A novel population of embryonic endothelial derived progenitors contributes to multiple mesodermal lineages during development and muscle regeneration

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CHAPTER I
1. GENERAL INTRODUCTION

1.1. SKELETAL MUSCLE DEVELOPMENT

It is generally accepted that all the skeletal muscles in the vertebrate body, with the exception of some craniofacial muscles, come from progenitors present in the somites [1]. The somites are derived from the mesoderm which, in the body (excluding the head), is subdivided into four compartments: the axial, paraxial, intermediate and lateral plate mesoderm. Somites are transient units which form in a cranio-caudal succession by segmentation of the paraxial mesoderm on either sides of the notochord and neural tube [2]. 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 (figure 1). The sclerotome contributes to the cartilage and bone of the vertebral column and ribs; the dermomyotome gives rise to the overlying derm of the back and to the skeletal muscles of the body and limbs. Myogenesis in the head follows a different route; some of the muscles of the head are derived from anterior, unsegmented paraxial mesoderm and from prechordal mesoderm [3]. Skeletal muscle in the mouse is established from embryonic day 8.5 to E18.5 (birth at around 19 days) with a further maturation during the postnatal period for about 3 weeks.
At the very onset of myogenesis (from E8.0 in the mouse) cells delaminate from the edges (lips) of the epithelial dermomyotome, initially mainly from the epaxial lip and later from the other edges and the hypaxial lip. Delaminating cells, already expressing the myogenic determination factors Myf5 (myogenic factor 5) and Mrf4 (myogenic regulatory factor 4) move under the dermomyotome and rapidly differentiate into the skeletal muscle of the early myotome [4] (figure 2).

Figure 1. Schematic representation of somitogenesis.

At certain axial levels, Pax3 positive cells delaminate and migrate from the hypaxial dermomyotome to form muscle masses elsewhere, for example to form limb muscles. Some of these cells retain their Pax-positive progenitor cell status, contributing to later muscle growth.
At later stages, starting from E10.5 in the mouse, the central region of the dermomyotome loses its epithelial structure and Pax3 / Pax7 positive cells enter into the muscle mass of the myotome (figure 2). These provide progenitor cells for the subsequent growth of the muscles of the trunk [5].

**Figure 2. 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. They proliferate and do not express markers of myogenic differentiation.

As depicted in figure 3, 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, during a phase which is usually referred as primary myogenesis, myogenic progenitors that have migrated from the dermomyotome to the limb start to differentiate into
multinucleated muscle fibers, called primary fibers. 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. At the end of this phase, satellite cells can be morphologically identified as mononucleated cells lying between the fiber membrane and the basal lamina.

Figure 3. 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 in the dermomyotome originate muscle precursors (embryonic and fetal myoblasts and satellite cells). Embryonic and fetal myoblasts originate respectively primary and secondary muscle fibers.
1.1.1. GENETICS OF VERTEBRATE MYOGENESIS

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 include *Myf5*, *Mrf4* (also known as *Myf6*), *Myod1* (Myogenic differentiation 1, also known as MyoD) and *Myog* (myogenin) within nascent and differentiating myoblasts. MRFs are responsible, in concert 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 [7].

The majority of studies performed to define the exact hierarchy of these different regulatory genes have used gene knockout studies in the mouse. Within the MRF family, studies have suggested that *Myf5* and *Myod1* act redundantly and upstream of *Myog*. Combined inactivation of *Myf5* and *Myod1* results in complete absence of skeletal muscle formation [8], while deletion of either gene alone results in relatively normal myogenesis, after transient defects [9, 10]. *Myog* mutants initiate myogenesis normally, but harbour defects in the differentiation of myocytes and myofibers [11, 12] and have
widespread muscle loss, suggesting less functional redundancy than other MRFs. Surprisingly, conditional inactivation of Myog postnatally seems to have no phenotypic consequence for muscle formation and homeostasis, suggesting that Myog is not important for the adult phase of myogenesis [13].

Initial studies of the Mrf4 knockout mouse suggested that, similarly to Myog, Mrf4 acts downstream of the redundant activities of Myf5 and Myod1. However, recently, it has emerged that the original gene targeting of the Myf5 locus resulted in the concomitant loss of function of the neighbouring Mrf4 gene [14]. Rescue of Mrf4 function in Myf5;Myod1 double mutant embryos partially restore embryonic myogenesis, suggesting that Mrf4 may have some role in embryonic myogenesis.

Lineage ablation experiments in the mouse have recently shed light on the mechanistic basis for the functional redundancy of Myf5 and Myod1 in myotome formation [15, 16]. Those experiments used a conditional cell ablation approach by expressing diphteria toxin within the Myf5-positive cell subset, coupled with lineage tracing, to demonstrate that Myf5 is expressed only in a subset of myoblasts that contribute to the early myotome. When Myf5 expressing cells are ablated in vivo, myogenesis can be fully rescued by a Myf5-independent cell population, with a concomitant expansion of the Myod1-expressing myoblast population. Those experiments demonstrate that redundancy can act through the existence of two distinct lineages of muscle cells, one specified by Myod1
and the other by Myf5; either of them can compensate for the loss of the other.

To summarize, it is currently agreed that skeletal muscle determination and acquisition of a myoblast precursor fate depend on Myf5, Myod1 and Mrf4; while Myog, Myod1 and Mrf4 are required for terminal skeletal muscle differentiation (see figure 4).

The primary myotome, composed of differentiated, mononucleated myocytes, forms in a Pax3/Pax7 independent way [17]. At this stage Myf5 and Mrf4, independently of Pax3 and Pax7, regulate the entry of cells into the myogenic program. However, subsequent hypaxial activation of Myf5 is Pax3 dependent in the trunk and limb [18]. Genetic data have provided insight into the regulatory genes that act upstream of the myogenic factors. Pax3 and Pax7 are important regulators of myogenic progenitor cells. Their role in different aspects of myogenesis has been widely studied: Pax3 is required for myogenic specification upstream of Myod1 [19], somite segmentation and dermomyotome formation [2, 20], limb musculature development [21, 22] and Myod1 and Myf5 expression [18, 23], while Pax7 has shown to be necessary for the maintenance of adult satellite cells [24, 25].

During maturation of the somite, cells expressing both those Pax genes move from the central region of the dermomyotome to the myotome, where they activate Myf5 and Myod1 and contribute to the growth of skeletal muscle [5, 17]. These cells, besides differentiating into skeletal muscle fibers during
embryogenesis, are present as a reserve cell population within the growing muscle mass during prenatal and postnatal life, supporting the continued growth of muscles.

In the double *Pax3* / *Pax7* mutant, these cells fail to enter the myogenic program, which leads to a major defect in skeletal muscle development: the only muscle fibers present are derived from the early myotome [5].

Myogenesis in the head does not strictly depend on *Pax3* and *Pax7*. *Pax3* is not expressed in head muscles and *Pax7*, even though is present in the myogenic cells of the branchial arches that give rise to facial muscles, is expressed after *Myf5* and *Myod1* [26] and, since the *Pax7* knockout mouse has no defect in head muscles, seems not to be critical in head muscle formation [27].

![Figure 4. Scheme summarizing the genetic hierarchy in vertebrate myogenesis.](image-url)
1.1.2. MYOGENIC SIGNAL INDUCTION IN THE DEVELOPING SOMITE

During development, tissues surrounding paraxial mesoderm and developing somites determine the context of “myogenesis” by secreting signals. A number of studies, mainly featuring chick as a model system, used tissue ablation and reconstitution to identify the notochord, neural tube and the overlying surface ectoderm as sources of activating signals for primary myogenesis, and the lateral plate mesoderm as a source of negative regulators of myogenic induction. These signals can act on different regions of the dermomyotome. Axial structures, like neural tube and notochord, act on epaxial dermomyotome to initiate myogenesis. By contrast, hypaxial precursors do not need signals from axial structures for correct myogenic specification; they rely on the overlying surface ectoderm and adjacent lateral plate mesoderm [28].

In the epaxial muscle, Pax3, Myf5 and Mrf4 can induce Myod1 independently [19]. By contrast, in the hypaxial dermomyotome Pax3 directly activates Myf5 expression, which in turn induces Myod1 expression [18] (see figure 5).

Several other molecules involved in this signalling have been identified. Canonical Wnt signals from the neural tube and surface ectoderm are required for myogenic induction. WNT1, produced in the embryonic dorsal neural tube, induces myogenesis through direct activation of Myf5, while WNT7A, originating from the dorsal ectoderm, preferentially activates
Myod1. Wnt signals seem to be transduced directly in muscle progenitors by Frizzled receptors, activating the canonical β-catenin pathway within the epaxial dermomyotome. It has been shown that activated β-catenin can directly induce Myf5 expression [29]. It remains to be genetically identified which Wnt ligand specifically trigger myogenesis in vivo. A recent report has used explants of presomitic mesoderm to identify a role for non canonical, β-catenin independent Wnt signalling in direct induction of myogenesis. The expression of Myod1 depends on non canonical Wnt signalling probably emanating from the dorsal ectoderm, requires PKC and acts through Pax3 transcriptional activity [30].

**Figure 5.** Signalling pathways involved in myogenic induction.
Sonic hedgehog (SHH) is a fundamental myogenic regulator. SHH is secreted from the notochord and ventral neural tube (see figure 1). Mouse embryos lacking SHH show altered expression of muscle specific transcription factors, in particular Myf5 [31, 32]. Nevertheless, SHH might be dispensable for the initiation of myogenesis both in hypaxial and epaxial muscles. SHH seems to act in both the epaxial and hypaxial somitic compartments mainly as a survival and proliferation factor for myogenic precursor cells rather than a primary inducer of myogenesis.

The SIX protein family also have a role in myogenesis. SIX1 (sine oculis related homeobox 1) and SIX4 regulate Mrf4 in the epaxial dermomyotome [33] and, acting together with the cofactors EYA1 (eyes absent 1 homologue) and EYA2, induce Pax3 in the hypaxial dermomyotome [33, 34].
1.1.3. MYOGENESIS IN THE HEAD

*Pax3* and *Pax7* play important roles prenatally and perinatally in regulating muscles in the body, but head myogenesis has evolved with a distinct transcriptional code.

Head muscles have diverse functions including facial expression, feeding and eye movements, and they are classified broadly into three groups: 1) tongue and posterior neck muscles; 2) pharyngeal muscles including those in the jaw, anterior neck and face and 3) extraocular muscles (EOMs). The majority of these muscles are derived from cranial paraxial mesoderm (CPM) comprising paraxial head (PHM) and the more anterior prechordal (PCM) mesoderm [35-39].

CPM, which is contiguous with somitic mesoderm, gives rise to EOMs and those in the pharyngeal arches (PAs). In addition to the genetic diversity among the muscle groups that originate from these regions, a cellular complexity characterises muscle progenitors in the first PA. A subset of progenitors in the first PA migrates ventrally to contribute to cardiac development in the anterior heart field [36-38]. A second resident population contributes to first PA skeletal myogenesis [40-42]. Furthermore, a number of markers of skeletal myogenesis, such as the transcription factors Tbx1 and Pitx2, are shared with cardiogenesis [36, 37]. These evidences demonstrate that divergent myogenic programmes (skeletal and cardiac) arise from PA muscle progenitors. By contrast, extraocular progenitors give rise to a specialized subset of seven muscle
groups in the mouse (six in human). Interestingly, in Duchenne muscular dystrophy, where essentially all skeletal muscle groups are affected, the EOMs are spared [43]. Extraocular muscles have an obligate requirement for Myf5 or Mrf4 for initiating myogenic fate; in the absence of those MRFs, muscle progenitors are rapidly lost by apoptosis. This does not happen for somite or PA-derived muscle progenitors. Therefore, EOM progenitors employ a distinct combination of genetic tools from those used elsewhere. Myogenesis in pharyngeal muscles operates using a genetic code which is distinct from the one in the body and the EOMs. Here, myogenesis in severely impaired in Tbx1:Myf5 double mutant [39]. In summary, skeletal myogenesis is defined by complementary genetic pathways, governed by Pax3 in the body, and Tbx1 in pharyngeal muscles; however, in EOMs this complementary pathway is absent (see figure 6). The reason why different transcriptional codes are employed in skeletal muscles in distinct locations is still not completely understood.
Figure 6. Core and complementary genetic networks regulating skeletal muscle stem cell fates.

