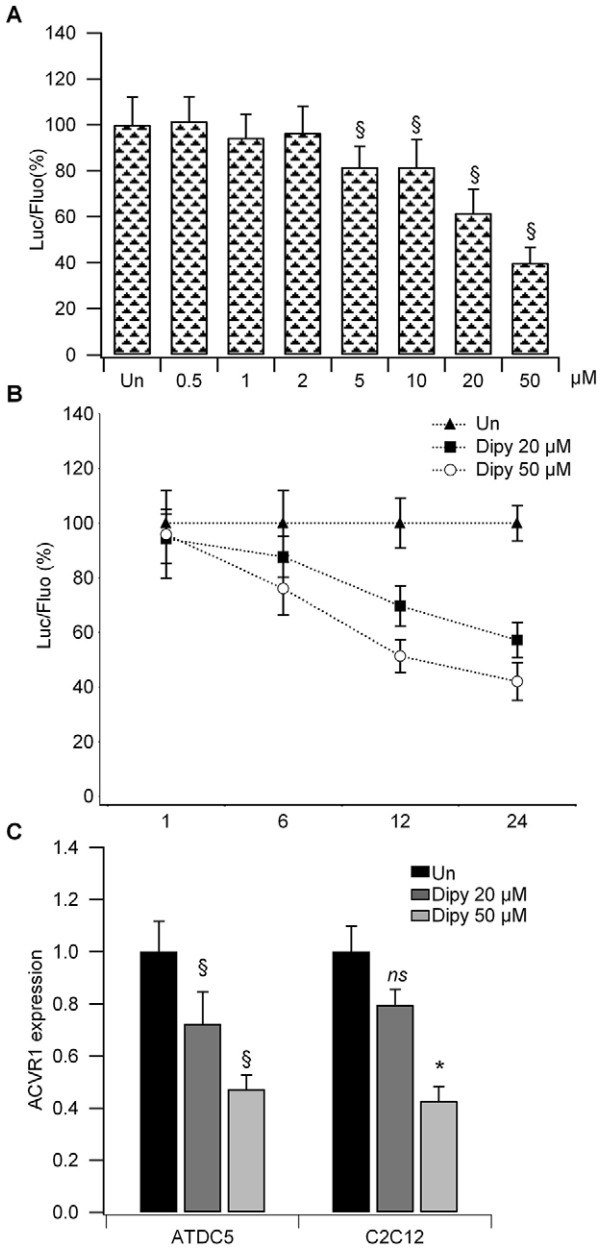

Fig. 1. Cellular assays of Dipy treatment. (A) Dose-response curve of Dipy on the luciferase reporter gene controlled by the promoter region of *ACVR1* in ATDC5 cells (Pr2.9-Luc). The ratio of luciferase (Luc)/fluorescence (Fluo) was normalized to that obtained with DMSO [untreated (Un); value 100]. Bar graph represents the mean and s.d. of three independent experiments. $^{\S}P<0.001$. (B) Time course of Dipy treatment in ATDC5 Pr2.9-Luc clones. The ratio of luciferase/fluorescence was normalized to that obtained with DMSO (Un; value 100) for each time point. (C) Effect of Dipy on the expression of *ACVR1* mRNA in native ATDC5 and C2C12 cells. Values were normalized on *GAPDH* and *β2M* and compared to expression level measured in cells treated with DMSO (Un). Bar graphs represent the mean and s.d. of at least three experiments, $^{*}P<0.01$, $^{\S}P<0.001$, ns, non-significant.

Normalization of the luciferase activity and monitoring of cell viability were obtained as described for the primary screening.

In accordance with the inhibitory effect of Dipy on the promoter of the *ACVR1* gene, we found that Dipy was able to downregulate the expression of *ACVR1* mRNA, as assessed by reverse-transcription quantitative PCR (RT-qPCR), both in native ATDC5 and C2C12 (mouse myoblast cell line) cells (Fig. 1C). After 24 h of treatment, we observed a gene-expression reduction of nearly 20% at 20 µM and 60% at 50 µM.

The effect of Dipy on the expression of genes encoding other type I and II receptors of the BMP family was also tested (Fig. S3). The highest degree of mRNA reduction was exerted on *ACVR1* (*Alk2*) but was also observed with *Alk3* and *BMPRII*. *Alk5*, involved in the growth differentiation factor (GDF)–transforming growth factor β (TGF-β) signaling cascade, and *Alk4*, *ActRIIa* and *ActRIIb* showed a low level of expression that was not affected by Dipy. Other type I receptors, such as *Alk1*, *Alk6* and *Alk7*, were not expressed in ATDC5 cells.

Effect of dipyridamole on the Smad-dependent BMP pathway

In order to test the effect of Dipy on the activation state of the Smad-dependent BMP signaling pathway, we generated ATDC5 clones stably expressing the luciferase reporter gene under the control of a minimal promoter carrying a BMP-responsive element (BRE-Luc) isolated from *Id1*, a well-known BMP target gene (Monteiro et al., 2008). Cells were treated with Dipy in the presence of BMP2 for 6 h. As reported in Fig. 2A, Dipy weakened the amplitude of the activation induced by BMP2 in a dose-dependent manner. Consistently, we found a downregulation in the mRNA expression of native *Id1*, *Id2* and *Id3* target genes, as assessed by RT-qPCR in ATDC5 cells (Fig. 2B), and a significant reduction in the phosphorylation state of the Smad1/5 proteins both in ATDC5 and C2C12 cells (Fig. 2C,D and Table S2 for immunoblot densitometric analysis).

Effect of dipyridamole on chondrogenic differentiation

The heterotopic bone that forms in individuals with FOP derives from an endochondral ossification process. ATDC5 cells are able to differentiate into mature chondrocytes when grown in three-dimensional (3D) cultures in differentiating medium (Tare et al., 2005).

ATDC5 cells were induced to develop 3D pellets in the presence of differentiating medium (DM), with and without Dipy (50 µM). After 3 weeks of culture, pellets were embedded in paraffin, and histological sections stained with Alcian Blue to verify the deposition of glycosaminoglycans typical of the cartilage extracellular matrix. As shown in Fig. 3A (left panels), compared to what was observed in proliferative medium (PM), pellets grown in DM are characterized by the presence of cells with peculiar morphology, with typical lacunae embedded in the extracellular matrix. By contrast, pellets grown in the presence of Dipy, both in PM and DM, showed the presence of small and undifferentiated cells (Fig. 3A, right panels).

The result was confirmed in ATDC5 cells cultured in alginate spheres. In the presence of inductive medium, we observed changes in cell morphology correlating with the differentiation state (Fig. 3B, upper panels). By contrast, treatment with Dipy induced a significant reduction of extracellular-matrix deposition as assessed by Alcian Blue staining of sections (Fig. 3B, left panels) and reduced expression of matrix proteins Sox9 and collagen II (Col II) as assessed by immunohistochemical analysis with specific antibodies (Fig. 3B, central and right panels, respectively).

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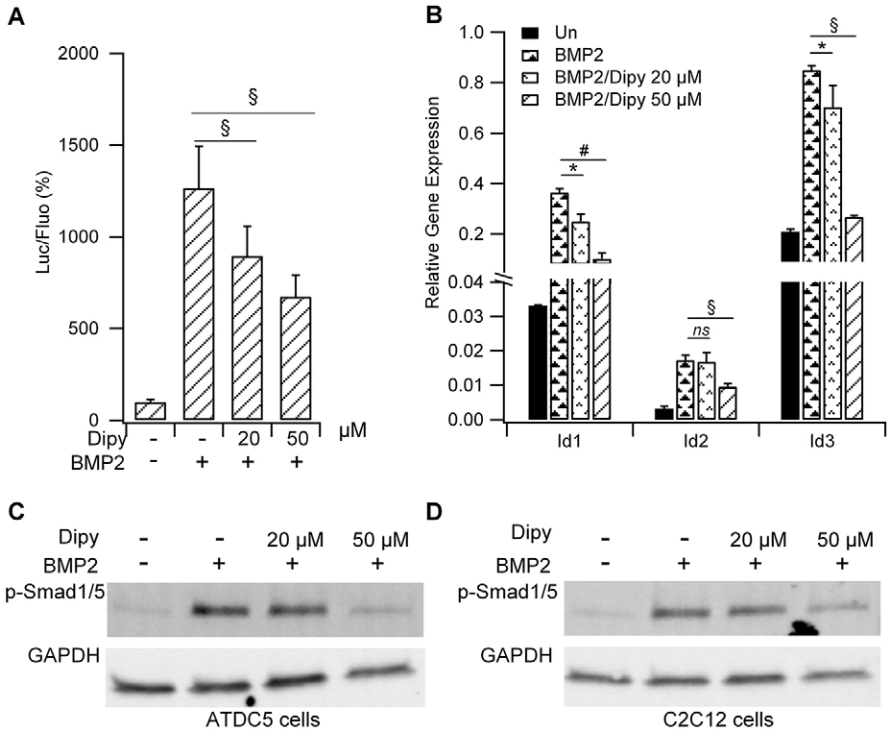

Fig. 2. Effect of Dipy on the BMP-mediated signaling pathway. (A) Luciferase activity measured in ATDC5 BRE-Luc cells treated with the indicated doses of Dipy and activated with [50 ng/ml] BMP2. The ratio of luciferase (Luc)/fluorescence (Fluo) was normalized to that obtained with DMSO (value 100). Bar graph represents the mean and s.d. of three independent experiments. $^{§}P<0.001$. (B) Effect of Dipy on the expression level of *Id1*, *Id2* and *Id3* BMP target genes in native ATDC5 cells. Values were normalized on the *β2M* reference gene (relative quantification by the ΔC_t method: ratio reference/target=$2^{\Delta Ct}$). Bars represent the mean and s.d. of three independent experiments. ns, non-significant; $^{*}P<0.05$, $^{\#}P<0.01$, $^{§}P<0.001$. (C,D) Effect of Dipy on the activation of the Smad-dependent pathway. ATDC5 (C) and C2C12 (D) cells were treated with Dipy and activated with [200 ng/ml] BMP2 for 1 h.