Myogenesis in the somites (trunk and limbs) relies heavily on Pax3 to complement the core myogenic regulatory network – Myf5, Mrf4 and Myod1. Extraocular muscles (EOM) have dispensed with this complementary regulatory pathway, hence, in absence of Myf5 and Mrf4, Myod1 expression is compromised and EOMs do not form. Similarly to somitic myogenesis, PA muscles rely on a complementary genetic pathway: Tbx1 cooperates with Myf5, and in their combined absence Myod1 expression is dramatically reduced and PA muscles are essentially all missing. Pitx2 null as well as Myf5:Mrf4 double mutants lack EOMs [44]. With low penetrance, some EOMs are observed in Myf5:Mrf4 double mutants and some PA muscle is observed in Tbx1:Myf5 double knockouts. In these cases, it is possible that Pitx2 rescues Myod1 expression [39].
1.2. MUSCLE REGENERATION

Skeletal muscle is the largest tissue in the human body, composing 40-50% of total human body mass [45]. The main functions of skeletal muscle are locomotor activity, postural behaviour and breathing. Skeletal muscles consist of muscle cells, networks of nerves and blood vessels, and connective tissue that connect individual fibers into bundles, which form the muscle. However, skeletal muscle can be injured after direct trauma (for example intense physical activities, lacerations) or resulting from indirect causes, such as innate genetic defects or neurological disfunctions. The normal muscular activity can also generate a certain degree of damage to the muscle. If 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. Indeed, upon muscle injury, a finely regulated set of cellular responses is activated, resulting in the regeneration of a well-innervated, fully vascularised and functionally contractile muscle apparatus.

The last years have seen significant advances in the comprehension of the cellular and molecular pathways involved in muscle regeneration. The identification and generation of rodent models for acute and chronic muscular damage has played a major role in those advancements. In particular, the discovery of satellite cells and the progress in the study of their biology has led to a significant insight into muscle regeneration
mechanisms. More recently, the identification of multipotent stem cells capable of myogenic differentiation during the course of muscle regeneration has extended our view on the muscle regeneration process and has opened new perspectives for the development of novel therapies.

1.2.1. BASIC CONCEPTS OF SKELETAL MUSCLE BIOLOGY

Adult mammalian skeletal muscle is composed by multinucleated postmitotic muscle fibers, which are the basic contractile units [46]. Newly formed multinucleated fibers exhibit central nucleation, and once the nuclei move to a subsarcolemmal position they are called myofibers [45]. The myofibers are individually surrounded by a connective tissue layer and grouped into fascicles (bundles) to form a skeletal muscle (see figure 7). 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 [45]. 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 [47].

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 determines 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 [48]. The contraction of myofibers is transformed into movement by myo-tendinous 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.
Figure 7. Scheme of skeletal muscle and associated structures.
Satellite cells are located between the basement membrane and the plasmalemma of the myofibre. Image in upper right is a section of the tibialis anterior muscle of a mouse which vasculature is labeled with a perfused dye, and satellite cells are revealed due to a transgenic strategy. Note the close proximity of the vessel and satellite cell. The vessel is formed of endothelial cells which form the lumen, separated from pericytes (located intermittently along vessels) by a basement membrane. The fluorescent image (upper right) shows a satellite cell marked with cytoplasmic GFP expression. Here the entire satellite cell is aligned along the vessel, and the two are separated by a basement membrane. Three connective tissue layers (epimysium, perimysium, endomysium) can be distinguished in skeletal muscle; these form the network and associated basement membranes in which myofibres regenerate after injury.
1.2.2. MORPHOLOGICAL FEATURES OF SKELETAL MUSCLE REGENERATION

Skeletal muscle in adult mammals is a relatively stable tissue which undergoes a little turnover of nuclei [49]. Normal everyday activity, with a certain variability, elicits only a slow turnover of its constituent muscle fibers. Estimations calculate that in a normal adult rat muscle no more than 1-2% of myonuclei are replaced every week. However, adult skeletal muscle possess a remarkable regenerative capacity. Large number of new myofibers are normally formed in only a few days after acute muscle damage. After an acute damage which causes destruction of the skeletal muscle in the adult mouse, regeneration is usually completed and homeostasis is established within 3-4 weeks after injury.

The healing of skeletal muscle in response to trauma depends on the type of injury, and on the severity. However, in general, the healing process consists of three phases: the destruction phase, the repair phase, and the remodelling phase [50, 51]. 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. This early phase of muscle injury is usually accompanied by activation of mononucleated cells, principally inflammatory and myogenic cells. Neutrophils are the first inflammatory cells to invade the injured muscle. After neutrophil infiltration and about 48 hours
post injury, macrophages become the predominant inflammatory cell type within the site of injury [52].

During the repair phase, the necrotic debris is phagocytosed and regeneration of myofibers takes place through the action of muscle stem cells (satellite cells and other putative adult myogenic progenitors) [53]. Cell proliferation is an important event necessary for muscle regeneration; the expansion of myogenic cells provides a sufficient source of new myonuclei for muscle repair [54]. The mammalian skeletal muscle regeneration process is characterized by typical and well-defined histological features. On muscle cross-sections, these fundamental morphological characteristics are newly formed myofibers of small calibre and with centrally nucleated myonuclei. Fiber splitting or branching is also a characteristic feature of muscle regeneration and is probably due to the incomplete fusion of fibers regenerating within the same basal lamina [55].

In the last phase remodelling 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. However, in some cases, reorganization and contraction of unstructured connective tissue takes place, resulting in scar tissue and subsequent incomplete skeletal muscle regeneration [51].
1.2.3. ANIMAL MODELS OF MUSCLE INJURY

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 [56]. 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 µL 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. A potential “caveat” to the use of this damage protocol are the unknown effects of CTX on various muscle cell types, including satellite cells. The most common way of obtaining a model of chronic muscular damage in mammals is to use genetic knockout mice. Several mouse models of muscular dystrophy will be discussed in paragraph 1.3.1. The mouse strain influences the efficiency of muscle regeneration: Swiss SJL/J mice display a more efficient muscle regeneration process than C57BL/6J, BALB/C and B6CBA/F1 strains, while A/J mice appear to be the least efficient [57].

1.2.4. SATELLITE CELLS IN MUSCLE REGENERATION

Satellite cells (SCs) were first identified in 1961 by Alexander Mauro, who observed by electron microscopy the presence of mononucleated cells intimately associated with skeletal muscle fibers of the frog [58]. SCs have been subsequently identified in skeletal muscles of other vertebrates, including humans, and their involvement in muscle regeneration became clear [59]. SCs are anatomically characterized by their position: directly juxtaposed to the plasma membrane, underneath the basal lamina of muscle fibers. SCs are present in healthy adult mammalian muscle as normally quiescent cells; they represent the 2.5% - 6% of all nuclei of a given muscle fiber. SCs express characteristic, although not unique, markers. A marker which is expressed in the vast majority of quiescent SCs
is Myf5. The majority of other markers are expressed in both quiescent and activated SCs. In the mouse, the most widely used of those markers is Pax7, which is essential for SC specification and survival. A number of other markers of quiescent and activated SCs have been identified; some of them are Pax3 (only in a subset of muscles), Syndecan-3 and -4, VCAM-1, CD34 and M-Cadherin (in mice and not in humans), CD56, c-met, and others [60]. During muscle regeneration, quiescent SCs migrate to the site of injury, up-regulate the myogenic regulatory factors MyoD and Myf5 and become proliferative [53, 61, 62]. From this point, SCs are also known as myoblasts or myogenic precursor cells. Subsequent differentiation of the myoblasts is marked by the downregulation of Pax7 [63] and upregulation of the MRFs Mrf4 and Myog [61]. Eventually, these differentiated myoblasts form multinucleated myofibers (hyperplasia) or fuse to the damaged myofibers (hypertrophy) for muscle regeneration [54]. Some of the activated SCs do not proliferate or differentiate, but instead self-renew and replenish the satellite cell pool. This feature is what characterizes a stem cell. Two different mechanisms have been proposed for self-renewal of SCs: asymmetric and symmetric. An asymmetric cell division results in two different daughter cells, one beginning to differentiate while the other remaining quiescent and self-renewing. Recent research pointed to the asymmetric model of division of SCs in skeletal muscles [64, 65]. Notch signalling is thought to regulate the asymmetric division process. Kuang et al. also
demonstrated that approximately 10% of Pax7+ mouse SCs had never expressed Myf5 and that these cells remain adherent to the basal lamina during mitosis, generating one Pax7+ Myf5- satellite stem cell and one Pax7+ Myf5+ satellite cell derived progenitor, eventually destined to differentiate [64].

**Figure 8.** Schematic representation of satellite cell myogenic progression.

Upon activation, satellite cells, 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 differentiate upregulating Myogenin (Myog+), and eventually fusing to form multinucleated myofibers. Some SCs self-renew by downregulating MyoD. Dashed lines represent possible, but still not proven connections.
1.2.5. 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 adult musculature, the neuronal compartment and various mesenchymal tissues can differentiate into the myogenic lineage.

*Haematopoietic 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*). Bone marrow from this transgenic mouse was transplanted into immunodeficient mice. After injury to the host muscle, donor derived nuclei were detected in regenerated fibers, demonstrating that murine bone marrow contains progenitors that can be recruited to injured muscle through the circulation, where they participate in muscle repair. However, this event occurred at a very low frequency and required the induction of
extensive muscle regeneration [66]. Several studies have followed, trying to identify a rare progenitor endowed with high myogenic activity, but so far those studies have had relatively modest success. A group has described a subpopulation of circulating cells co-expressing CD133 (a marker of haematopoietic stem cells) and myogenic markers, which can contribute to muscle repair and to the replenishment of satellite cell pool when transplanted into dystrophic and immunodeficient mice [67, 68]. To date, several subpopulations of haematopoietic 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.

**Neural stem cells.**

Neural stem cells (both murine and human) are the only ectoderm-derived stem cells that are able to differentiate into skeletal muscle when co-cultured with myoblasts or transplanted into regenerating skeletal muscle [69].

On the other hand, many types of mesoderm-derived stem or progenitor cells (other than haematopoietic 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) [60, 70, 71].
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 (see table 1).

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Origin</th>
<th>Proliferation rate</th>
<th>In vitro myogenic differentiation</th>
<th>In vivo Dystrophin expression</th>
<th>Systemic delivery</th>
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<tr>
<td>CD133+</td>
<td>Blood/ Skeletal muscle</td>
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<td>Induced by muscle cells / spontaneous</td>
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<td>Low</td>
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<td>MABs</td>
<td>Vessel wall</td>
<td>High</td>
<td>Induced by muscle cells / spontaneous</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>MADSCs</td>
<td>Adipose tissue</td>
<td>High</td>
<td>Spontaneous</td>
<td>Yes</td>
<td>Not done</td>
</tr>
<tr>
<td>MAPCs</td>
<td>Vessel wall</td>
<td>High</td>
<td>Induced by aza-cytidine</td>
<td>Not tested</td>
<td>Not done</td>
</tr>
<tr>
<td>MDSCs</td>
<td>Skeletal muscle</td>
<td>High</td>
<td>Induced by muscle cells</td>
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<tr>
<td>MECs</td>
<td>Vessel wall</td>
<td>High</td>
<td>Spontaneous</td>
<td>Yes</td>
<td>Not done</td>
</tr>
<tr>
<td>MSCs</td>
<td>Vessel wall</td>
<td>High</td>
<td>Induced by aza-cytidine</td>
<td>Yes</td>
<td>Not done</td>
</tr>
</tbody>
</table>

Table 1. Properties of non-SC myogenic progenitors
Mesoangioblasts.

Among the vessel wall-derived myogenic progenitors reported in table 2, probably the candidate which has the major therapeutic potential is the mesoangioblast, 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 [72]. 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 [73, 74]. 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. When wild-type or dystrophic, genetically corrected MABs are intra-arterially transplanted into mouse models of muscular dystrophy, they induce a dramatic morphological and functional
amelioration of the dystrophic phenotype [75, 76]. Intra-arterial delivery of wild type postnatal canine MABs into golden retriever dogs which model Duchenne muscular dystrophy also resulted in extensive recovery of dystrophin expression and improvement of muscle morphology and function [77]. MABs isolated from postnatal human skeletal muscle were shown to represent a subset of pericytes, expressing genes like alkaline phosphatase, and to differentiate into myotubes in vitro with high efficiency. When transplanted into dystrophic immunodeficient mice they gave rise to dystrophin-positive myofibers [72].

Other mesoderm-derived progenitors
Mesenchymal stem cells (MSCs) have been shown to be capable of differentiating into skeletal muscle in vitro and in vivo [78]. Pax3 activation enables the in vitro differentiation of murine and human MSCs into MyoD+ myogenic cells. However it was recently demonstrated that, despite good engraftment of MSCs into dystrophic muscle, this was not accompanied by functional muscle recovery [79].

Multipotent adult progenitor cells (MAPCs) were isolated from adult bone marrow, co-purifying with MSCs. Like MSC they are CD45− and CD34−, but MAPCs are also CD117− and CD133+ [80]. They could differentiate at the single cell level into mesenchymal cells, but also visceral mesoderm, endoderm and neuroectoderm-like cells. When injected into an early
blastocyst, single MAPCs contributed to most, if not all, somatic cell types. On transplantation into a non-irradiated host, MAPCs engrafted and differentiated to the haematopoietic lineage, in addition to the epithelium of liver, lung and gut. Furthermore, they could proliferate extensively without obvious senescence or loss of differentiation potential.

MAPCs thus represent a more primitive progenitor cell population than MSCs, demonstrating remarkable differentiation capability along the epithelial, endothelial, neuronal, myogenic, hematopoietic, osteogenic, hepatic, chondrogenic, and adipogenic lineages. MAPCs thus embody a unique class of adult stem cells that emulate the broad biological plasticity characteristic of embryonic stem (ES) cells, while maintaining the characteristics that make adult stem cells more amenable to therapeutic application. MAPCs have been reported to be capable of prolonged culture without loss of differentiation potential, and of showing efficient, long-term engraftment and differentiation along multiple developmental lineages in nonobese diabetic (NOD)-severe combined immunodeficient (SCID) mice without evidence of teratoma formation. Based on these findings, there is great interest in evaluating the therapeutic value of MAPCs for a variety of human genetic and degenerative diseases [81]. Nevertheless, despite their great theoretical potential, at the moment there is little data on MAPC capacity of repairing damaged skeletal muscle in vivo. Furthermore, it is not clear if the pluripotency-like characteristics
of MAPC are intrinsic properties of an unique cell population or if it is a property acquired with cell culture [82].

Myoendothelial cells (MECs) were identified in human adult skeletal muscle, representing less than 0.5% of the total mononucleated cells [83]. 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.

PICs are a recently described population of muscle-resident interstitial stem cells, which have been identified on the basis of marker expression (they are positive for the cell stress mediator PW1, but negative for Pax7) and anatomical localization. Satellite cells are also positive for PW1. PICs are myogenic and bipotential in vitro, generating smooth and skeletal muscle. When injected in the damaged muscle of immunodeficient mice, PICs contributed to myofiber formation, recolonized their niche and gave rise to satellite cells. PICs myogenic activity is dependent on Pax7 [84].
PICs are abundant in the perinatal period, declining in number until about 2-3 weeks after birth, maintaining a 1:1 approximate ratio with satellite cells. During muscle regeneration, PW1+ cells increase in number, exceeding the number of Pax7+ cells. However, a precise physiological role for PICs in myogenesis is not yet clearly defined [84].

Multipotent adipose-derived stem cells (MADSCs) were isolated from adipose tissue; they were shown to possess multilineage differentiation potential (being able to differentiate into adipocytes, osteoblasts and myoblasts), self renewal and capacity of engrafting and repairing dystrophic skeletal muscle in mice [85, 86]. However, the antigen profile of MADSCs suggest a great similarity to MSCs, therefore it is unclear if they represent a unique cell population.

MDSCs are a population of early myogenic progenitor cells which have, in contrast to SCs, multi-lineage potential. Transplantation of MDSCs into skeletal muscle gives better recovery compared to SCs. One of the advantages of MDSCs is their prolonged proliferation in vivo, combined with a strong tendency for self-renewal and immune tolerance. However, there is a lack of evidence for their long-term self-renewal capacity and their efficacy in dystrophic mice [87].
1.3. MUSCULAR DYSTROPHIES

The muscular dystrophies are inherited myogenic disorders characterized by progressive muscle wasting and weakness of variable distribution and severity [43], leading to mobility limitations and, in the most severe forms, heart and/or respiratory failure. Although several forms of dystrophy are associated with mutations in various genes coding for proteins associated with the muscle membrane, others are not (figure 9).

**Figure 9. Schematic representation of various proteins involved in different forms of muscular dystrophy.**

bm=basement membrane; pm=plasma membrane; NMJ=neuromuscular junction; AChR=acetylcholine receptor [88]

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. Other forms are characterized by variable degrees and distribution of muscle wasting and weakness. On those basis, six major forms can be delineated, with the addition of congenital dystrophies, in which muscle weakness is more generalized.

- Duchenne and Becker muscular dystrophy;
- Emery-Dreifuss muscular dystrophy;
- Distal muscular dystrophy;
- Facioscapulohumeral muscular dystrophy;
- Oculopharyngeal muscular dystrophy;
- Limb-girdle muscular dystrophy.

The mode of inheritance is X-linked recessive for Duchenne and Becker forms, and is variable for the others, depending on the gene which is involved.

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 [89].
About two thirds of all DMD patients have large deletions in the dystrophin gene, mainly involving two hot spots located around exons 45-53 and exons 2-30. As a consequence, the essential C-terminal region that binds to the DGC is often truncated. In cases where portions of the central part of the protein are missing but the C-terminal region is maintained, the protein is partially functional. The resulting disease is milder and defined as Becker muscular dystrophy [90]. In one third of DMD cases, the disease is instead caused by small deletions or point mutations that introduce a stop codon, resulting in very little or no dystrophin production, either because of transcript or protein instability.

1.3.1. ANIMAL MODELS FOR MUSCULAR DYSTROPHIES

In contrast to most neuromuscular diseases, several distinct mammalian models are available for muscular dystrophies. 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 [91]. Although mdx mice are normal at birth, skeletal muscles show extensive signs of muscle degeneration by 3 – 5 weeks of age. This acute muscle degeneration phase is accompanied by an effective regeneration process leading to a transient muscle hypertrophy. After this period, the
degeneration / regeneration activity continues at lower and relatively constant levels throughout the life span of the animal. In the older animals (around 15 months) muscle regeneration process is defective and the mice become extremely weak and die before wild-type littermates [92]. 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 [93].

Another widely used murine model for muscular dystrophy is the $\alpha$-sarcoglycan ($\alpha$SG) knockout mouse, which models a human autosomal recessive disorder caused by a mutation in the same gene, limb-girdle muscular dystrophy 2D (LGMD-2D) [94]. Sarcoglycans are a group of single pass transmembrane glycoproteins, which form a complex with the DGC. In contrast to other sarcoglycans ($\beta$-, $\gamma$-, $\delta$-), expression of $\alpha$-sarcoglycan is specifically restricted to striated muscle fibers. $\alpha$SG-ko mice display a progressive muscular dystrophy very reminiscent of human LGMD-2D. Histological changes appear at 1 week of age and extend to most skeletal muscles, and comprise extensive central nucleation, connective tissue proliferation, increasing variability of muscle fiber diameter and presence of necrotic fibers. The early onset, and the severity and persistence of the pathology distinguish the $\alpha$SG-ko model from the mdx mice [94].

Recent studies have used canine models for DMD [77]. 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 [95]. 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 [96]. Myocardial involvement is much more evident in the golden retriever than in other animal models, resembling very closely the cardiac complications present in DMD patients. To summarize, despite the less severity of the disease, mouse models of muscular dystrophy have several advantages, namely a large number of papers published on them, resulting in good understanding of the pathology; the reproducibility of the phenotype and the fact that they have already been used to demonstrate the efficacy of several potential treatment strategies, including pharmaceuticals as well and gene and cell therapy. In contrast, dog models closely resemble the human disease, but they are only starting now to be appropriately characterized.
1.3.2. TREATMENT OF MUSCULAR DYSTROPHIES

There is actually no resolutive treatment for muscular dystrophies. Treatment is generally aimed at controlling the onset of symptoms to maximize the quality of life.

The therapeutic protocols currently in use are based on corticosteroid administration. They provide some delay in the progression of the disease, improving muscle strength and function in the short term, but long term treatment is associated with clinically significant adverse effects [97].

Several pharmacological strategies have been attempted in the past years. Some of them, like administration of protease inhibitors, or drugs regulating calcium homeostasis, or acting on protein or lipid metabolism, did not yield favourable outcomes in clinical trials and therefore were not translated into clinical practice [98]. Recently, despite good results obtained in mice [99], a clinical trial using a novel small molecular agent designed to make ribosomes less sensitive to stop codons has been stopped due to a lack of effectiveness in humans.

There are many avenues of research currently under investigation, including gene therapy, stem cell therapy and pharmacological strategies.

Any exogenous tool (gene / cell therapy) used to improve muscle regeneration in muscular dystrophies has to face the fact that muscle is the most abundant tissue of the body. To induce a significative amelioration of the disease all muscles have to be treated, including cardiac and respiratory muscles; it
is clear that this result can be achieved only with a systemic delivery of the therapeutic agent.

Gene therapy in muscular dystrophy targets the genetic defects, attempting to overcome the pathological mutation by providing the muscle with the correct form of the gene, or by correcting the splicing with exon-skipping vectors, or drugs. A challenging factor in this field is the size and complexity of the causative genes. Several gene therapy strategies are being pursued. Naked DNA plasmids can be used as vectors; they have the advantage to be safe and non-toxic, but the efficiency of the transgene delivery is low. A clinical trial that uses high pressure intravascular delivery of plasmid DNA containing full length human dystrophin is ongoing [100]. Adenovirus and adenoassociated (AAV) virus have been used over the recent years as viral vectors of gene therapy for DMD. They can deliver transgenes in a relatively efficient way, but the disadvantage is that they trigger an acute inflammatory response caused by their capsid proteins. Furthermore, AAV 4.7 kb genome is too small for the insertion of the entire dystrophin gene. To overcome this problem, truncated but still partially functional mini- and micro- dystrophin genes have been produced, by removal of most of the middle rod domain and portions of the amino- and carboxyl terminals of the dystrophin gene. Some success has been obtained in mice, especially using AAV vectors, probably because of the milder immune response [101, 102]. A successful clinical trial of gene transfer of α-sarcoglycan genes with an AAV vectors in patients with
LGMD2D was recently performed [103], and others are ongoing. Exon skipping approaches generally use anti-sense oligonucleotides to restore the reading frame of the mutated gene to allow the expression of internally deleted but partially functional dystrophin proteins, thus theoretically changing a Duchenne phenotype into a Becker phenotype [104, 105]. This approach has some disadvantages: it requires repetitive administrations (since it modifies only the process of mRNA splicing), and different antisense oligonucleotides are required for different mutations.

Stem cell therapy is probably the most attractive approach for the treatment of DMD and other muscular dystrophies; research in this direction has moved very rapidly in the last years. In order to achieve clinical relevance a candidate stem cell population must be easily obtained, remaining capable of efficient myogenic conversion upon isolation; must be suitable for systemic delivery and when transplanted must integrate into the musculature leading to the functional correction of the dystrophic phenotype. General consensus in the field identifies satellite cells as the primary, if not only, physiologically relevant population that contributes significantly to muscle regeneration. However, because satellite cells are not currently amenable for distribution through the vasculature, they do not constitute a viable option to treat DMD. Stem cell populations with myogenic potential can be derived from multiple regions of the body, although their origin is generally still controversial. Paragraph 1.2.5. contains a summary of the most important stem cell
populations endowed with myogenic properties. The cell types that fulfill the most criteria for use in the treatment of muscular dystrophy are vascular-associated stem cells, and in particular the mesoangioblast [60, 70, 76, 77]. A phase I/II clinical trial with mesoangioblast allotransplantation is currently ongoing. However, it is still critical to better understand the physiological properties of mesoangioblast and other stem cells to improve their clinical use.

Recent studies have used a combination of nitric oxide (NO), a molecule whose beneficial effects on muscle repair and regeneration are well known, and non steroidal anti-inflammatory drugs (NSAIDs), like flurbiprofen and ibuprofen [106, 107]. This treatment have numerous advantages: it does not have the severe side effects of corticosteroids, it has already been approved for use in humans, it is effective on oral administration and thus it is well suited for long term treatment. Most importantly, co-administration of NO and NSAIDs proved to be effective in mice, opening the possibility of immediate clinical testing in humans.

The future for the treatment of muscular dystrophy will probably see the combination of stem cell-, gene- and pharmacological based therapies. In some cases the synergistic effects of combinatorial therapy have already been demonstrated. For example, the treatment of dystrophic mice with nitric oxide plus NSAIDs greatly improved the homing and engrafting of mesoangioblast stem cells to skeletal muscle, increasing their therapeutic effectiveness [106].
1.4. VASCULAR DEVELOPMENT: VASCULOGENESIS AND ENDOTHELIAL PROGENITOR CELLS

Two different mechanisms are mainly responsible for vascularization: vasculogenesis and angiogenesis.

Vasculogenesis in the embryo is a mechanism consisting of determination and differentiation of endothelial progenitor cells (or angioblasts) from the mesodermal blood islands and their \textit{de novo} organization into a primitive vascular blood plexus. This process starts at around E7.5 in the mouse embryo. The essential steps in this process have been defined as the following: 1) the birth of angioblasts; 2) angioblast aggregation; 3) elongation of angioblasts into cord-like structures; 4) the organization of isolated vascular segments into capillary-like networks; and, contemporarily with step four, 5) endothelialization and lumenization. Blood vessels formed by vasculogenesis are initially free of smooth muscle cells, pericytes and other associated cells [108].

In the prenatal period, the circulation of the extraembryonic yolk sac is generated by vasculogenesis, followed by formation of the primitive heart and a primary vascular plexus within the embryo. Furthermore, all organs developing from the mesoderm and endoderm (e.g. liver, spleen and myocardium) are primary vascularized by vasculogenesis [109].