In accordance, RT-qPCR analysis on mRNA extracted from cells cultured in alginate spheres showed that the expression level of *ACVR1* and markers of cartilage differentiation [*Runx2*, *Sox9*, *Col II* and collagen X (*Col X*)] was downregulated upon Dipy treatment compared to untreated cells (Fig. 4).

Effect of dipyridamole on osteogenic differentiation

We also investigated the effect of Dipy on the osteoblastic transformation of C2C12 cells upon BMP2 induction (Katagiri et al., 1994). As shown in Fig. 5A and B, Dipy caused a dose-dependent reduction in alkaline phosphatase activity without significantly affecting cell viability (Fig. S4). The effect was accompanied by a downregulation of the mRNA of markers typical of the osteoblastic differentiation – Runx2, osterix and osteocalcin – which was statistically significant at the highest dose (Fig. 5C). During the differentiation process, in the presence of Dipy, we confirmed the reduction in the expression of *ACVR1* mRNA.

Effect of dipyridamole on heterotopic ossification in a BMP-induced mouse model

We examined the effect of Dipy on a BMP-induced model of HO *in vivo*. C57BL/6 2-month-old mice were injected with BMP2 intramuscularly in the quadriceps and treated with vehicle or 10 mg/kg (body weight) Dipy, administered daily by intra-peritoneal (IP) injection as described in Wang et al. (2013), according to two different experimental protocols as schematically represented in Fig. S5. Serum concentration of Dipy in mice was assessed according to Oshrine et al. (2005), and results were comparable to what was described in the same work (not shown).

Ossicle formation and HO volume were evaluated by µCT scan after 10 (n=6 for each group, Fig. S5 protocol A) and 21 (n=11 for each group, Fig. S5 protocol A) days of treatment. After 10 days of treatment, we observed highly variable volumes of HO (mineralized volume, mm^3) in control mice and no significant difference in HO volume was observed in treated mice compared to controls (Fig. 6A,B). By contrast, after 21 days of treatment, µCT scans showed a significant reduction of HO volume in mice treated with Dipy compared to controls (Fig. 6C,D). Histological analysis revealed that HO lesions (Fig. 7A) in treated mice were reduced, possibly due to a delay in maturation. In particular, Toluidine Blue staining indicated a reduced deposition of cartilage matrix, also at 10 days of treatment (Fig. S6), whereas Alizarin Red staining at 21 days and quantification of the area of calcium deposition showed a decrease in lesions of Dipy-treated mice (Fig. 7B,C), in agreement with the µCT scan results.

Smad1/5 phosphorylation in the injured tissue was assessed at the two different time points, 10 and 21 days, by immunofluorescence with a specific anti-phospho-Smad1/5 (p-Smad1/5) antibody.

We observed that, at 10 days after injury, the number of cells showing Smad phosphorylation was higher than at 21 days. This is consistent with the ongoing osteogenic differentiation of the HO lesions at the early time point, when the extent of mature heterotopic ossification was still comparable in untreated and treated mice, as described above. This is also indicated by the shape and intensity of p-Smad staining per cell. Interestingly, the effect of Dipy at this stage was already detectable as a statistically significant reduction in the expression of p-Smad1/5 (Fig. S7). This decrease was still present as a trend at 21 days of treatment (Fig. S7B), when the overall number of p-Smad1/5-positive cells was reduced in the lesions of both control and treated mice.

When Dipy was administered to the mice starting from 10 days after the ossification trigger by BMP2 (Fig. S5, protocol B), the µCT-scan analysis of ectopic lesions (Fig. S8A) showed a trend of reduction (P=0.074) of the HO volume increase between 10 and 21 days (Fig. S8B,C). Histological analysis revealed that, at day 21, calcium deposition was also significantly reduced as assessed by Alizarin Red staining (Fig. S9A) and corresponding quantification (Fig. S9B).

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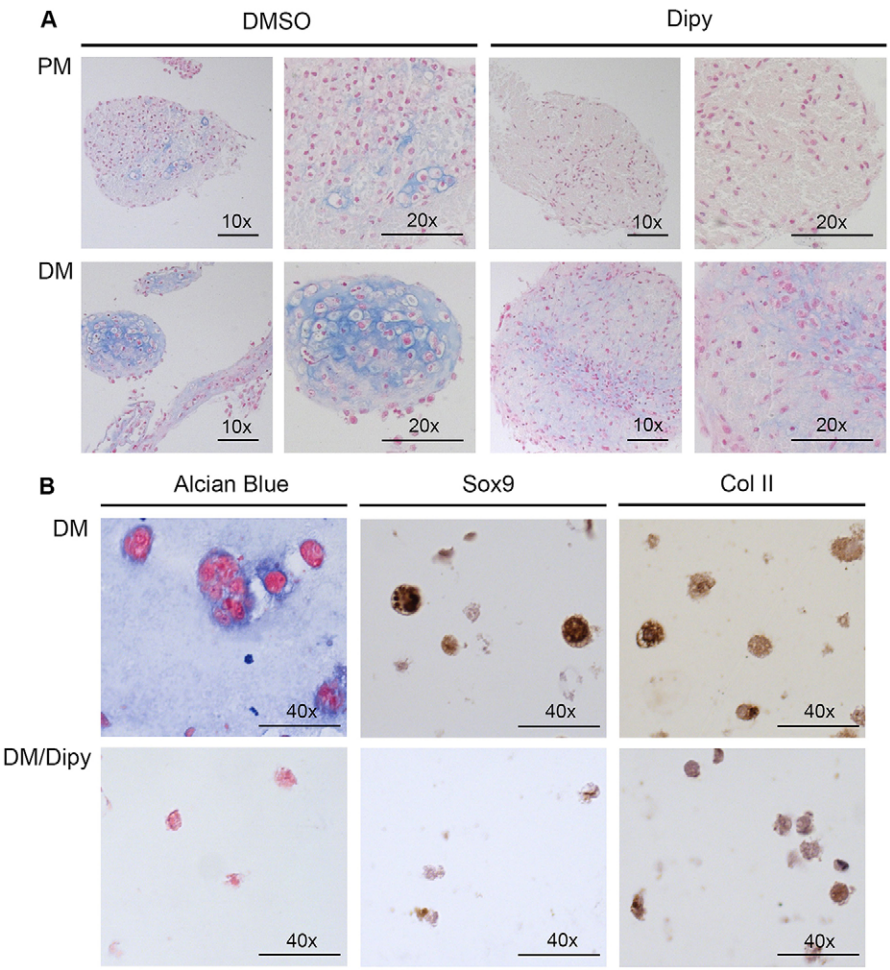

Fig. 3. Histological analysis of ATDC5 chondrogenic differentiation. (A) Alcian Blue staining of sections from ATDC5 cell 3D cultures grown in proliferative medium (PM, upper panels) and in differentiation medium (DM, lower panels), in the presence of 50 µM Dipy or DMSO. Scale bars: 100 µm (10×) and 50 µm (20×). (B) Histological and immunohistochemical analysis of ATDC5 cells cultured as alginate spheres grown in DM in the presence of DMSO or 50 µM Dipy (upper and lower panels, respectively). Scale bars: 25 µm.

DISCUSSION

To date, no therapy is available to prevent or control HO in FOP patients. Therefore, intense work is being carried out to find potential therapeutic intervention essentially based on inhibition of BMP signaling using different approaches (Kaplan et al., 2013).

The rationale basis of a therapeutic approach for FOP is that small molecules might function as inhibitors, thus correcting the hyper-functioning BMP signaling pathway(s), either by inhibiting directly the receptor function or the transcriptional or post-transcriptional expression of the encoding gene, which will in turn result in the quantitative reduction of the receptor protein.

Following the identification of dorsomorphin as an inhibitor of BMP type I receptors, through HTS in zebrafish (Yu et al., 2008b), other inhibitors have been described (Yu et al., 2008a; Cuny et al., 2008; Hao et al., 2010). Previously published work demonstrated that treatment of bone-marrow-derived mesenchymal stem cells (MSCs) with RAR-γ agonists negatively regulates BMP signaling. This is due to the reduction of the intracellular concentration of p-Smads by a post-translational mechanism of degradation, supporting the idea that quantitative reduction of components of this pathway might cause a reduction of signaling function (Sanvitale et al., 2013; Shimono et al., 2011; Sheng et al., 2010).