Angiogenesis is responsible for the further expansion and networking of primary vessels; it implies new capillary formation from existing blood vessels [110]. Two types of angiogenesis
exist: a) sprouting angiogenesis (the properly called angiogenesis), consisting of growth of endothelial sprouts by migration, proliferation, three-dimensional organization and tube formation of endothelial cells; and b) non-sprouting angiogenesis (also called “intussusception”): the division of vessels by transluminal invagination and pillar formation through interstitial tissue [111].

The “dogma” that in the postnatal period new blood vessels only develop by angiogenesis has generally been accepted until the late 1990s. Recently, new findings have challenged this conviction. The group of Asahara has described a population of mononuclear cells from human peripheral blood that showed similar antigenic characteristic to embryonic angioblasts [112, 113]. These “adult endothelial progenitor cells” were shown to be capable of de novo formation of capillary structures by migration and proliferation, besides being involved in physiological, pathological and pathophysiological neovascularization.

These results led to the concept of postnatal neovascularization via vasculogenesis from endothelial stem and progenitor cells. As a result, the current view of adult vessel development consists in a combination of vasculogenesis and angiogenesis [114].
Figure 9. Visual representation of vasculogenesis and angiogenesis.
Panel A: Vasculogenesis.  Panel B: Sprouting angiogenesis (arrowhead);
Non-sprouting angiogenesis (arrow)
1.4.1. ENDOTHELIAL TO MESENCHYMAL TRANSDIFFERENTIATION (EndMT) AND ENDOTHELIAL PLASTICITY

During embryogenesis, endothelial cells arise from hemangioblasts (a mesodermal progenitor with both endothelial and haematopoietic potential) [115], while smooth muscle cells arise from local mesenchyme and the neural crest [116]. 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 [117]. Later, there results were confirmed by the demonstration that endothelial cells and smooth muscle cells, derived from a single embryonic progenitor, integrate into pre-existing vasculature [118]. Postnatally, such common vascular progenitors have not been described yet. However, reciprocal plasticity between endothelial and mesenchymal lineages has been suggested. 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 [119].

The first description of endothelial to mesenchymal transdifferentiation (EndMT) was the process of heart valves formation during embryonic development [120, 121]. A specific subpopulation of cells, the cardiogenic mesoderm, gives rise to the first two types of heart cells, the myocardial and endocardial
cells. The latter acquire endothelial markers, like VE-Cadherin, CD31, Tie-1, Tie-2 or VEGFRI/II. Endothelial/endocardial cells within the atrio-ventricular region give rise to the mesenchymal heart cushion cells, which form the mesenchymal portion of cardiac septa and valves. To accomplish this, endocardial cells undergo an endothelial to mesenchymal transformation, a process which has been shown in mammals to be largely dependent on TGF-β signalling and on β-catenin [120, 122].

The in vivo postnatal role of EndMT has long been unclear. Recent evidences support a role for EndMT in cardiovascular fibrosis. It has been demonstrated that fibroblasts emerging during cardiac fibrosis originate from endothelial cells, suggesting an EndMT similar to events that occur during formation of the atrioventricular cushion in the embryonic heart [123]. This process is induced by TGF-β and inhibited by BMP-7. A similar mechanism may be involved also in renal fibrosis.

Other studies have shown that EndMT in the tumor microenvironment generates carcinoma-associated fibroblasts and may be essential for cancer progression [124, 125]. EndMT is also implicated in atherosclerosis, pulmonary hypertension and wound healing [126, 127]. Furthermore, very recent data show that expression of constitutely active activin-like kinase 2 (ALK2) in endothelial cells cause EndMT and acquisition of a stem-cell like phenotype; this mechanism is involved in fibrodysplasia ossificans progressiva (FOP), in which heterotopic chondrocytes and osteoblasts express endothelial markers and are derived from endothelial cells.
1.4.2. HAEMOGENIC ENDOTHELIUM

Mammalian intraembryonic hematopoiesis takes place in the dorsal aorta starting at embryonic day E10.5 in the mouse, during a developmental period when it is flanked by the gonado-mesonephric columns (the AGM region) [129]. Within this region, haematopoietic cells can be detected attached to the internal walls of the aorta as intra-luminal cell clusters, and have been described to emerge from the aortic endothelium by a “budding” process [130].

Until recently, two apparently conflicting theories tried to explain the origin of haematopoietic cells during embryonic development. The first one proposed that haematopoietic cells arise from haemangioblasts, common precursors for both endothelium and haematopoietic system. Support for this theory came from the identification, during mouse embryonic stem cell differentiation, of a clonal precursor which gives rise to blast colonies with both endothelial and haematopoietic components [131]. Later, other studies demonstrated the existence of this bipotential precursor in vivo, though not clarifying the precise mechanism for generation of haematopoietic cells from the haemangioblast [132].

Another theory provided evidence for the in vivo existence of a specialized subpopulation of differentiated endothelial cells that have haematopoietic potential, which were called haemogenic
endothelium. Those were described as endothelial cells that transiently possess the ability to generate haematopoietic stem cells during vertebrate development [130, 133]. A recent study provided a link between these two precursor populations, demonstrating that the haemangioblast generates haematopoietic cells through the formation of a haemogenic endothelium intermediate [115].
1.5. THE CRE-LOXP RECOMBINATION SYSTEM

Cre is a 38 kDa recombinase protein from bacteriophage P1 which mediates intramolecular (excisive or inversional) and intermolecular (integrative) site specific recombination between loxP sites [134]. A loxP (locus of X-over Phage 1) site consists of 34 bp: two 13 bp inverted repeats separated by an 8 bp asymmetric spacer region. The recombination occurs in the asymmetric spacer region, which is also responsible for the directionality of the site. Two loxP sequences in opposite orientation to each other invert the intervening piece of DNA; two sites in direct orientation dictate excision of the intervening DNA between the sites, leaving one loxP site behind.

In terms of applicative potential, currently the Cre/LoxP system is simply the major tool to create conditional somatic genome modifications in vivo. In fact, this precise removal of DNA can be used to eliminate an endogenous gene or transgene, or activate a transgene. As opposed to conventional gene targeting, that produces permanent mutations in the germ line and, thus, in every cell of the animal, Cre/LoxP technology allows the conditional generation of predetermined genetic alterations in selected somatic cells.

The tissue specificity of the gene knockout or activation is achieved by directing Cre expression to the cell type of interest, using tissue-specific promoters. Temporal control can be obtained by using ligand-inducible Cre recombinases. The creation of those genetic tools came from the observation that
the activity of a number of proteins can be controlled by a ligand when fused to the ligand-binding domain of a steroid hormone receptor [135]. The most widely used inducible Cre recombinase is currently the CreER<sup>T2</sup> variant. This is a fusion protein which comprises the Cre recombinase and a mutated version of the human estrogen receptor, which is insensitive to endogenous β-estradiol but activated by the synthetic ER antagonist 4-hydroxytamoxifen [136]. The current model of action proposes that, in the absence of the ligand, the chimeric Cre recombinase is retained in the cytoplasm; binding of the ligand results in the translocation of the Cre recombinase into the nucleus where it can recombine its loxP-flanked DNA substrate. In other words, ligand binding appears to regulate primarily the localization of the recombinase rather than its enzymatic activity.

The Cre/LoxP system is a tool for tissue- and time-specific knockout of genes that cannot be investigated in differentiated tissues because of their early embryonic lethality in mice with conventional knockouts. It can also be used for the removal of a transgene, which was overexpressed in a specific tissue, at a certain time point to study the invert effect of a downregulation of the transgene in a time course experiment. Cre’s excising capability can also be used to turn on, at a given time, an exogenous gene by cutting out an intervening stop sequence between the promoter and the coding region of the transgene.
Figure 10. Cre-mediated DNA recombination.
The loxP site is represented by black triangles. A DNA segment flanked by two directly repeated loxP sites is excised by Cre as a covalently closed circular molecule. DNA recombination is conservative, so no gain or loss of nucleotides occur during the recombination reaction.

1.5.1. MOLECULAR FATE MAPPING IN THE MOUSE

Cell fate analyses are greatly informative for our understanding of normal development, and provide an important framework for analyzing genotype-phenotype relationships uncovered by mutagenesis. Before the advent of site-specific recombinases, the vast majority of vertebrate fate maps have been plotted in avian and amphibian systems, because of the ease of manipulating tissue in ovo. Employed methods include the
injection of retroviral, fluorescent or vital dye lineage tracers, or the grafting of quail cells into chick embryos. Because mouse embryos develop *in utero*, they are less accessible, making these established tracing methods more difficult. To circumvent this obstacle, site-specific recombinase based strategies have been developed, to genetically (and non-invasively) activate lineage tracers in mice.

Those systems generally involve two mouse strains that are intercrossed:

a) a recombinase mouse, generally expressing a constitutive or inducible Cre recombinase in a gene-specific fashion;

b) a reporter mouse, harbouring a transgene that indicates that a recombination event has occurred, and provides a permanent record of this event by transforming it into a heritable lineage marker.

The basic elements contained within a reporter transgene are mainly two. One is a reporter gene itself, functionally silenced by insertion of a floxed (flanked by loxP sites) STOP cassette, containing, for instance, a head-to-tail array of four SV40 polyA sequences coupled with translational stop codons in all reading frames [137]. The second element is a widely active promoter capable of driving reporter expression ideally in all cell types and at all stages of development, such that, following a recombination event in any given cell, that cell and all progeny cells will be marked, regardless of subsequent fate specification. In double transgenic animals (recombinase;
reporter) excision of the STOP cassette in the reporter transgene will occur only in the recombinase-expressing cells, thereby activating the reporter gene. Because the excision event activating the reporter is at the level of chromosomal DNA, it is stably inherited to progeny cells, thereby marking these cells and revealing their contribution to embryonic and adult tissues [138]. By using an inducible Cre recombinase, it is possible to temporally restrict cell labelling, in order to selectively label specific subpopulations of cells.

A number of useful reporter mice are available. Each reporter molecule contained within them has advantages and disadvantages. For example, nuclear-localized β-gal facilitates the identification of individual cells; and green fluorescent protein (GFP) or other color variant fluorescent proteins can be used to visualize live cells, and even to isolate them by fluorescence-activated cell sorting.
1.6. AIM OF THE THESIS

Vessel-associated multipotent mesodermal progenitors have been recently isolated from several dissociated and cultured embryonic, fetal, perinatal and adult tissues. Despite the increasingly recognized medical value of these progenitor cells, among which are mesoangioblasts, these indirect extraction methods have precluded the understanding of their native identity, tissue distribution and frequency. Furthermore, despite the fact that the role of angiogenesis in muscle repair has been extensively studied and is now well defined, the extent and biological significance of vessel-associated progenitors’ direct contribution to the formation of muscle fibers and other mesodermal lineages during development or muscle regeneration is less clear.

We tried to address these questions by using a genetic lineage tracing approach in the mouse. Chapter II describes the results of this study, which is also my Ph.D. project.

We have generated a transgenic mouse expressing an inducible form of Cre recombinase (CRE-ERT²) under the control of an endothelial specific promoter (Vascular Endothelial Cadherin, or VE-Cad, promoter). This mouse was crossed with different lines of reporter mice (R26R, Rosa-EYFP, Rosa-NZG). Cre recombination was induced in a narrow window during embryogenesis to label a specific cell subpopulation, at a point in which embryonic mesoangioblasts should be present in vivo, corresponding to the time of isolation.
We have studied the contribution of embryonic endothelial progenitors to muscle and other mesodermal lineages during development. We have also investigated the distribution of endothelial derived haematopoietic cells in the early stages of definitive haematopoiesis in the embryo. Furthermore, we have isolated endothelial-derived cells both from the embryo and from adult muscle, to check their multi-lineage differentiation potential \textit{in vitro} and their myogenic reconstitution capability \textit{in vivo}. Finally, we have analyzed the role of endothelial derived progenitors in muscle regeneration.
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CHAPTER II
2. A NOVEL POPULATION OF EMBRYONIC ENDOTHELIAL DERIVED PROGENITORS CONTRIBUTES TO MULTIPLE MESODERMAL LINEAGES DURING DEVELOPMENT AND MUSCLE REGENERATION

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2.1. ABSTRACT

Several investigators have reported the isolation of vessel-associated multipotent mesodermal progenitors from diverse dissociated and cultured embryonic, fetal, perinatal and adult tissues. Despite the increasingly recognized medical value of these progenitor cells, among which are mesoangioblasts, these indirect extraction methods have precluded the understanding of their native identity, tissue distribution and frequency. We addressed this question by using a genetic lineage tracing approach.

We labelled embryonic VE-Cadherin+ endothelial cells in the E8.5-E9 time window. At embryonic, foetal and perinatal stages, we detected labelled skeletal muscle, smooth muscle and dermis cells originating from embryonic endothelium as part of their in vivo normal developmental fate. We excluded that this contribution derives from a somitic intermediate progenitor.

Consistently, we observed a small number of endothelial-derived muscle fibers in the adult skeletal muscle. We also found labelled subsets of pericytes and PICs (PW1+ interstitial progenitors), but we did not detect any labelled satellite cell. Following muscle damage, cells derived from embryonic endothelial progenitors participate to myogenic regenerative response, generating myofibers and macrophages.

FACS-isolated endothelial derived cells are myogenic in vitro and are able to differentiate in several mesodermal tissues,
including SMA (smooth muscle actin) positive cells, AP (alkaline phosphatase) positive osteoblast-like cells, adipose tissue and endothelial networks. Intra-muscular injection of isolated endothelial derived cells results in colonization and reconstitution of skeletal muscle tissue in both wild type and dystrophic mice.  

Moreover, we demonstrated that haematopoietic cells originated by endothelial to haematopoietic transition in the yolk sac emerge abluminally from vessels in the body of the embryo and migrate into the mesenchyme. We also identified a novel population of cells that express haematopoietic and mesenchymal markers and are originated by this process. These data suggest the existence of a previously unrecognized endothelial-derived progenitor cell population, which could represent the \textit{in vivo} counterpart of embryonic mesoangioblasts. These progenitor cells contribute to smooth and skeletal muscle development, as part of their normal fate, and generate cells that persist in the adult life, participating to muscle regeneration.
2.2. MATERIALS AND METHODS

Transgenic mice generation and genotyping.
To generate Tg:VeCadCREERT\(^2\) mice, we have produced a pBlueScript-based construct comprising a 2.5-kbp 5' flanking region of the VECD gene (VECDp), directing endothelial specific gene expression [1, 2] of a Tamoxifen-inducible form of Cre recombinase (CRE-ERT\(^2\)) [3]. A 4-kbp fragment, present in the first intron of VECD gene (VECD-IE), which has been demonstrated to carry enhancer activity [4], has been cloned upstream of the Cre-ERT\(^2\) transgene. A scheme of the 11 kbp fragment used for transgenic mice production is outlined in figure 11.

Transgenic animals were generated by injecting the purified linearized construct represented in figure 11 into the pronuclei of fertilized oocytes. A total of 7 independent transgenic lines were obtained. Those lines were tested by crossing with R26R and R26R-EYFP reporter mice, containing respectively a cytoplasmic lacZ gene and a cytoplasmic EYFP gene; the expression of both of them is dependent on Cre expression, as reporter genes are cloned downstream of a floxed stop
cassette. 5 lines were found to express β-gal or EYFP, and 2 among them were further selected for good expression and therefore were used for the following experiments.

Mice were kept as heterozygous, and were genotyped by using the following couples of primers:
ICre2FW (5’-AGATGCCAGGACATCAGGAACCTG-3’) and ICre2REV (5’-ATCAGCCACACCAGACAGAGATC-3’), both mapping on the CRE-ERT2 sequence, generating a 250 bp band; VeCadFW (5’- ACAAGGAACAAATAACAGGAACC-3’), mapping in the VECDp, and ICre2REV, generating a 1.600 bp band. Other transgenic mice used in this work have been published: VE-Cadherin(PAC)-CreERT2 [5]; R26R [6]; R26R-EYFP [7]; R26-NZG [8].

All experiments were performed under internal regulations for animal care and handling (IACUC 355).

**Tamoxifen injection.**

*Tg:VeCadCREERT2* and *VE-Cadherin(PAC)-CreERT2* mice were alternatively crossed with R26R, R26R-EYFP or R26-NZG mice.

Cre activity was induced during embryogenesis by one single intraperitoneal injection of pregnant females at day 8.5 post coitum with 200 µl tamoxifen solution (SIGMA, T5648; 10 mg/mL in corn oil).
**Embryos and foetuses.**
Dissected E9.5 – E15.5 embryos and yolk sacs were fixed with a 4% solution of paraformaldehyde (PFA) in PBS at 4°C. The duration of the fixing step was dependent on the stage of the embryo (1 hour for E9.5, 1.5 hours for E10.5, 2 hours for E11.5 and E12.5, 2.5 hours for E13.5, 3 hours for E15.5). 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 finally embedded in OCT and sectioned using a Leica 1850UV cryostat (10μM sections were made).
E17.5 and P0 foetuses were dissected and skin was removed. Anterior and posterior limbs were further dissected, fixed, dehydrated and embedded similarly to E15.5 embryos.

**X-Gal staining.**
To perform whole-mount X-Gal staining, dissected muscles and embryos were fixed with a 4% solution of paraformaldehyde (PFA) in PBS at 4°C, for the appropriate duration (see above). After that, samples were washed in PBS and incubated in X-Gal staining solution (1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactosidase, 5 mM K₄Fe(CN)₆, 5 mM K₃Fe(CN)₆, 2 mM MgCl₂ in PBS) O/N at 37°C. Subsequently, samples were washed, dehydrated and embedded in OCT as described above. To perform X-Gal staining on sections, the duration of the incubation in X-Gal staining solution was 2-3 hours at 37°C.
Immunohistochemistry.
Endogenous peroxidases were blocked by treating the sections with a solution of H$_2$O$_2$ 30% in methanol for 30’. Sections were then permeabilized by incubation in a solution of 0.2% Triton, 1% BSA in PBS for 30’ at room temperature, and subsequently blocking was done by incubation in donkey serum 10% in PBS for 30’ at r.t. Sections were incubated overnight with primary antibody at 4°C and washed in PBS. Treatment with a biotinylated secondary antibody (DAKO) for 1 hour at r.t. was followed by ABC Elite Kit (Vectastain). Finally, enzymatic reaction with SIGMAFAST DAB (3,3’-Diaminobenzidine tetrahydrochloride) (SIGMA) to reveal the signal was followed under observation at the microscope and stopped when signal reached the desired intensity.

Immunofluorescence and antibodies.
Muscle or embryo sections and cultured cells were fixed with a 4% solution of paraformaldehyde (PFA) in PBS at 4°C (unless already fixed prior to embedding). After washes in PBS, they were blocked/permeabilized with a 10% donkey serum, 0.2% Triton solution in PBS for 1 hour at r.t. Primary antibody was incubated for 1 hour at r.t. or O/N at 4°C. Secondary antibodies used in this work were AlexaFluor 488, 546, 594 or 647 (Molecular Probes – Invitrogen), made in the appropriate species. Variations to the standard immunofluorescence protocol were introduced according to requirements for specific antibodies. Additional steps introduced were a permeabilization
step in Methanol at \(-20^\circ\text{C}\) for 6'; an antigen demasking step (often required for nuclear antigens), involving incubation in a 10 mM solution of sodium citrate at 100°C for 10'; or modifications in the blocking/permeabilization procedure (involving different concentrations of serum or adding of bovine serum albumin – BSA).

Immunofluorescence was carried out using the following antibodies: \(\beta\)-galactosidase goat anti-mouse (1:300, DBA) or rabbit anti-mouse (1:2000, Cappel-MP); Laminin rabbit anti-mouse (1:300, SIGMA) or chicken anti-mouse (1:500, Abcam); NG2 rabbit anti-mouse (1:300, Chemicon); CD31/PECAM1 monoclonal mouse anti-mouse (1:2, clone MEC13.3, gift from E. Dejana); Pax3 monoclonal mouse anti-mouse (1:2, Developmental Studies Hybridoma Bank - DHSB); Pax7 monoclonal mouse anti-mouse (1:2, DHSB); Smooth Muscle Actin (1:200, SIGMA); MyHC monoclonal mouse anti-mouse/MF20 (1:2, DHSB); GFP rabbit anti-mouse (1:300, Invitrogen) or monoclonal mouse anti-mouse (1:100, Molecular Probes); MyoD monoclonal mouse anti-mouse (1:100, DAKO); Myf-5 rabbit anti-mouse (1:300, Santa Cruz); SGCA rabbit anti-mouse (1:50, SIGMA); Collagen-I monoclonal mouse anti-mouse (1:100, Abcam); CD68 monoclonal mouse anti-mouse (1:100, Abd Serotec); PW1 rabbit anti-mouse (1:3000, gift from D. Sassoon); Desmin rabbit anti-mouse (1:300, SIGMA); CD45-PE rat anti-mouse, clone 30-F11 (BD); CD45.2-PE mouse anti-mouse, clone 104 (BioLegend); CD41-PE rat anti-mouse, clone MWReg30 (BD).
Image acquisition and manipulation.
Fluorescent and phase contrast images were taken using the following microscopes:

- Nikon microscope Eclipse E600 with Plan Fluor lenses 4x/0.13, 10x/0.33, 20x/0.50, 40x/0.75. Image acquisition was done using the Nikon digital camera DXM1200 and the acquisition software Nikon ACT-1.

- Leica AF6000. Image acquisition was performed using the DFC350 FX camera and the Leica AF600 acquisition software.

The imaging medium was PBS buffer; all images were recorded at room temperature. Images were assembled in panels using Adobe Photoshop CS4. Images showing double or multiple fluorescence were first acquired separately using appropriate filters, then the different layers were merged using Adobe Photoshop CS4.

Flow cytometry.
Endothelial derived cells were isolated from E13.5 embryos, after removal of the head, liver and viscera. Embryos were cut in small pieces and dissociated with 0.15 mg/mL collagenase type V (Sigma), 0.4 mg/mL Dispase (Gibco), 0.1 mg/mL Dnase I (Roche) in a buffered solution (Hank's Balanced Salt Solution – HBSS; BioWhittaker) supplemented with 15 mM HEPES, 15 mM glucose, 1.5 mM MgSO<sub>4</sub>, 0.3% (W/V) bovine serum albumin (BSA), pH 7.4. Dissociation reaction was performed at 37°C, for 20’, for 4-5 cycles. After spin and filtration (using 40
µm filters), cells were resuspended in either DMEM (SIGMA) 20% FBS, 20 mM HEPES, 2 mM EDTA or PBS 2%FBS, 2mM EDTA (in case of antibody staining).

Endothelial derived cells were also isolated from adult muscle. Dissected muscles were trimmed in small pieces, and dissociated with collagenase D 0.1% (Roche) and Trypsin 0.25% (Gibco) or 0.4 mg/mL Dispase (Gibco). Dissociation reaction was performed at 37°C, for 20’, for 2-3 cycles. Resuspended mononucleated cells were filtered with 70 µM and 40 µM filters. Cells were resuspended in either DMEM 20% FBS, 20 mM HEPES, 2 mM EDTA or PBS 2%FBS, 2mM EDTA (in case of antibody staining).

Cell sorting was performed using the MoFLo system (DAKO). FACS analysis was carried out using the BD FacsCANTO system. Data were analyzed by FACSDIVA software (BD).

The following antibodies were used for FACS analysis and cell sorting: α7-Integrin unconjugated mouse anti-mouse, clone 3C12 (MBL); CD45-PE rat anti-mouse, clone 30-F11 (BD); CD45-APC rat anti-mouse, clone 30-F11 (BD); CD31-APC rat anti-mouse, clone MEC13.3 (BD); CD34-Alexa Fluor 647 rat anti-mouse, clone RAM34 (eBioscience); CD41-PE rat anti-mouse, clone MWRReg30 (BD); SCA-1-APC rat anti-mouse, clone D7 (eBioscience); NG2 unconjugated rabbit anti-mouse (Chemicon); PE rat IgG isotype control (BD); APC rat IgG isotype control (BD). When using unconjugated antibodies, primary antibody reaction was followed by secondary antibody
Donkey anti-rabbit Alexa Fluor 647 or Donkey anti-mouse Alexa Fluor 647.

7-Aminoactinomycin D (SIGMA) was used for dead cells detection.

**Cell cultures and differentiation assays.**

Sorted cells were resuspended in growth medium (DMEM 20%FBS with addition of bFGF [5 ng/mL] and β-mercaptoethanol [50 µg/mL]) and spotted onto calf skin collagen (SIGMA)-coated dishes, at a density of approximately 5 x 10^5 cells/cm². Cells were kept in a low oxygen incubator and cultured with growth medium for proliferation.

Sorted cells were induced to differentiate to smooth muscle by treatment with TGF-β1 (SIGMA) and to bone-like cells by treatment with BMP-2 (Peprotech) as described in [9].

To evaluate alkaline phosphatase expression in BMP-2 treated cells, we performed an alkaline phosphatase histochemical reaction. PFA 4%-fixed cells were washed twice with NMNT solution (NaCl 100 mM, Tris HCl 9,6mM, Tris 90 mM, MgCl₂ 50 mM, Tween 0,1% in H₂O). After that, cells were incubated in NMNT + NBT [4,5 µl/mL] + BCIP [3,5 µl/mL] for 1 hour in the dark at 20-22°C.