In the current work, we introduced an HTS approach aimed at identifying potential therapeutic candidates acting by modulation of the *ACVR1* gene expression. The primary screening was made possible by the generation of a cell system consisting of murine ATDC5 cells stably expressing the luciferase gene controlled by the 2.9-kb promoter region of *ACVR1* that was previously identified and functionally characterized by our group (Giacopelli et al., 2013). Our method was able to pinpoint molecules with both positive and negative effects. However, in the context of FOP pathogenesis, in which activating mutations of *ACVR1* cause an inappropriate BMP-mediated signaling, our interest was focused on molecules able to reduce the expression level of the gene.

In addition to the primary screening, our approach included assays to confirm the effect of candidate molecules on the different steps of the BMP pathway, on chondrogenic and osteogenic differentiation processes and on HO *in vivo*. The experimental procedure described in this workflow can also be exploited to test compounds able to affect BMP signaling, even when discovered by other cell-based HTS assays or by *in silico* virtual screening approaches.

An advantageous approach to search for innovative treatments for rare disease in a relative short time is to perform an HTS approach with a drug repositioning purpose (Muthyala, 2011; Li and Jones, 2012; Sardana et al., 2011; Yamamoto et al., 2013). To this aim, we screened a library of 1280 FDA-approved compounds. We identified a list of interesting molecules with positive or negative effect and decided to focus on the candidate with the most significant effect as a transcriptional inhibitor, dipyridamole.

Validation assays confirmed a specific negative effect of Dipy on the *ACVR1* gene expression and demonstrated that such an effect resulted in the attenuation of the entire BMP-specific signaling pathway. This was demonstrated by the reduction of BMP2-induced activation tested by the luciferase reporter gene under the control of BMP-responsive element (BRE-Luc). Consistently, this effect was

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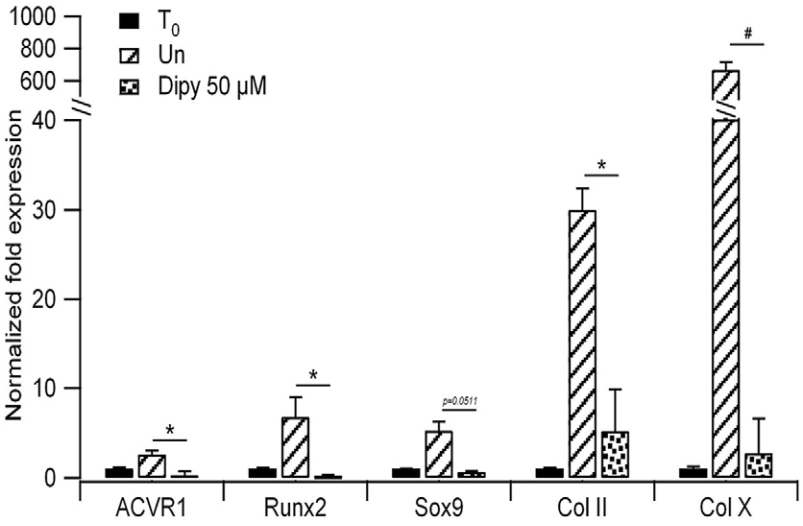

Fig. 4. Effect of Dipy on the expression of chondrogenic markers. RT-qPCR on RNA extracted from ATDC5 cells cultured as alginate spheres for 14 days in differentiation medium. Bars show mean and s.d. of three independent experiments. Expression levels were normalized on *GAPDH* and *18S* and compared to that of cells at T_0 (cells harvested at the beginning of the differentiation protocol). Un, untreated cells. ns, non-significant; **P*<0.05, §*P*<0.001.

confirmed as reduced expression of the Smad-signaling target genes *Id1*, *Id2* and *Id3*, and as reduced phosphorylation of Smad1/5 mediators.

Although the highest effect of Dipy treatment was found to impact *ACVR1* (*Alk2*) expression, we found that Dipy could also affect the expression of other BMP receptors that can synergistically contribute to the downregulation of the overall BMP signaling, such as, among type I receptors, Alk3 or, among type II, BMPRII, which cooperates with ACVR1 as a type I partner in the receptor complex. It is of interest to note that Dipy did not affect the expression of specific receptors, such as *Alk5*, which is involved in cascade mediated by TGF-β type I, or *Alk4*, *ActRIIa* and *ActIIb*, which intervene in GDF-BMP signaling. This finding suggests that the downregulating effect of Dipy is mainly, but not exclusively, exerted on Alk2 (ACVR1), possibly because of common regulatory mechanisms for the expression of molecules belonging to the same family and participating in common pathways.

Because HO in FOP derives from an endochondral ossification process (Kaplan et al., 1993; Medici and Olsen, 2012; Shore, 2012), we set up assays to evaluate both chondrogenesis and osteogenesis. To simulate differentiation *in vitro*, we took advantage of the ability of ATDC5 cells to differentiate towards mature chondrocytes in 3D cultures, with cell morphological changes and deposition of glycosaminoglycans typical of the cartilage extracellular matrix. Using this assay, we observed that Dipy could inhibit chondrogenic differentiation.

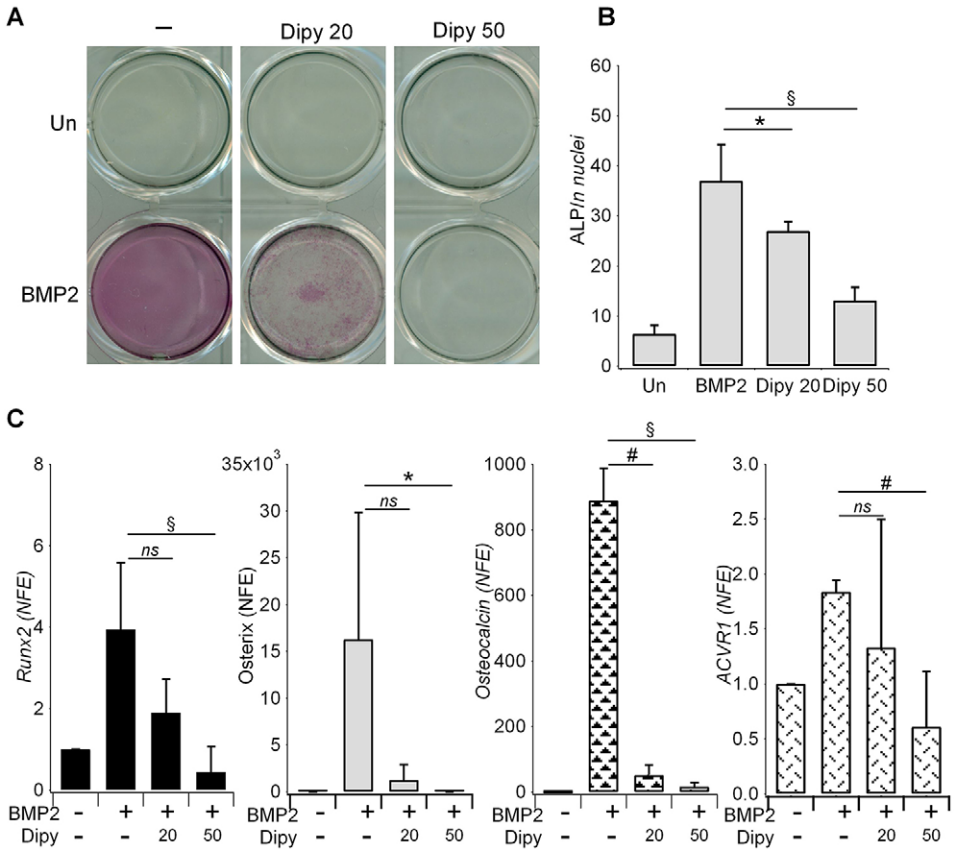

Fig. 5. Effect of Dipy on osteoblastic differentiation of C2C12 cells. (A) Specific staining for alkaline phosphatase activity in C2C12 native cells cultured for 6 days in the presence of [200 ng/ml] BMP2, ±Dipy as indicated (20 or 50 µM). Un, untreated. (B) ALP activity measured in C2C12 cells treated as in A, normalized against the number of nuclei (cells) obtained by automated count after Hoechst staining in each well (see Materials and Methods and Fig. S3). (C) Gene expression level of osteogenic marker genes in C2C12 untreated or treated with Dipy (20 or 50 µM). Bar graphs represent mean and s.d. of three independent experiments. mRNA levels were normalized on *GAPDH* and *18S* and compared to that measured in untreated cells (value 1). NFE, normalized fold expression; ns, non-significant; **P*<0.05, #*P*<0.01, §*P*<0.001.