Spontaneous myogenic differentiation was assessed by culturing cells onto matrigel coated plastic support in differentiation promoting medium (complete medium supplemented with 2% Horse Serum – Biowhittaker). Myogenic differentiation capacity was also assessed by a co-culture
assay. Sorted endothelial derived cells were infected with a third generation lentiviral vector directing LacZ expression (pRRL.sin.PPT.CMV.nLacZ.pre). C2C12 myoblasts (ATCC) were cultured as described in [10]. Infected endothelial derived cells were cultured with a fourfold excess of unlabeled C2C12 myoblasts. For both assays, after 3 to 5 days cells were fixed with PFA 4% and stained with X-Gal and/or with immunofluorescence for MF20.

For endothelial network formation assay, cells were plated onto matrigel coated wells in differentiation promoting medium, with addition of VEGF 1:1000.

For adipogenic differentiation, we used the STEMPRO Adipogenesis Differentiation Kit (Gibco). Endothelial derived cells were seeded in a 12-well plate at 2x10^4 cells/cm^2; the day after, growth medium was replaced by Adipogenesis Differentiation Medium. Cultures were re-fed every 3-4 days.

To confirm adipogenic differentiation, we performed an Oil Red O histochemical staining. PFA 4%-fixed cells were rinsed twice, in deionized water; after that, they were rinsed in 60% isopropanol for 2’, then stained in Oil Red O working solution for 30’ at room temperature, and finally rinsed again in water to stop reaction.

**Muscle regeneration assays.**

To evaluate the ability of endothelial derived cells to participate in the *in vivo* regeneration process, acute injury was performed on *tibialis anterior* (TA), *gastrocnemius*, *quadriiceps*, *triceps*
*brachialis* muscles by injecting 25 µl (TA and *triceps brachialis*) or 50 µl (*gastrocnemius* and *quadriceps*) of 10 µM cardiotoxin (CTX, SIGMA) intra-muscularly. Mice were sacrificed 5, 10, 15, 20 or 30 days after the CTX injection. Muscles were collected and either sectioned for histological analysis or dissociated for flow cytometry.

**In vivo engraftment assay.**

Sorted endothelial derived cells from E13.5 embryo or adult muscle of *Tg:VeCadCREERT²; R26R-EYFP* or *VE-Cadherin(PAC)-CreERT²; R26R-EYFP* were cultured for 3-4 passages and infected with the pRRL.sin.PPT.CMV.nLacZ.pre lentivirus. 5 – 10 x 10⁵ cells were intra-muscularly injected into the TA muscle of wild type mice of the same background, which were injured with CTX 2 days before. Injected muscles were harvested 6 days later, stained for X-Gal and subsequently sectioned.

Endothelial derived cells (either infected or not infected) were I/M injected also in the TA muscle of alpha sarcoglycan (α-SG) knockout mice [11]. At least 5 x 10⁵ cells were injected. Mice were treated with Tacrolimus (subcutaneous injection, 2.5 mg/kg/day, starting 3 days before injection of cells until the end of the experiment) for immunosuppression, and were sacrificed 12 days after injection of cells. Muscles were recovered for histology.
2.3. RESULTS

Generation of the VeCadCREERT² transgenic mouse.

In order to genetically label and follow the fate of different subsets of endothelial derived cell populations during embryonic and adult development, we generated a construct comprising a gene encoding for an inducible form of Cre recombinase (CRE-ERT²) under the control of VE-Cadherin (Cdh5, or CD144) regulatory sequences (see Materials and Methods). VE-Cadherin is a very well studied gene, known to be expressed exclusively in endothelial progenitors and differentiated cells from early developmental stages [1, 2, 4]. To evaluate the functionality of the construct, we performed an *in vitro* assay in H5V murine endothelial cell line. Transfection of a plasmid in which GFP is under the control of Ve-Cadherin regulatory sequences resulted in GFP expression (see figure 1A). When H5V cells were transfected with the plasmid used for transgenic mice generation, together with pGAP plasmid, addition of 4-hydroxytamoxifen resulted in excision of the DsRed expression cassette from the pGAP plasmid, allowing to detect GFP fluorescence signal.

We obtained 7 founders (see figure 1B) which were screened for transgene transmission capability, and specificity and efficiency of Cre expression. Two of the 7 original founders (#9618 and #9623) were not able to transmit the transgene to the progeny, therefore were eliminated from further screening.
We tested the efficiency of endothelial-specific Cre recombination during embryogenesis in double heterozygous VeCadCREERT²; R26R mice. We induced Cre recombination with one single intra-peritoneal Tamoxifen injection in the pregnant mother at embryonic day E8.5, since for later experiments we wanted to avoid labelling the yolk sac mesoderm, which transiently expresses VE-Cadherin at E7.5 [12]. Panel 1C shows whole mount X-Gal staining of E12.5 embryos. All the transgenic lines displayed endothelial-specific Cre expression (which was detected by X-Gal staining). One line (#9620) had a very weak Cre expression (not shown), and although it was able to transmit the transgene, it did so to a small percentage of the progeny, and therefore was eliminated. Of the other 4 remaining lines, line #9616 had a normal transgene transmission and the strongest Cre expression. For these reasons, line #9616 was selected for further experiments and line #9621, #9624 and #9625 were kept as a backup. To confirm that Cre recombination was specifically directed to the endothelium, we sectioned VeCadCREERT²; R26R and VeCadCREERT²; R26R-EYFP E9.5 – E11.5 embryos (after 1 Tamoxifen injection at E8.5). Immunofluorescence analysis revealed colocalization of β-gal or EYFP with endothelial specific markers (CD31/Pecam1 or CD34); see panel 1D. No β-gal+ or EYFP+; CD31- or CD34- cells were detected. The single E8.5 Tamoxifen injection protocol resulted in a Cre recombination efficiency of approximately 30-40% (EYFP+ cells on the total CD31+ cells) in these conditions.
We used another endothelial specific Cre transgenic line in our experiments. We tested VE-Cadherin(PAC)-CreERT² mouse [5] in the same way, and we confirmed that it displays a very strong, endothelial specific Cre expression and recombination (figure 1C) which results in approximately 80%-90% recombination efficiency, even with 1 single E8.5 Tamoxifen injection (figure 1E). No EYFP+ CD31- cells were detected in VE-Cadherin(PAC)-CreERT² embryos at E9.5, immediately after Cre recombination. We also tested, in both Cre lines, whether our single E8.5 Tamoxifen injection protocol resulted in correct vascular labelling in the yolk sac. E9.5, E10.5 and E11.5 embryos displayed no EYFP+ cells in the yolk sac mesoderm, while CD31+ vessels were also EYFP+ (not shown). We conclude that both Cre lines used in this work display correct, endothelial specific, Cre expression, although with different efficiency. Moreover, a single E8.5 Tamoxifen injection is sufficient to induce endothelial-specific recombination.

**Endothelial derived progenitors contribute to embryonic and foetal myogenesis.**

Reciprocal plasticity between endothelial and mesenchymal lineages has been suggested. To evaluate the contribution of embryonic endothelial derived progenitors to non-endothelial mesodermal lineages, several developmental stages during embryonic and foetal development were analyzed. Since there are several reports that describe the isolation of vessel-associated progenitors endowed with myogenic potency [13,
14], we started our analysis by looking for endothelial-derived myogenic cells.

Both Cre lines (VeCadCREERT² and VE-Cadherin(PAC)-CreERT²) were used for this analysis. We detected the presence of rare single-nucleated MF20+ EYFP+ CD31-myoblasts in E13.5 embryos. In E12.5 embryos those cells were more rare, and we were able to detect them only using the VE-Cadherin(PAC)-CreERT² line. Conversely, in E15.5 fetuses MF20+ EYFP+ CD31- cells were more abundant in both lines. At later foetal stages (E17.5 and P0) we analyzed the foetal forelimbs and hindlimbs and the presence of endothelial-derived myofibers was unequivocally detected using both Cre lines (see figure 2A, 2C). Longitudinal hindlimb sections were made and β-gal+ MF20+ CD31- or EYFP+ desmin+ CD31-multi-nucleated myofibers were detected (see figure 2D). However, at later foetal stages, endothelial derived myofibers were less in number than those observed at E15.5. A graph summarizing the result of the quantification of endothelial derived myogenic cells during development is shown in figure 2B. It is clear that a “peak” in endothelial derived myogenesis is observed at E15.5 stage. At this stage, endothelial derived myofibers are approximately 3.5 – 4.5 % of the total MF20+ cells. A possible reason for the observed decrease is the limit of the detection system. Cell fusion events which take place during secondary myogenesis (and adult life), may lead to a dilution of the reporter proteins and therefore to the impossibility to detect them, at least with those methods. MF20+ EYFP+
CD31- cells were detected in forelimb, hindlimb and trunk muscles without any apparent bias. In some cases, those cells were also observed in muscles of the head. Figure 2E show a section of the mandible of a E15.5 foetus, in which a EYFP+ myogenic cell is present. As described in the introduction, head myogenesis is controlled by a unique set of genes, and in general follows completely different mechanisms in comparison to myogenesis in the rest of the body. This observation suggests that the process of endothelial derived myogenesis may be unique in all the districts of the body, in contrast to classical myogenesis.

**Endothelial derived progenitors do not enter the somitic lineage.**

We checked whether the contribution that we observed to myogenesis by embryonic endothelial derived progenitors was due to somitic intermediates. As shown in figure 3, no EYFP+ cells at E9.5 colocalize with Pax3 staining. Conversely, every EYFP+ cell is also CD31+ at this stage. Later stages were also analyzed and no EYFP+ cells at E10.5, E11.5 or E12.5 (not shown) were found to colocalize with markers of somitic populations (Pax3, Myf5, MyoD) in both VeCadCREERT² and VE-Cadherin(PAC)-CreERT² Cre lines. Therefore, we conclude that cells that express Ve-Cadherin at E8.5 enter the myogenic lineage without going through a somitic intermediate as part of their normal, unperturbed fate.
Endothelial derived progenitors contribute to multiple mesodermal lineages during development.

We analyzed late embryonic (E13.5) to foetal (E15.5-E17.5) stages to evaluate the contribution of VE-Cadherin+ progenitors to mesodermal lineages other than skeletal muscle. We detected EYFP+ CD31- cells in the smooth muscle layer of big vessels (dorsal aorta, cardinal vein, mesenteric artery) which were also positive for smooth muscle actin (SMA) or NG2 (see figure 4A), markers of the smooth muscle lineage at those stages. Those cells accounted for approximately 0.5-3% of the total SMA+ or NG2+ periendothelial, smooth muscle cells. This finding was somewhat unexpected, since to our knowledge there are no reports that document a developmental relationship between endothelial progenitors and smooth muscle cells, although, interestingly, there are reports that suggest the existence of a common progenitor for smooth muscle of the dorsal aorta and skeletal muscle of the myotome [16], and others that show that circulating endothelial progenitor cells can give rise to smooth muscle-like progeny in vitro [17].

By transversally sectioning the forelimbs of E17.5 foetuses, we observed that the dermal layer surrounding the developing fingers contained a remarkably high number of EYFP+ endothelial derived cells (see figure 4B), some of which were also CD31-. By staining with the dermal marker Collagen I, it was detected that EYFP+ CD31- cells in the dermal layer were indeed positive for Collagen I, meaning that endothelial derived cells had entered the dermal lineage. EYFP+ CD31- CollagenI+
cells in the developing foetal fingers were quantified and accounted approximately for 2-4% of the total Collagen I+ cells. Examination of the haematopoietic regions in the embryo and foetus revealed, as expected, the presence of numerous cells belonging to the haematopoietic lineage, thus confirming the many recent reports that supported the evidence for the existence of a specialized endothelial population in the embryo (haemogenic endothelium), virtually responsible for the origin of all adult blood [18-20]. Endothelial-derived haematopoietic cells are present in the E11.5 AGM region (see figure 4D) and are evident inside the lumen of big vessels (figure 4A), meaning that they entered the circulation. During embryogenesis, they colonize the foetal secondary haematopoietic organs. In order of abundance, at E15.5 endothelial-derived haematopoietic cells are present in the foetal liver, in the thymic primordia and in the spleen (figure 4D). The heart displays a high degree of EYFP positivity starting from early developmental stages, since endocardium (an endothelial subpopulation), covers almost its entire internal surface, and it is VE-Cadherin+, so most of its cells are found labeled. However, we did not detect any EYFP+ cardiomyocytes. Figure 4C shows a section of a E15.5 foetal heart. It is known that endocardial cushions, from which originate AV valves and part of the septum, arise from a subset of endothelial cells that undergo epithelial (or endothelial) to mesenchymal transition [21, 22]. In our model, at E15.5, almost all the fibroblast-like CD31- cells of the endocardial cushions are EYFP+ (as shown in the inset of figure 4C), thus confirming
previous data obtained with other endothelial lineage tracer models.