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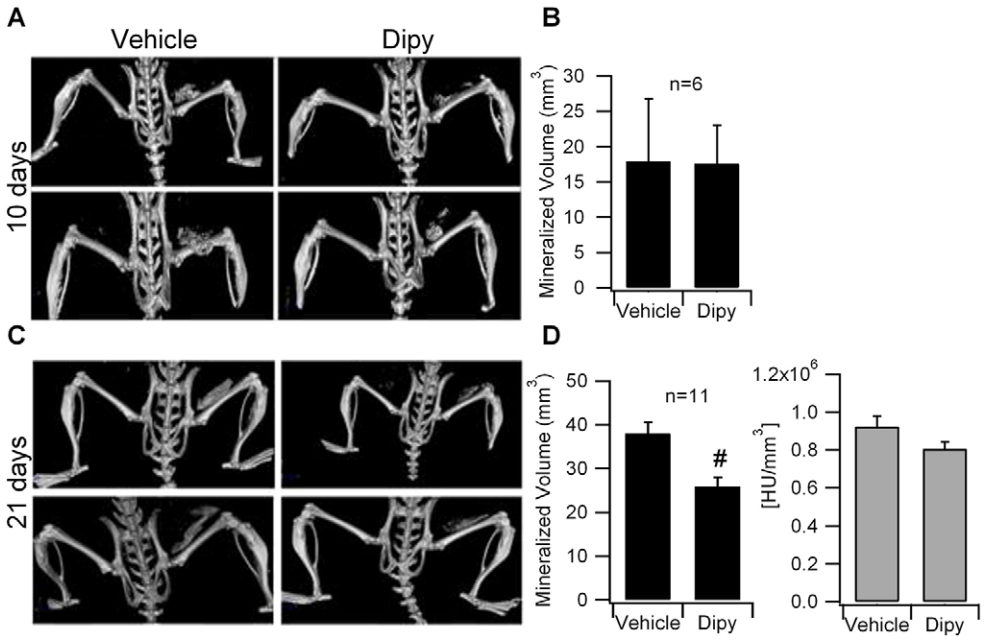

Fig. 6. Effect of Dipy on HO *in vivo*. (A) Micro-computerized tomography (μCT) scans of C57BL/6 mice treated with vehicle or Dipy (10 mg/kg body weight) for 10 days. (B) Quantification of the mineralized ossicle volume. *n*=6 mice/condition. (C) μCT scans of C57BL/6 mice after 21 days of treatment with vehicle or Dipy (10 mg/kg body weight). (D) Quantification of the mineralized ossicle volume (left panel) and lesion bone density (HU/mm^3, right panel). *n*=11 mice/condition. Bars represent mean and s.e.m. $^{\#}P<0.01$. HU, Hounsfield unit.

C2C12 cells were used to evaluate osteogenic differentiation, which was inhibited by Dipy as indicated by the reduction in the alkaline phosphatase (ALP) activity and expression of different markers (Runx2, osterix and osteocalcin).

The effect of Dipy was also verified *in vivo* in a BMP-induced mouse model of HO (Medici et al., 2010). During the induction of the ectopic ossification process, triggered by the implantation of a BMP2-embedded Matrigel coupled to cardiotoxin (CTX) injection, muscle fibers degenerate and the site of injury is infiltrated by different populations of inflammatory cells that contribute to the orchestration of the subsequent repair/differentiation process (Zordan et al., 2014; Rigamonti et al., 2014). During the first week after injury, progenitor cells of different origin are then recruited to the site of the lesion (Bentzinger et al., 2013) and committed towards the endochondral ossification process by the local presence of BMP2. At 10 days, HO lesions are not completely differentiated, and recruitment and activation of cells is ongoing: this was consistent with our finding that, at this stage, the overall number of cells able to respond by activating a specific BMP2/Smad-dependent signaling was higher than what was observed at 21 days after BMP2 induction.

At the earliest time point, the effect of treatment with Dipy became evident as a statistically significant decrease in the number of cells expressing an activated BMP/Smad pathway inside the HO lesions that finally resulted in the reduction of the volume of mineralized heterotopic ossicles, of decreased deposition of extracellular matrix and of a reduced area of calcified nodules that we observed after 21 days of treatment.

Most interestingly, Dipy effect was evident also when mice were treated after the establishment of heterotopic ossification, and resulted in a reduced calcium deposition within the ectopic bone and a decreased mineralization. However, concerning the choice of an *in vivo* prevention strategy versus a treatment on established/ongoing lesions, it is important to consider that the course of FOP is episodic with quiescent phases, lasting even for years, and acute phases that can be triggered by several types of recognizable stimuli (trauma, vaccinations, infection, iatrogenic harms etc.), but that can also occur apparently spontaneously or, more likely, without a recognizable trigger. In this context, a treatment for FOP is ideally a drug that can be administered chronically or for long periods of time in order to prevent the occurrence of even unpredictable flare-ups, thus counteracting their consequences.

Dipy is a commercially available drug that was introduced on the market more than 50 years ago as a coronary vasodilator (Kadatz, 1959). At present, it is widely used as an antithrombotic and vasodilator agent both as monotherapy and in combination with aspirin to prevent secondary stroke or transient ischemic attack (Gresele et al., 2011; Balakumar et al., 2014; de Vos-Koppelaar et al., 2014). At the pharmacological level, Dipy acts by different mechanisms. By inhibiting the activity of phosphodiesterases 5 and 3 (PDE5 and PDE3), it increases the intracellular level of cyclic adenosine monophosphate (cAMP), which is a potent inhibitor of platelet activation, and of cyclic guanine monophosphate (cGMP), which has a vasodilator effect on smooth muscle, thus potentiating the platelet inhibitory actions of prostacyclin (PGI2) (Gresele et al., 2011; de Vos-Koppelaar et al., 2014; Kim and Liao, 2008; Yip and Benavente, 2011). Moreover, Dipy inhibits the re-uptake of adenosine by blocking the equilibrative nucleoside transporters (ENTs), thus increasing plasma levels of this nucleoside, which also plays a role in inhibiting platelet aggregation (Kim and Liao, 2008; Visser et al., 2005; Dresse et al., 1982; German et al., 1989), regulation of vascular tone, vasodilation, immunity and inflammation (Kim and Liao, 2008).

It should be noted that the effect of Dipy in our *in vitro* assay of C2C12 cells was not in accordance with other *in vitro* experiments performed to correlate adenosine level to osteoblast differentiation (Costa et al., 2011; He et al., 2013). This discrepancy might be due to differences in the cellular model and experimental conditions. Moreover, very recently, Mediero and colleagues (2015) reported that local daily injection of Dipy administered with a collagen sponge was able to induce bone regeneration and proposed this treatment as an alternative to recombinant human BMP2 (rhBMP2). At difference with the above report, in the BMP-induced *in vivo* model that we used, HO was locally triggered in quadriceps muscles, whereas treatment with Dipy was systemic, by daily IP injection. Moreover, it has been shown that, in humans, blood cells tend to accumulate the drug (Serebruany et al., 2009), and Dipy is able to inhibit at the mRNA level the production of TNF-α and

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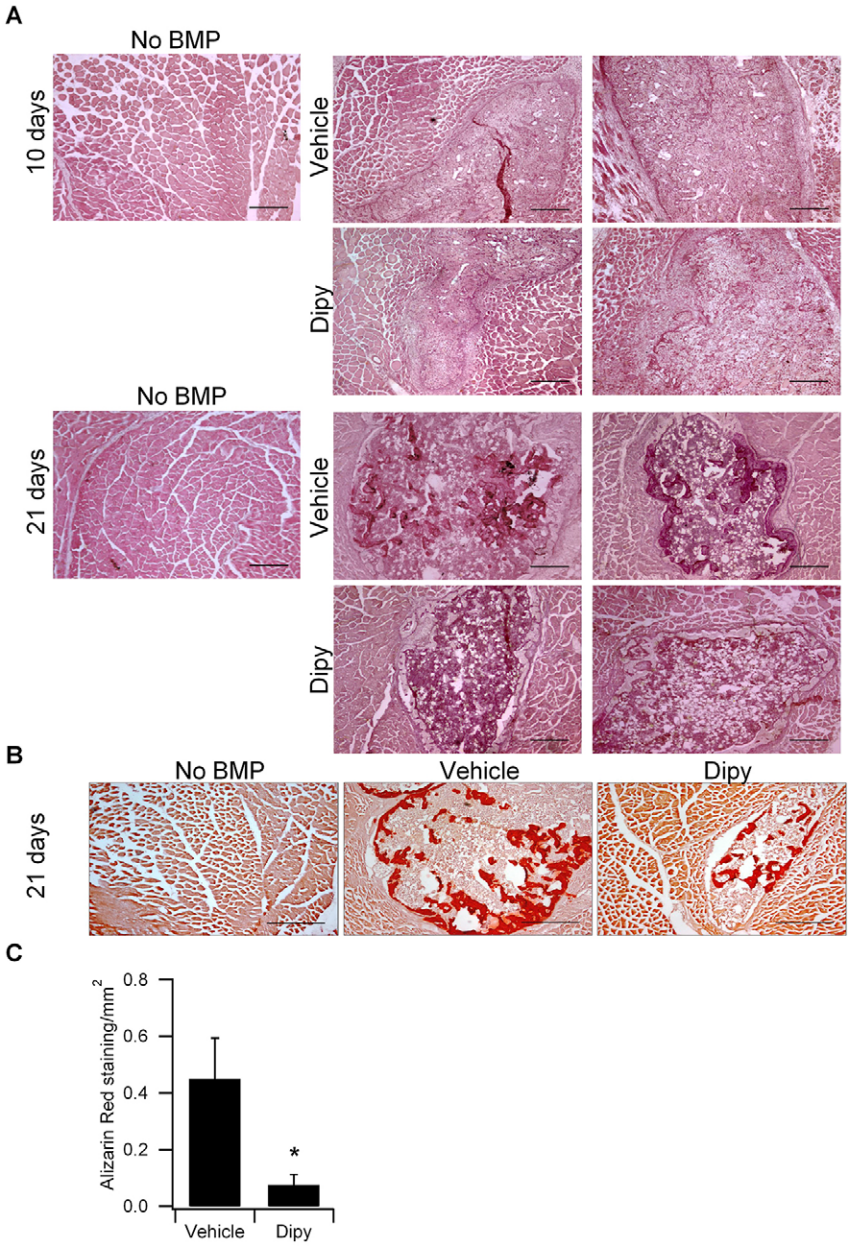

Fig. 7. Chronic Dipy treatment decreases BMP2-induced HO lesions and calcium deposition. (A) Hematoxylin and eosin (H&E) staining showing lesions in muscle injected with BMP-2 and cardiotoxin in mice treated with vector or Dipy for 10 or 21 days. No lesions appear in muscle damaged without BMP. Scale bars: 1 mm. (B) Alizarin Red S staining showing calcium deposition in tissue sections of muscle injected with BMP2 and cardiotoxin in mice treated with the vehicle or Dipy for 21 days. Scale bars: 500 μm. (C) Graph representing the relative quantification of the Alizarin-Red-S-positive area. Data represent mean±s.e.m. *$P \leq 0.05$ (n=3, Dipy versus control).

MMP-9 of peripheral blood mononuclear cells (PBMCs) and derived macrophages (Massaro et al., 2013). Because several types of immune cells are recruited at the site of HO lesions, this might contribute to the overall effect observed in our HO model.

Several observations suggest a role of an immune-mediated response in FOP pathogenesis, in particular a possible relevant contribution to the episodic neo-formation of ectopic bone. In humans, FOP flare-ups can be triggered or exacerbated by trauma, immunizations, medical procedures and infections, all of which are conditions in which the immune response is solicited/stimulated (for a review, see Kaplan et al., 2016). Both in humans and in animal models, histological examination of early pre-osseous lesions has clearly demonstrated that several types of immune cells, such as lymphocytes, monocytes/macrophages and mast cells, are readily recruited to these sites (Kaplan et al., 1993; Chakkalakal et al., 2012). In addition, it has been demonstrated that *in vivo* targeted ablation of macrophages or of macrophages and mast cells concomitantly leads to a significant reduction in the ectopic bone formation in FOP mouse models (Kan et al., 2009; Convente et al., 2015).

The deregulated BMP signaling in cells harboring the mutated *ACVR1* gene might contribute to the amplification of inflammatory pathways (Convente et al., 2015); moreover, it has been recently demonstrated that the presence of FOP mutations specifically confers to the mutated receptor the ability to respond to activin A (Hatsell et al., 2015; Hino et al., 2015). Activin A is a ligand member of the TGF-β superfamily that is rapidly released during inflammation and considered a crucial mediator of inflammation and immunity. Its activity is stimulated by inflammatory cytokines, Toll-like receptor ligands and oxidative stress, and it is involved in regulating growth and maturation of mast cells, monocyte/macrophage differentiation, and interaction between natural killer (NK) and dendritic cells (Funaba et al., 2003; Ogawa and Funaba, 2011; Aleman-Muench and Soldevila, 2012; Seeger et al., 2014).

Given the known pleiotropic effect of Dipy, with anti-inflammatory, anti-oxidant and anti-proliferative properties, and

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the complexity of the action of a drug *in vivo*, related to its absorption, metabolism and distribution, we cannot exclude that the observed decrease of ectopic ossification in our *in vivo* model might depend on the involvement of different pathways. However, we showed that Dipy is also able to affect specifically the Smad-dependent pathway in HO lesions of treated mice.

Summarizing, the overall effect of Dipy on the process of HO *in vivo* might be mediated by different mechanisms of action, such as the metabolic effect of extracellular adenosine, regulatory properties on differentiation and activation of immune cells, and anti-inflammatory action.

In conclusion, our study implicates this molecule as a candidate drug for the treatment of FOP, considering the great advantage that Dipy is already widely used in the therapy of cardiovascular disorders, and that safety and adverse effect profiles have already been evaluated and established.

MATERIALS AND METHODS

Chemicals and reagents

The Prestwick Chemical Library was purchased from Prestwick Chemical (Illkirch-Graffenstaden, France) and supplied in a special academic format with the 1280 FDA-approved compounds at 10 mM concentration in DMSO, in 16 96-well format plates, each containing 80 compounds.

Resveratrol (CAS no. 501-36-0, Enzo Life Sciences, Farmingdale, NY, USA) and dipyridamole (CAS no. 58-32-2, Sigma, Buchs, SG, Switzerland) were dissolved in DMSO and prepared as 1 M and 200 mM stock solutions, respectively.

Recombinant human BMP2 [rhBMP2; Chinese hamster ovary (CHO)-derived, R&D Systems, Minneapolis, MN, USA] was prepared as a 100 µg/ml stock solution in 4 mM HCl containing 0.1% bovine serum albumin (BSA; Sigma-Aldrich, Buchs, SG, Switzerland).

Antibodies for western blot analyses were: anti-p-Smad1/5 (13820S, Cell Signaling, Danvers, MA, USA), anti-GAPDH (MAB3749, Millipore, Billerica, MA, USA), and horseradish peroxidase (HRP)-conjugated anti-rabbit and anti-mouse secondary antibodies (Dako, Glostrup, Denmark). For immunohistochemical analyses, the following antibodies were used: rabbit anti-bovine polyclonal antibody anti-collagen type II (AB746P, Millipore, Billerica, MA, USA), rabbit polyclonal antibody anti-Sox9 (AB5535, Millipore, Billerica, MA, USA), and anti-rabbit (K4002) and anti-mouse (K4000) EnVision System-HRP Labelled Polymer (Dako, Glostrup, Denmark).

Expression plasmid preparation

The isolation of the genomic region, corresponding to the *ACVR1* promoter, was previously described by our group (Giacopelli et al., 2013). The whole 2.9-kb genomic fragment was subcloned in the pGL4.17 vector (Promega Corporation, Madison, WI, USA) upstream of the luciferase coding sequence as a reporter gene; this expression plasmid carries the Neomycin-resistance gene for selection of stable transfectants. The obtained reporter construct is reported as Pr2.9-Luc throughout the present work. A second reporter gene construct was also prepared by isolating a minimal promoter containing the BMP-responsive element (BRE) of the *Id1* gene from the pGL3-$(BRE)_2$Luc plasmid (kindly provided by Dr Peter ten Djike, Leiden University Medical Center, Leiden, The Netherlands) (Monteiro et al., 2008). BRE was transferred in to the pGL4.17 vector upstream of the luciferase reporter gene (referred to as BRE-Luc) plasmid, carrying the Neomycin-resistance gene that allowed the generation of stable transfectants as described below.

Cell culture

ATDC5 cells (mouse chondrogenic cell line derived from teratocarcinoma) were obtained from the Cell Bank of the Riken BioResource Center upon material transfer agreement (MTA); C2C12 myoblasts were purchased from the ATCC Cell Biology Collection (LGC standards, Bury, Lancashire, UK). ATDC5 cells were routinely cultured in complete medium consisting of 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium (DMEM/F12), containing 5% fetal bovine serum (FBS, Gibco, Thermo Fisher Scientifics, Waltham, MA, USA). C2C12 cells were cultured in DMEM containing 10% FBS. Both culture media were supplemented with 2 mM glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin (EuroClone® S.p.a., Pero, MI, Italy), and cells were maintained at 37°C in a humidified atmosphere with 5% CO_2. Where indicated, in depletion media, FBS was replaced by 0.1% BSA (Sigma-Aldrich, Buchs, SG, Switzerland).

Transfection and generation of the cellular system

For stable transfection, ATDC5 cells were plated in 100-mm dishes at a density of $2\times10^4/cm^2$. The next day, cells were transfected with 30 µg of the Pr2.9-Luc and of the BRE-Luc constructs, using the Lipofectamine 2000 reagent protocol (Invitrogen, Thermo Fisher Scientifics, Waltham, MA, USA). After 24 h and for 2 weeks, transfected cells were maintained in complete medium containing 400 µg/ml of Neomycin/G418 (Sigma-Aldrich, Buchs, SG, Switzerland) as selective agent. Thereafter, Neomycin-resistant clones were picked up and expanded. For each clone, 1×10^5 cells were collected after every cell-culture passage and lysed to evaluate the Luciferase activity with the ONE-Glo™ Luciferase Reporter Assay (Promega Corporation, Madison, WI, USA) according to the manufacturer's instruction. Clones showing stable expression of the reporter gene over the time course were considered suitable for our purposes and used to set up the culture and treatment protocols in 96-well format plates.

Screening of the Prestwick Chemical Library in ATDC5 cells

A selected clone of ATDC5 Pr2.9-Luc was seeded into 96-well plates in depletion medium (3×10^5 cells/well). After overnight culture, cells were treated with compounds at the final concentration of 20 µM and 2 µM. We tested 80 molecules in each plate; cells in columns 1 and 12 were treated with 1% DMSO as neutral control, resveratrol (10 µM) as positive control and dipyridamole (20 µM) as negative control (8 wells for DMSO, four wells for each control).