**Endothelial derived haematopoietic cells emerge from embryonic vessels in an abluminal direction and migrate into the mesenchyme, co-expressing mesodermal markers.**

We analyzed the distribution of endothelial-derived haematopoietic cells in E9.5 to E12.5 VE-Cadherin(PAC)-CreERT²; R26R-EYFP embryos. By inducing Cre recombination at E8.5 we were able to avoid labelling of yolk sac-derived primitive haematopoiesis (VE-Cadherin is expressed transiently in the yolk sac mesoderm at E7.5), concentrating our analysis on endothelial-derived intraembryonic definitive haematopoiesis.

At E9.5, we did not detect any EYFP+ CD45+ or EYFP+CD41+ cell in the body of the embryo. Rare CD45+ cells that co-express CD31+ are present, as well as CD41+ cells (not shown). Conversely, in the E9.5 yolk sac extraembryonic blood islands we identified EYFP+ CD41+ round cells emerging from the yolk sac endothelium. Staining for CD31 reveals that those round haematopoietic cells are CD31+ and therefore retain an endothelial identity (see figure 5B).

Starting at E10.5, we were able to detect EYFP+ CD45+ CD31 (low) cells, which appeared to come out from vessels in an abluminal direction, and enter the surrounding mesenchyme. This phenomenon was observed in vessels throughout the whole embryo, and not only from vitelline artery as described in
a recent paper [15]. Figure 5A shows EYFP+ CD45+ cells in the developing limb bud and in the mesenchyme surrounding the somites. Some EYFP+ CD41+ SMA- cells, exiting vessels and entering the mesenchyme, have been detected as well. Approximately, 1 EYFP+ CD41+ was detected every 10 EYFP+ CD45+.

At E11.5, vessels release EYFP+ CD45+ CD31(low) cells in the mesenchyme with more frequency. Clusters of those endothelial-derived haematopoietic cells can be seen budding out from the dorsal aorta in figure 5A. Isolated EYFP+ CD45+ CD31 (low) and EYFP+ CD41+ SMA- cells can also be seen in the mesenchyme.

We checked whether endothelial-derived haematopoietic cells expressed mesenchymal markers. At E10.5 and E11.5 stages, we detected EYFP+ CD45+ cells that co-express SMA (figure 5B). Those cells account for approximately 30% of the total EYFP+ CD45+ cells. Rare EYFP+ CD45- SMA+ cells were also detected. No EYFP+ CD41+ SMA+ cells were found.

At E12.5, EYFP+ CD45+ cells migrating in the mesenchyme are abundant and can be detected throughout the whole embryo. Those cells appear to preferentially localize at sites of tissue remodelling, such as the developing limbs, the brain or between somites. At this stage, EYFP+ CD41+ are rarely found in the mesenchyme; the majority of those cells is present in the embryonic liver.
Endothelial derived progenitors participate in muscle regeneration.

Next, we investigated the fate of embryonic endothelial derived cells in the adult postnatal development. We labelled the same subset of cells as those of the embryonic-foetal analysis by inducing Cre recombination with 1 single injection of Tamoxifen at E8.5. Figure 6A shows a schematic outline of the experiments on postnatal development. We focused our postnatal analysis on muscle. Whole mount X-Gal staining of adult muscles shows extensive vascular labelling (see figure 6B); subsequent sectioning and immunofluorescence analysis confirms that most (>80%) of the CD31+ vessels is labelled using VE-Cadherin(PAC)-CreERT² line.

We characterized the non-endothelial cell populations, derived by embryonic endothelial progenitors, in the adult muscle at different stages. The developmental origin of satellite cells had remained controversial for a long time. Recent evidences support that satellite cells are derived from Pax3/Pax7 progenitors in the somites [23, 24]. However, experimental evidences suggested that satellite cells may be derived from cells associated from the embryonic or adult vasculature [25]. We could not find any Pax7+;β-Gal+ or Pax7+;EYFP+ satellite cell in perinatal, juvenile or adult mice, at least in basal conditions, without muscle damage.

A small percentage (<1%) of muscle fibers was found to be labelled in undamaged adult muscle of VE-Cadherin(PAC)-CreERT²; R26R-EYFP mice (figure 6B). Those EYFP+ fibers
have been detected only in *pectoralis* and *triceps brachialis* muscles. Immunofluorescence analysis in the same mice revealed that the adult muscle contained many EYFP+ interstitial cells which were indeed CD31-. A subset of NG2+ CD31- pericytes was found to colocalize with EYFP staining. NG2+ CD31- EYFP+ cells accounted for approximately 10% of the total NG2+ cells, depending on the muscle (they were more abundant in *pectoralis* and *triceps brachialis* muscles compared to hindlimb). Moreover, a significant subset of PW1+ CD31-interstitial cells colocalized with EYFP signal in undamaged adult muscle (figure 6B). In these conditions, more than 80% of PW1+ CD31- interstitial cells was also EYFP+. Since PICs are known to be abundant in muscles of juvenile mice, we also checked P10 and P21 stages. Surprisingly, no PW1+ interstitial cells were found to be positive for EYFP at those stages (not shown). NG2+ CD31- EYFP+ interstitial cells behave in a similar way, as we were able to detect them only in adult muscles. These results indicate that embryonic endothelium contribute to subsets of myogenic progenitors (pericytes and PICs) other than satellite cells during unperturbed development. To analyze the fate of endothelial derived cells in a regenerative setting, an acute injury and consequent regeneration were induced in muscles (*tibialis anterior*, *gastrocnemius*, *quadriceps* and *triceps brachialis*) of adult mice by cardiotoxin (CTX) intramuscular injection. Mice were sacrificed 5,10 or 21 days after CTX injection. In the regeneration experiments we chose to use R26R-NZG over R26R-EYFP as the reporter transgene
because of technical issues. Muscle damage causes a significative background in immunofluorescence, however in these conditions nuclear β-gal can still be detected with good sensitivity and specificity. We were able to detect regenerating, centrally nucleated β-Gal+ myofibers 5 and 10 days after CTX damage (see figure 6C). Endothelial-derived muscle fibers accounted for 1-2% of all myofibers in regenerating muscles. CD68+ β-Gal+ macrophages are also present. Analysis at later time points is still ongoing. We are also setting up multiple damage experiments to evaluate the fate of endothelial derived cells after several degeneration-regeneration cycles.

**Isolation and in vitro characterization of endothelial derived cells.**

By crossing VE-Cadherin(PAC)-CreERT² mice with R26R-EYFP reporters, we were able to isolate endothelial derived EYFP+ cells by fluorescence activated cell sorting. We chose two main protocols, as summarized in figure 7A. Endothelial derived cells were isolated from E13.5 embryos and from CTX-damaged or undamaged adult muscle. EYFP+ cells accounted for approximately 1-2% of total cells in the E13.5 embryo, or 26-28% of total mononucleated cells in adult undamaged muscle (figure 7B). After isolation, embryonic (figure 7C) or adult (not shown) cells were grown in culture in conditions suited for mesoangioblasts [26]. Immunofluorescence for EYFP confirmed that isolated cells were indeed positive for EYFP. Embryonic cells grow as a
mixed population of fibroblast-like cells and small round-shaped cells, some of which are adherent and others are in suspension (and are smaller than the adherent ones). Adult cells are also a mixed population of fibroblast-like and round shaped cells, but they lack the non-adherent component.

Endothelial derived cells were exposed to several differentiating stimuli. Figure 7D shows the results of differentiation experiments using embryonic cells. When treated with TGF-β, most of the cells became positive for smooth muscle actin (SMA). After treatment, more than 80% of the cells are SMA+ (embryonic and adult), while in normal conditions less than 10% of the embryonic cells is SMA+; by contrast, 40-50% of adult cells from undamaged muscle are SMA+.

Embryonic and adult cells expressed alkaline phosphatase (a marker of osteoblasts) when treated with BMP-2. Adult cells differentiate in bone-like cells with more efficiency (>90% of AP+ cells) compared with embryonic cells (60-70% of AP+ cells). Less than 5% of embryonic and adult untreated cells are AP+.

When treated with VEGF, embryonic and adult cells formed endothelial-like networks (although embryonic cells are more efficient). Culturing embryonic and adult cells in adipogenic medium induced the appearance of adipocytes containing Oil Red O – positive fat droplets.

Both embryonic and adult cells are unable to differentiate spontaneously into multinucleated myotubes, although a small number of single nucleated myosin-positive cells is detectable.
(data not shown). When co-cultured with C2C12 murine myoblasts (after infection with a lentivirus directing expression of nLacZ), thus exposing cells to a myogenic inductive environment, embryonic and adult cells fuse into multinucleated, MF20+ myotubes. Single nucleated MF20+ β-Gal+ cells are also detectable, indicating that endothelial derived cells, besides fusing, are also able of directly differentiating into myogenic cells. Embryonic cells are approximately three to four times more efficient than adult cells in forming multinucleated myotubes.

We analyzed the phenotype of freshly isolated, embryonic and adult, endothelial derived cells by FACS. For analysis of embryonic cells, we separately analyzed cells isolated from the liver and from the rest of the embryo. Liver cells should most likely represent a fraction enriched in haematopoietic cells. According to this, 70% of the liver EYFP+ cells express CD45, compared to around 30% of the total embryo cells (see figure 7E). Furthermore, CD41 was more expressed (20%) in liver cells than total embryo cells (3,5%), accordingly to histological data. By contrast, 70% of the total embryo cells express CD31, while only 30% of the liver cells do. NG2 is only expressed by total embryo cells. Both fractions express CD34 and SCA-1. Accordingly to FACS data, liver cells and total embryo cells have different morphologies when grown in culture.

For the phenotype analysis of adult cells, we compared undamaged muscle with injured, regenerative muscle after 10 or 21 days from CTX damage. Undamaged muscle expresses
\( \alpha-7 \) integrin at high levels, while CD45 expression is low. Interestingly, around 20% of EYFP+ cells is also NG2+. NG2 in the adult muscle is a pericyte-specific marker; this is in agreement with the histological data previously shown. After 10 days from CTX damage, the fraction of EYFP+ CD45+ is greatly increased; at the same time, \( \alpha-7 \) integrin+ cells diminish. 21 days after injury, \( \alpha-7 \) integrin and CD45 populations go back to a basal-like situation.

**Intra-muscular injection of endothelial derived cells results in colonization and reconstitution of skeletal muscle tissue.**

We checked whether endothelial derived cells, after isolation and *in vitro* expansion, could be able to repair damaged skeletal muscle tissue both in acute and chronic injury settings.

Embryonic endothelial derived cells were infected with a lentivirus expressing nuclear LacZ and injected into previously injured *tibialis anterior* muscles of wild type mice. We detected donor derived X-Gal+ nuclei inside centrally nucleated, regenerating myofibers, meaning that donor cells had differentiated or fused into regenerating myofibers. Donor-derived nuclei localize preferentially in a central position typical of myonuclei inside regenerating myofibers. Alternatively, donor-derived nuclei can be detected inside myofibers in a peripheral position typical of satellite cells (note that the fiber in figure 8A-II and II’ contains X-Gal+ nuclei both in central and in
peripheral positions; the one in the satellite cell-like position is lying underneath basal lamina).

Embryonic and adult cells were injected into tibialis anterior and gastrocnemius muscles of immunosuppressed alpha-sarcoglycan (α-SG) knockout mice, a mouse model of limb-girdle muscular dystrophy. nLacZ-infected cells were detected inside regenerating myofibers, but usually those cells caused a very high immune response even in immunosuppressed mice (probably due to the presence of β-Gal and EYFP, which are strong immunogens). Injection of not infected cells resulted in the appearance of large clusters of α-sarcoglycan positive, regenerating myofibers, that are undetectable in uninjected control mice and therefore are originated by donor cells (figure 8B). We then conclude that endothelial derived cells are able to morphologically restore α-sarcoglycan expression in α-SG knockout mouse model of muscular dystrophy.
2.4. DISCUSSION

Embryonic endothelium is a tissue with unique properties; in fact, it resembles more a group of similar tissues (or cell subpopulations) with distinguishing characteristics rather than a single tissue. Using the Cre/LoxP genetic lineage tracing technology, recent work on haematopoiesis has shed light on the existence of haemogenic endothelium [18-20], a specialized endothelial population that is present in the vertebrate embryo but not in the adult, and gives rise to haematopoietic stem cells and virtually all adult blood. The process by which endothelial cells give rise to blood cells has been demonstrated to be not an asymmetric cell division but rather an unique type of cell transition [27]. Cardiac endocardium is another endothelial subpopulation that in the embryo displays a peculiar behaviour: it undergoes endothelial to mesenchymal cell transition to give rise to the mesenchymal cells that form the atrioventricular cushions, the primordia of the valves and septa of the adult heart [21, 22]. Adult endothelial cells can also undergo endothelial to mesenchymal transition, although the postnatal role of this process has long been unclear. Recent evidences support the idea that postnatal endothelial to mesenchymal transition may have a role in pathological settings like cardiac fibrosis, in which endothelial cells recapitulate the events that occur during formation of the embryonic heart cushions [28], and fibrodysplasia ossificans progressiva, in which endothelial cells give rise to heterotopic chondrocytes and osteoblasts [29].
Several investigators have reported the isolation of vessel-associated stem cells with multilineage mesodermal differentiation potential [30, 31] among which are mesoangioblasts (MABs), myo-endothelial cells (MECs) and multipotent adult progenitor cells (MAPCs). Embryonic mesoangioblasts have been isolated from the dorsal aorta of early stage (E9.5) mouse embryos [13]. Embryonic mesoangioblasts express endothelial and haematopoietic markers, but not myogenic markers; they display an in vitro multilineage differentiation potential, being able to differentiate into smooth muscle cells, osteoblasts, adipocytes, skeletal muscle (when co-cultured with myoblasts) and cardiocytes (when co-cultured with rat neonatal cardiocytes). When transplanted into dystrophic mice and dogs, those cells could functionally reconstitute with high efficiency damaged skeletal muscle [32, 33]. Importantly, when delivered through the vasculature, mesoangioblasts can cross the vessel wall and home to the site of muscle damage.