After 24 h, we measured the effect of the compounds on both cell viability and Luciferase activity by using the ONE-Glo™+Tox Luciferase Reporter and Cell Viability Assay (Promega Corporation, Madison, WI, USA) as suggested by the manufacturer. In brief, 20 µl of the CellTiter-Fluor Reagent were added *in situ* to living cells; after 1 h at 37°C, a fluorescent signal proportional to the number of viable cells in the culture well was measured by Glomax Multi Detection System (Promega Corporation, Madison, WI, USA). 100 µl of the second ONE-Glo Reagent were then added directly to each well to allow cell lysis and detection of the luciferase signal (Glomax Multi Detection System, Promega Corporation, Madison, WI, USA).

Fluorescence (fluo) and luminescence (*ACVR1* promoter activity; Lum) raw data were handled with the Instinct Software (Promega Corporation, Madison, WI, USA) and analyzed as an Excel spreadsheet. Cell viability (Vi) was first evaluated by comparing the fluo signal obtained in cells treated with compounds ($fluoC_x$) versus that of cells exposed to the vehicle [$Vi=(fluoC_x/\text{average } fluo_{DMSO})\times100$, with $0\geq Vi\geq100$]. In parallel, the effect of compounds (E) on *ACVR1* transcriptional activity was evaluated as follows: first, by normalizing luminescence signal over the fluorescence signal for each test well ($N_x=LumC_x/fluoC_x$) and for the neutral control ($N_{DMSO}=Lum_{DMSO}/fluo_{DMSO}$), then by comparing the normalized values of compounds with that of the vehicle [$E=(N_x/\text{average}N_{DMSO})\times100$].

RNA extraction and quantitative RT-PCR (RT-qPCR)

For expression studies, treated and untreated cells (ATDC5 and C2C12) were harvested and total RNA was isolated by using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA), according to the provided protocol.

RNA was quantified with a Nanodrop Spectrophotometer (Thermo Scientific, Thermo Fisher Scientifics, Waltham, MA, USA), and first-strand cDNA was synthesized by the Advantage RT-for-PCR Kit (Becton) from 200 ng of total RNA.

Expression of endogenous *ACVR1* gene and of selected markers was evaluated through RT-qPCR using specific TaqMan Gene Expression Assay (Life Technologies, Thermo Fisher Scientifics, Waltham, MA, USA) (see Table S3 for specification). Samples were measured in triplicate and the results were normalized on reference genes *18S*, *GAPDH* and β2-microglobulin (*β2M*), depending on the cell line. qPCR was run on the IQ5

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instrument from Bio-Rad and data analysis was performed using the provided Bio-Rad iQ5 software for Gene Expression Study.

Western blot

For detection of p-Smad, 1.2×10^6 cells were plated in 100-mm dishes in 1:1 complete/depletion medium for ATDC5 cells, and in depletion medium for C2C12 (DMEM containing 1% FBS). The next day, serum-starved cells were treated with Dipy for 24 h and, where indicated, with BMP2 (R&D Systems, Minneapolis, MN, USA) 200 ng/ml for 1 h. Cells were then washed once with PBS and lysed in 1× RIPA buffer (50 mM Tris HCl pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholic, 0.1% SDS) containing phosphatase and protease inhibitors (PhosSTOP cocktail and Complete tablets, Roche, Basel, Switzerland). Protein concentration was determined by the Pierce™ BCA Protein Assay Kit (Thermo Scientific, Thermo Fisher Scientifics, Waltham, MA, USA) according to the manufacturer's protocol and 15 µg of total lysates run onto precasted 4-15% Mini Protean®TGX-gels (Bio-Rad, Hercules, CA, USA). Proteins were transferred onto PVDF membrane (Millipore, Billerica, MA, USA) and probed with the indicated primary antibody at 4°C overnight. After incubation with HRP-conjugated secondary antibodies, protein bands were revealed by chemiluminescence with the ECL kit (Pierce, Thermo Fisher Scientifics, Waltham, MA, USA) and detected with the ChemiDoc instrument (Bio-Rad, Hercules, CA, USA). Densitometric analysis of western blot signals was performed by using the ImageJ software.

Culture in 3D pellets

ATDC5 cells were trypsinized from monolayer cultures and 1 ml of cell suspension with 5×10^5 cells in DMEM was added to 15 ml polycarbonate sterile tubes according to Tare et al. (2005). The cell suspension was centrifuged at 400 ***g*** for 10 min at 4°C to obtain pellets that were cultured both in standard complete medium and in chondrogenic medium containing 10 ng/ml TGF-β3 (Calbiochem, Millipore, Billerica, MA, USA), 10^{-8} M dexamethasone (Sigma-Aldrich, Buchs, SG, Switzerland), 100 mM ascorbate-2-phosphate (Sigma-Aldrich, Buchs, SG, Switzerland), 1× insulin-trasferrin-selenium (ITS) solution (Life Technologies, Thermo Fisher Scientifics, Waltham, MA, USA). Pellets were cultured for 21 days in a humidified incubator at 37°C and 5% CO_2. Pellets were swirled within to allow medium access to all sides and prevent adhesion to the inner walls of the tube. Once compact pellets were formed, both proliferative and differentiating media were replaced every 3 days and thereafter over the culture period. Three pellets from each group were harvested and processed for histological analysis.

Culture in alginate spheres

ATDC5 cells were cultured in alginate spheres according to Culbert et al. (2014). Briefly, cell suspensions at 6.7×10^6 cells/ml in 1.2% alginate acid sodium salt (Sigma-Aldrich, Buchs, SG, Switzerland) solution were extruded through 16-gauge needles as ∼30 µl drops in 30 ml of 102 mM $CaCl_2$ (Sigma-Aldrich, Buchs, SG, Switzerland) in order to allow sphere formation. After drop solidification, cells/alginate spheres were washed with PBS and cultured in chondrogenic medium, replenishing every 3 days. A number of alginate spheres for each condition were formalin-fixed and processed for histological stainings and immunohistochemical assays. In parallel, spheres were also incubated with 55 mM sodium citrate (Sigma, Buchs, SG, Switzerland) to recover cells for total RNA extraction and expression analysis of markers specific for chondrogenesis such as *Runx2*, *Sox9*, *Col II*, *Col X*, aggrecan and also *ACVR1* by RT-qPCR with TaqMan Assays probes (Life Technologies, Thermo Fisher Scientifics, Waltham, MA, USA) (see Table S3 for specification).

Histological analysis

Cell aggregates were fixed with 4% formaldehyde (Santa Cruz Biotechnology, Dallas, TX, USA) in PBS for 10-15 min, and embedded in paraffin according to standard protocols. Paraffin sections (5 µm) were obtained by microtome, dewaxed and rehydrated with decreasing ethanol solutions. For histological analysis, sections were stained with Alcian Blue 8GX (Sigma-Aldrich, Buchs, SG, Switzerland) following established procedures and viewed in transmitted and polarized light microscopy.

Immunohistochemistry

Dewaxed and rehydrated sections were incubated with 3% hydrogen peroxide in methanol for 30 min to inhibit endogenous peroxidase activity, rinsed in PBS/0.2% Triton X-100, then were subjected to digestion with 1 mg/ml hyaluronidase in PBS, pH 6.0 for 15 min at 37°C prior to use. Sections were exposed to normal goat serum (Dako, Glostrup, Denmark) 1 h before incubation with the primary antibodies (24 h, 4°C). Slides were then washed with PBS (four times for 5 min each) and incubated with the HRP-conjugated secondary antibodies for 1 h at room temperature (RT). The peroxidase reaction was developed using 3,3′-diaminobenzidine tetrahydrochloride (DAB) as chromogens. After rinsing in distilled water, sections were dehydrated in increasing ethanol solutions, cleared in xylene and mounted.

C2C12 cell culture and osteogenic differentiation

In order to induce C2C12 differentiation towards the osteoblastic lineage (Katagiri et al., 1994), 1.2×10^4 cells were seeded in 6-well plates and cultured in complete medium supplemented with 5% FBS (low-mitogen medium). The day after, cells were treated with 300 ng/ml BMP2 (R&D Systems, Minneapolis, MN, USA) for 6 days. Where indicated, Dipy (50 µM) was also added to both standard and differentiating media.

Cells were processed to evaluate the alkaline phosphatase (ALP) enzymatic activity by the Alkaline Phosphatase (Sigma Diagnostics, Buchs, SG, Switzerland) kit following the manufacturer's instructions and total RNA was extracted to evaluate the expression of *Runx2*, osterix and osteocalcin by RT-qPCR with TaqMan Assays probes. In order to quantify the ALP activity in C2C12 cells induced by BMP2 treatment, 5×10^3 cells were plated in CellCarrier-96-well™ microplates (Perkin Elmer, Waltham, MA, USA) and cultured in the presence of BMP2±Dipy as described. After 6 days, Hoechst 33342 Nuclear Stain (ENZ-51031-HOE33342, Enzo Life Sciences) was added to the culture medium at a 1:1000 dilution and incubated for 20 min. Cells were then visualized with the NIKON Ti Eclipse microscope; 16 640×490-µm fields for each well and condition were acquired and analyzed by the NIS-Elements AR software to obtain an automated count of the present nuclei. The number of nuclei has been used to normalize the ALP activity measured as follows. After analysis, cells were washed with PBS and incubated with 200 µl of the Alkaline Phosphatase Yellow liquid substrate system (nNPP) (Sigma, Buchs, SG, Switzerland). Reaction was stopped with 60 µl 3 M NaOH and ALP activity measured at 405 nm by Mithras LB940 plate reader (Berthold Technologies).

Heterotopic ossification *in vivo*

0.05 µg/µl of BMP2 (Peprotech, Rocky Hill, NJ, USA) in 200 µl growth-factor-reduced Matrigel (BD Biosciences) were injected intramuscularly in the quadriceps of C57BL/6 2-month-old mice (11 mice/group). The contralateral muscle was used as internal control and injected with Matrigel only. Both quadriceps were injected with 50 µl cardiotoxin 5 µM (CTX from *Naja mossambica mossambica*, Sigma-Aldrich, Buchs, SG, Switzerland) to increase muscle damage. Animals were anesthetized by inhalation of 2-bromo-2-chloro-1,1,1-trifluoroethane, ≥99% (CAS no. 151-67-7, Sigma-Aldrich, Buchs, SG, Switzerland) before the injection. 10 mg/kg (body weight) dipyridamole was administered daily IP to the treated animals (n=11, for Protocol A; n=5 for Protocol B, see Fig. S5) in a solution composed of 10% ethanol, 5% 2-pyrolidone, 12-15% propylene glycol, 10% Cremophor ELP, saline to 100% (Wang et al., 2013). Control mice (n=11, for Protocol A; n=5 for Protocol B, see Fig. S5) received the injection solution without drug. Mice were housed at the San Raffaele Institute SPF animal facility and were kept in pathogen-free conditions. All procedures were in accordance with Italian law and were performed under internal regulations for animal care and handling.

In vivo CT imaging of heterotopic ossification

At day 10 and 21 after BMP injection, *in vivo* micro-computerized tomography (µCT) scans were carried out to assess progression of ossification and any effect on the normal skeletal structure. *In vivo* µCT imaging was performed using the IVIS SpectrumCT Pre-clinical *in vivo* imaging system (Perkin-Elmer, Waltham, MA, USA). CT images were acquired without any contrast medium with the following parameters: x-ray

tube voltage=50 kV, tube current=1 mA, x-ray focal spot size=50 µm. The CT images calibrated in Hounsfield unit (HU) were reconstructed with a voxel size of 75 μm^3. Threshold-based image segmentation were performed to obtain a 3D reconstruction and quantification of the ossification.

The total mineralized volume V=N×voxel size (mm^3) was quantified using MIPAV (medical imaging processing analysis and visualization) and MATLAB software, where N is the number of voxels corresponding to bone derived from the image segmentation procedure. The bone density quantification was calculated by using the following formula: $\sum Ni=1\ HUi/V$.

Morphological and histochemical analysis of the heterotopic ossification

At 21 days after BMP injection, muscles were collected and processed for further morphological and histological analyses. BMP/Matrigel-injected and Matrigel-injected quadriceps from treated and control mice were frozen in liquid-nitrogen-cooled isopentane, to allow preparation of 10-µm-thick sections.

Muscle sections were stained with hematoxylin and eosin (H&E) (Sigma-Aldrich, Buchs, SG, Switzerland) or Toluidine Blue (Bio-Optica, Milano, Italy) or Alizarin Red (Sigma-Aldrich, Buchs, SG, Switzerland) according to the manufacturers' instructions. Images were acquired using a Nikon Eclipse E600 microscope (Nikon, Tokyo, Japan). To quantify heterotopic ossification, images of Alizarin-Red-stained sections were subsequently analyzed using the batch mode of the ImageJ vs1.49 macro. The color thresholding algorithm used by this macro is based on an algorithm written by G. Landini (version v1.8) available at http://www.mecourse.com/landinig/software/software.html.

Immunofluorescence on muscle sections

For immunofluorescence, 8-µm sections from OCT-embedded muscles were fixed with 4% PFA in PBS. They were permeabilized with a 0.2% Triton X-100, 1% BSA solution in PBS for 30 min at RT and then blocked in 10% serum, 1% BSA solution in PBS for 30 min before incubation with the primary antibody p-Smad1/5 (1:800; Cell Signaling, Danvers, MA, USA), after a demasking step in sodium citrate 10 mM pH 6 for 10 min between fixation and blocking steps (2 h). Alexa-Fluor-546-conjugated antibody (1:500; Invitrogen, Thermo Fisher Scientifics, Waltham, MA, USA) was used as second-step reagents. Specimens were counterstained with DAPI (Sigma, Buchs, SG, Switzerland) and analyzed using a Zeiss LSM710 confocal microscope. Images showing double fluorescence were first acquired separately using appropriate filters, then the different layers were merged using Adobe Photoshop CS4.

Statistical analysis

All luciferase reporter gene assays were performed in triplicate and repeated independently at least twice (2-5 times). Z′ factor was evaluated by using the formula $[Z'=1-3\times(\sigma_s+\sigma_c)/|\mu_s-\mu_c|]$, where σ_s and σ_c are the s.d. of positive or negative sample and of the solvent (control) and μ_s and μ_c represent the average. Experiments to evaluate gene expression by RT-qPCR were performed in triplicate from at least two independent RNA extractions. Both the non-parametric Mann–Whitney test (Social Science Statistics) and the unpaired two-tailed Student's *t*-test (GraphPad *t*-test Calculator; http://graphpad.com/quickcalcs/ttest1.cfm) were applied to verify statistical significance of the observed variations. Significant differences were given as $^{*}P<0.05$, $^{\#}P<0.01$ or $^{\S}P<0.001$.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

S.C. conceived, designed, performed and analyzed the experiments, and prepared the manuscript; L.T. and B.C. performed differentiation assays and 3D cultures; F.G. performed and analyzed RT-qPCR experiments; M.T., M.S. and A.G. performed *in vivo* experiments; L.J.V.G. provided support for HTS protocols and critical reading of the manuscript; A.E.S. performed mouse µCT scan analyses; S.B. supervised *in vivo* experiments and critical discussion of data; R.B. and R.R. conceived and supervised the experiments and manuscript preparation.

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Supplementary information

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CHAPTER V. Final Discussion

5.1. Summary

Endothelial to mesenchymal transition (EndMT) is a complex biological process in which resident cells lose their endothelial markers, acquire a mesenchymal phenotype and develop migratory ability. EndMT is critical for cardiac development, particularly in the formation of the valves and septa of the heart. By using lineage-tracing strategy, we demonstrated that embryonic endothelial derived progenitors contribute to skeletal and smooth muscle development, and that an early treatment with nitric oxide donor molsidomine enhances eVE-Cad^{+} contribution to embryonic and fetal myogenesis.

Although EndMT has positive effects in embryonic development, it has been traditionally considered to have negative effects in disease.

Our group provided new insight on the EndMT occurring during unbalanced repair of skeletal muscle. We demonstrated that macrophages are necessary for efficient vascular remodeling in the injured muscle. We confirmed that polarized M2 macrophages directly modulate EndMT in endothelial progenitors.

In addition, we characterized this process by performing Next Generation Sequencing on the isolated endothelial progenitors and identify Flt4 as a new potential regulator of the transition.

We have further clarified the involvement of endothelial progenitor to heterotopic ossification (HO) in muscle. Finally, with the aim of finding a new therapeutic strategy for preventing HO, we developed a high-throughput screening (HTS) and found dipyridamole, a drug currently used as a platelet antiaggregant, as a possible inhibitor of the process both *in vitro* and *in vivo*.

5.2. Transitional significance and future perspectives

Muscle based disease are manifested in a multitude of manners, from hereditary to acquired disorders. The classic example is muscular dystrophy, of which there are several major types, which can appear from infancy to adulthood, and which affect over thousands worldwide people. Those diseases are gaining more and more clinical relevance because of the length of the individual lifespan due to improved medical treatments. However, patients suffering from such illnesses are currently facing with a relatively short list of options, which include long term drug therapy (which may allow a disease to be managed, but rarely cure it) or organ transplant, often with significant adverse effects. In the last decades, gene and cell therapy become of great interest in this field of research: in gene therapy, mutated genes are replaced with normal ones, while in stem cell therapy diseased cells are exchanged with healthy cells. Although these approach raised hopes for treatment for muscular genetic diseases, it seems that one single approach is not sufficient to obtain great results and that a definitive cure is likely to emerge as a combination of more approaches. At the moment there is a focus on the therapeutic use of engrafted stem cells to treat degenerative diseases or aging; however, such efforts are best complemented by advancing our understanding of the basic biology of stem cell activation. The first approach of cell therapy for muscle dystrophy was based on satellite cells (SCs), the highly myogenic resident stem cell of the muscle. In 1990 the first transplant in a DMD patient showed safety and promising results. However, these clinical trials produced no clinical benefit[1-5]. The limited results observed was

mainly due to the rapid death of SCs in the first days after transplantation, poor migratory capacity of SCs and the immune responses toward the injected SCs, that lead to complete rejections in one weeks. The demonstration that multipotent cell types (different from SCs) can differentiate into skeletal muscle *in vitro* and in *vivo* has created an alternative possibility for cell therapy. In the last years several progenitors endowed with myogenic potential. Among these there are mesoangioblasts (MABs), mesoderm derived stem cells associated with small vessels and originally described in the mouse embryonic dorsal aorta. Preclinical studies in both dystrophic mice and dogs provided evidence that MABs have the capacity to functionally ameliorate the dystrophic phenotype[6, 7]. Based on this data, MABs are in phase I/II clinical trial for the treatment of pediatric DMD patients and, nowadays, they are the only cell type, in addition to SCs, that has reached clinical experimentation[8]. However, in-depth studies on their developmental origin, physiological role and biological characteristics are still missing.

Another kind of approach that reaches importance in these years aims to develop new strategies to activate and mobilize the endogenous stem cells. Ideally therapeutic approaches based on advantage since they involve minimal manipulation, do not present problems related to the immune response and are less invasive. Moreover, they would be significantly less expensive and thus affordable to public health systems. Regarding the endogenous MABs, an optimal intervention would require a deeper knowledge of their role during normal development, or their relationship with other vessel-associated cells with therapeutic potential[9]. Understanding how these endogenous

progenitors choose their fate could help to identify novel ways for *in vivo* manipulation, not only in adult life, but also during embryonic and fetal development.

Endothelial cells can exhibit a wide range of phenotypic variability depending on local physiologic needs throughout the vascular tree [10]. For example, during embryogenesis, endocardial–endothelial cells might transdifferentiate into mesenchymal cells (EndMT) during the formation of endocardial cushion tissue in the early embryonic chick heart[11-13]. Importantly our laboratory has shown that the hemogenic endothelium, a specialized embryonic endothelial population, can indeed give rise both to hematopoietic cells and mesenchymal progenitor cells. bearing characteristics of embryonic MABs and are able to physiologically contribute to different mesodermal lineages in the embryo, including the skeletal muscle.

Still there is a therapeutic need of molecules that could activate mesodermal or circulating stem cells.

In the first study of the present study we used a genetic lineage tracing approach to investigate whether the effects of nitric oxide in muscle repair could derive from an improvement in the myogenic differentiation of embryonic VE-Cadherin-expressing (eVE-Cad^{+}) progenitors during embryogenesis.

Several studies in animal models of muscle dystrophy have demonstrated that nitric oxide donors provide several beneficial effects, including modulation of the activity of endogenous cell populations involved in muscle repair and the delay of muscle degeneration[14-16]. One specific NO donor, Molsidomine, already used in humans for treatment of angina, was shown to slow disease

progression and to re-establish the functional capability of the damaged muscle[17, 18]. We demonstrated that Molsidomine treatment during embryogenesis results in a greater contribution of eVE-Cad^{+}-derived cells (embryonic MABs) to both embryonic and fetal myogenesis, while not affecting endothelial network formation (Tirone et al.[19], see Chapter II). We also provide evidence that Molsidomine treatment affects several important signaling pathways in the embryonic endothelium, possibly resulting in the expansion of a CD45+ non-MPs eVE-Cad^{+} population, that we have previously shown to include mesoangioblast-like cells. Next step will be to study mouse models of dystrophy in order to analyze the effect of molsidomine in a context in which normal components of the skeletal muscle development are altered. These findings will bear great therapeutic relevance as stem cell loss in dystrophic muscles begins as early as late embryogenesis in affected individuals[20].

Although endothelial plasticity regulate embryonic development and open the possibility to find new therapeutic strategy for treating degenerative disease, in the adult transition from endothelial cells to mesenchymal phenotype plays a critical role in various pathological processes such as cancer and fibrosis[21]. In postnatal organism, tissue damage and inflammation can stimulate the mechanism to give rise to fibroblasts and myofibroblasts that form scar tissue during wound healing or fibrotic disease. EndMT-dependent fibrotic phenotype contributes to diseases such as systemic sclerosis, atherosclerosis, pulmonary hypertension, and cerebral cavernous malformations[22-26]. In 2014 our lab described the first evidence of EndMT occurring in adult skeletal muscle. After an acute damage,

infiltrating macrophages represent a relevant endogenous source of cytokines and angiogenic factors, which support neo-angiogenesis during muscle regeneration by sustaining the differentiation of endothelial-derived progenitors toward an endothelial fate and by preventing the formation of a fibrotic scar. By contrast, in pathogenic conditions, when failure of correct macrophages occurs, the fate of endothelial-derived progenitors can be skewed to a fibroblastic phenotype, further contributing to an abnormal tissue remodeling and concurring to the severity of many chronic diseases[27].

With the idea to uncover new data on gene expression profiling and identify signaling pathway that mediate the transition, we have further characterized this process by performing Next Generation Sequencing on endothelial progenitors isolated form the muscle in different EndMT-inducing conditions.. In addition, we have set *in vitro* co-culture model that reproduce the transition occurring *in vivo* (Online Figure 1) to understand which subpopulation of polarized macrophages are involved in the modulation of endothelial precursors during EndMT. We demonstrate that anti-inflammatory macrophages directly modulate EndMT in endothelial progenitors. In addition, based on RNA-sequencing experiments, we also postulate the role of Flt4 (VEGF3) protein. Flt4 have an important role in the mechanism for rapid conversion “tip to stalk” of endothelial cells, which is required at contact points during angiogenesis[28-30]. Our work suggests a model where the ligand VEGF-C, produced by macrophages, binds Flt4 and alloy restoring the Notch signaling pathway (Notch4-Dll4), which leads to decrease sensitivity to growth factors (TGF- β) and supports neovascularization. When macrophages

are depleted, the balance shift toward an increase sensitivity to TGFβ and a mesenchymal phenotype.

Genes expression changes on freshly isolated endothelial progenitor during EndMT has revealed that vascular cells could de-differentiate into mensenchymal intermediate and, through appropriate signals and microenvironment, thereby participate directly in the process of endochondral ossification (See Fig.3 Chapter III). Endochondral ossification in skeletal muscle is a debilitating condition that could occur in cases of inflammation associated with severe injury, surgery or genetic disease[31]. The most extreme case can be found in a genetic rare disease, known as Fibrodysplasia ossificans progressiva (FOP). Patients with FOP are characterized by congenital toe malformation and experience periodic episodes of acute inflammatory processes that cause tumor-like swellings in muscle tissue, which lead to the formation of cartilage and bone in the inflamed region. Activating mutations in ACVR1 gene are responsible for FOP (See Chapter 1.2.4). Until some years ago, little was known about the molecular mechanism and cellular candidate that mediate this process. The endothelial origin of heterotopic ossification is still unclear: several studies provide different candidate cells[32]. Some work suggested an endothelial origin, other studies disagree[33-35]. Our lineage tracing of endothelial progenitors and the RNAseq analysis, provide new insight into the potential role of EndMT in this process. Our data suggest that vascular cells contribute the new bone formation in muscle in a mice model of heterotopic ossification induced by recombinant BMP2 (see Chapter III).

Cai et al. recently shows that ECs obtained from FOP human induced pluripotent stem cells (hIPSCs) have a reduced expression of vascular endothelial growth factor and spontaneously transform into mesenchymal cells through EndMT [36]. The issue of cellular origins of HO is important not solely for the clinical implications, but also because it highlights the basic biological processes that govern bone formation. It is likely that more than one subpopulation of stem/progenitor cells contributes to HO, and further studies are necessary to identify the predominant contributing subpopulations. Nonetheless, is crucial to find cells that indirect contribute to this process: this may eventually identify new cellular targets for healing intervention or for delaying disease's progress.

No established pharmacological treatment is available for FOP. Early diagnosis prevents unnecessary interventions, such as biopsies or surgical operations that can exacerbate the progression of the disease, and high-dose glucocorticoids are used in the management of inflammatory flare-ups.

In collaboration with the group of R.Ravazzolo, we have performed a high throughput screening (HTS) of a library of drugs already approved by FDA, in order to identify modulators of ACVR1 gene expression. We found that dipyridamole, commonly used as an antithrombotic and vasodilator drug, has an inhibitory effect on ACVR1 expression, as well as on the whole BMP signaling pathway, and it is able to inhibit chondrogenesis and osteogenesis, both in cellular assays and in a BMP-induced HO mouse model (See Chapter III). Further study are needed to understand the complexity of the action of the drug *in vivo* since it is very probable that the overall

effect of Dipyridamole on the in vivo HO process is mediated by different mechanisms of action, such as the metabolic effect of extracellular adenosine, regulatory properties on differentiation and activation of immune cells, and anti-inflammatory action[37].

In conclusion, endothelium has been shown to be a crossroad of several cell lineages during development. Study of the plasticity of endothelial cells during embryogenesis can be exploited to investigate new strategies to mobilize endogenous stem/progenitor cells and improve their contribution to skeletal or smooth muscle, with the ultimate goal to treat chronic and degenerative disease, for example with molecules like nitric oxide.

In adult, EndMT, the hallmark of endothelial plasticity, has emerged as a pathologic process in several diseases. We are the first group that have analyzed the impact of this process during skeletal muscle regeneration and investigated in detail the molecular mechanism that controls the transition. Furthermore, the results of this project suggest that greater understanding of the mechanism involved and its pharmacological modulation may represent an innovative therapeutic approach for the devastating effects of muscle diseases characterized by fibrotic accumulation and inefficient healing.

Our hypothesis is that EndMT could evolve as new paradigm that could be exploited to restore the homeostatic capacity of the tissue, breaking the vicious circle that leads to persistent inflammation/necrosis, defective neo-angiogenesis, with the final loss of tissue integrity and function, as in the case of heterotopic ossification.

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