However, despite the growing clinical interest around mesoangioblasts and other vessel-associated stem cells, their precise developmental origin and physiological role are still unclear.

Several studies identify a close developmental relationship between endothelial, smooth and skeletal muscle cells in the embryo. Kardon G. et al. identified a common somitic progenitor for myogenic and endothelial cells in the chicken limb [34]. In the mouse, retrospective clonal analysis experiments have
demonstrated the existence of a common progenitor for the skeletal muscle of the myotome and the smooth muscle of dorsal aorta [16]. Furthermore, genetic lineage studies confirm that the Pax3-positive cells of the dermomyotome contribute to the blood vessels’ walls [16]; another study demonstrates that the Pax3:Foxc2 ratio determines the myogenic versus vascular cell fate choice in multipotent cells derived from the somite [35].

Other data suggested reciprocal plasticity between endothelial and mesenchymal lineages. Ten years ago, Yamashita J. et al. described embryonic vascular progenitor cells that differentiate into both endothelial and smooth muscle cells [36]. Later, Ferreira et al. showed that endothelial cells and smooth muscle cells, derived from a single embryonic progenitor, integrate into pre-existing vasculature [37]. To our knowledge, such common vascular progenitors have not been described postnatally yet.

Based on these data, we decided to analyze the fate of endothelial cells and progenitors by using a genetic lineage tracing approach. We chose to label cells in a narrow window during embryogenesis (between E8.5 and E9), corresponding to the time of isolation of embryonic mesoangioblasts.

**Endothelial progenitors contribute to skeletal and smooth muscle development**

We have shown that, starting from E12.5, endothelial-derived myogenic cells can be detected during embryogenesis. This contribution does not go through a somitic intermediate, as we never detected Pax3/7 positive cells in the dermomyotome or
Myf5/MyoD positive cells in the myotome. This finding implies that, as part of their normal fate, endothelial derived progenitors can enter the skeletal muscle lineage. The presence of single-nucleated endothelial derived myogenic cells suggests that endothelial derived progenitors can enter the myogenic lineage by a direct differentiation step, although fusion events during foetal and later development cannot be excluded.

With the techniques that we used, it is not possible to demonstrate whether VE-Cadherin+ endothelial cells directly differentiate to myoblasts/muscle fibers, or rather if intermediate progenitors are responsible for the myogenic contribution. An in vivo live imaging approach could theoretically answer this question, but it is very difficult to apply this kind of technique in mammals, due to the fact that the mouse embryo is not easily accessible and, moreover, it is opaque. Using an ex vivo approach or switching model organism to zebrafish (easily accessible, transparent) could represent possible solutions.

We also proved that endothelial derived cells enter the smooth muscle lineage during development. The contribution that we observe is restricted to the smooth muscle layer surrounding big vessels; we never saw labelling in other types of smooth muscle, such as the smooth muscle of the intestine. However, it is not surprising to find this labelling pattern, given the fact that the most part of the smooth muscle of the gut and viscera is known to be originated from the neural crest, which is a structure that forms at the dorsal tips of the neural epithelium,
and in the vertebrate embryo gives rise to migratory cells that colonise a wide range of tissues [47, 48]. Nevertheless, to our knowledge, this is the first experimental evidence that smooth muscle cells can be generated \textit{in vivo} from a progenitor derived from the endothelium. Furthermore, we detected endothelial-derived cells in the dermis at foetal stages, but not in the epidermis.

Our findings are not in contrast with the long-standing dogma that all muscles derive by somites; it has been demonstrated that during embryogenesis, dermomyotomal Pax3+ progenitors give rise not only to cells of the skeletal muscle lineage, but also endothelium, smooth muscle and dermis [38]: exactly the same lineages that we find labelled, originating from embryonic endothelial derived progenitors. Thus, we can hypothesize to have labelled a progenitor which has an endothelial phenotype (VE-Cadherin+) but is still multipotent, in a very similar way to a Pax3+ dermomyotomal progenitor.

Minasi \textit{et al.} for the first time hypothesized the existence of vessel-associated multipotent stem cells in the embryo [13], using transplantation experiments to test multipotency \textit{in vivo}. When transplanted into a chick host, mesoangioblasts gave rise to skeletal muscle fibers, smooth muscle cells, myocardial cells, dermal (but not epidermal) cells, cartilage and perichondrium. The distribution of endogenous endothelial derived cells that we observe is similar to the one observed with the mesoangioblast cell line transplantation: the only differences are that we did not detect any endothelial derived myocardial or cartilage cell.
However, it is of fundamental importance to distinguish between developmental fate and differentiation potency. The contribution observed with our genetic lineage tracing experiments did not involve any transplantation or cell culture, but rather reflects the natural and physiological developmental events that ultimately bring to cell fate choice. In any case, isolation or culture remove cells from their original niche and expose them to modifications. It is possible that cultured or transplanted cells display a broader multipotency differentiation range, or differentiate with more efficiency, because *in vitro* culture conditions, or simply isolation, enhance this hidden potential.

Indeed, we were able to isolate endothelial derived cells by FACS, in order to characterize their phenotype and multi-lineage differentiation capability. When isolated from the embryo, endothelial derived cells express endothelial and haematopoietic markers (as expected), but also the smooth muscle/pericyte marker NG2, thus confirming histological data. Embryo- and adult-derived cells are able to differentiate in several mesodermal lineages, including endothelial networks, smooth muscle, bone-like structures, adipocytes, and skeletal muscle, thus resembling the multi-lineage differentiation potency of embryonic mesoangioblasts. Endothelial derived cells can only differentiate into skeletal muscle when exposed to a myogenic environment; however, in these conditions they can not only fuse to myotubes, but they are also able to differentiate to single-nucleated myoblasts. Similarly to mesoangioblasts, when transplanted into damaged muscle,
endothelial derived cells can be recruited to reconstitute skeletal muscle fibers. These data suggest that the *in vitro* and *in vivo* myogenic and multi-lineage differentiation capability of vessel-associated stem cells, such as mesoangioblasts, are not a cell culture or transplantation artifact, but rather the consequence of a differentiation potential that takes part also during normal development, although in a less frequent way. Moreover, these data identify the embryonic endothelium as the developmental origin of embryonic mesoangioblasts.

**Haematopoietic cells derived from yolk sac haemogenic endothelium emerge abuminally from vessels in the body of the embryo, migrate through the mesenchyme and acquire a mesenchymal / haematopoietic phenotype**

The last 5 years have shown a significant advance in our understanding of intra-embryonic definitive hematopoiesis. Previous studies have demonstrated that definitive haematopoietic stem cells are not completely generated from the early yolk sac (in which takes place the first wave of embryonic haematopoiesis, at E7.0 in the mouse), but rather from later intraembryonic arterial sites [39-41]. Using different strategies, ranging from in vivo lineage tracing [18], live imaging in mouse and zebrafish [20, 27, 42], in vitro tracing and deletion of a critical haematopoietic transcription factor (Runx1) in mouse embryonic stem cells [43], several groups have demonstrated that a subset of endothelial cells is indeed
responsible for the generation of haematopoietic stem cells; in other words, it is “haemogenic”. The haemogenic capacity is embryonic, short-lived and restricted to a specific subset of endothelial cells. Only particular vascular beds are thought to be capable of haematopoietic emergence; those include the dorsal aorta (AGM region), yolk sac, placenta, vitelline and umbilical arteries. In avian embryos, endothelial derived haematopoietic cells have been detected both in intra-aortic clusters, when cells bud into the direction of the vessel lumen and form intraluminal clusters, and in the subaortic mesenchyme [39]. In contrast, almost without exception, in mammals the endothelial to haematopoietic transition (EHT) event that generates HSCs is known to occur in an intraluminal direction. Only very recently, abluminal endothelial to haematopoietic transition has been reported during vascular remodelling of the vitelline artery in the mouse [15].

We analyzed the distribution of endothelial derived haematopoietic cells, and the first surprising finding that we obtained was the detection of the abluminal emergence of haematopoietic cells from embryonic vessels. Starting from E10.5, those cells emerge from vessels throughout the whole body of the embryo, enter the mesenchyme and migrate through it to reach their destination. At E12.5, endothelial-derived haematopoietic cells are found preferentially at sites of tissue remodelling. We excluded that those cells could be derived from primitive yolk sac haematopoesis, since Cre recombination at E8.5 does not lead to labelling of yolk sac
mesoderm. Moreover, the appearance of those abluminally emerging cells is temporally concomitant to the emergence of “canonical” intraluminal haematopoietic clusters, which at E10-E10.5 are very rare (a recent paper quantified the number of endothelial to HSC transition events as 1.7 per embryo at this stage [20], although in an ex vivo model). These data strongly suggest that the abluminally emerging cells cannot represent haematopoietic cells that have already emerged intraluminally from intraembryonic haemogenic endothelium, entered the circulation and at some point extravasated to enter the mesenchyme. We hypothesized that those cells could undergo a de novo abluminal endothelial to haematopoietic transition in a similar way to what happens during remodelling of the vitelline artery [15]. However, analysis of E9.5 yolk sac (after Cre induction at E8.5) revealed the presence of round EYFP+ CD41+ haematopoietic cells emerging from yolk sac endothelium. CD41 marks the initiation of definitive haematopoiesis in the embryo [49]. It is likely that the mechanism by which those cells are generated is indeed endothelial to haematopoietic transition, as strongly suggested by the facts that at E8.5 VE-Cadherin expression is highly restricted to the endothelium and that EYFP+ haematopoietic cells in the yolk sac retain the expression of the endothelial marker CD31/Pecam1. Previous data suggested that the yolk sac vasculature is capable of haematopoietic generation [18,50], but in those works it is not clear whether the yolk sac endothelium generates definitive haematopoiesis, and
furthermore the timing of this process is not well defined. On the other hand, Samokhvalov et al. have recently demonstrated that at least some of the definitive HSC are originated by the yolk sac [41]. Our work confirms these data and suggests that yolk sac-derived definitive haemopoiesis originate by endothelial to haematopoietic transition in the yolk sac blood vessels. Haemogenic endothelium is present in the body of the embryo from E10.5. Our data also suggest that haemogenic endothelium is active in the yolk sac prior to the body. Since at E8.5 the yolk sac vasculature is already connected to the embryo body and the heart is beating, we can also hypothesize that the haematopoietic cells emerging abluminally from vessels in the body of the embryo (starting from E10.5) could be at least initially derived from the yolk sac, although we cannot exclude that vessels in the body of the embryo are capable of abluminal endothelial to haematopoietic transition. To our knowledge, this is the first report documenting extravasation and migration of post-EHT haematopoietic cells to the mesenchyme in mammals. Interestingly, in the zebrafish, cells in the aortic wall undergo abluminal EHT and move towards the axial vein to enter circulation. A subset of those abluminally emerging haematopoietic cells could represent HSC (CD41+). Another subset, surprisingly, expressed the mesenchymal marker smooth muscle actin (SMA). To further investigate the identity and fate of haematopoietic cells in the mesenchyme we should be able to
specifically label or at least isolate them, but with the tools that we have at the moment this is not possible.

We could hypothesize that at least a subset of the extravasating haematopoietic cells could be HSC that migrate through the mesenchyme to enter the circulation and/or reach secondary haematopoietic organs (liver, spleen, thymus). The SMA-expressing subset could instead represent a different and previously unrecognized cell population of endothelial derived haematopoietic cells that have acquired a mesenchymal fate. Although at the moment it can’t be directly demonstrated, we postulate that those cells could be responsible for the contribution that we observe to skeletal and smooth muscle, and possibly to dermis. The fact that we observe, even at early stages, EYFP+ CD45- SMA+ cells support the hypothesis that haematopoietic cells can emerge from vessels, migrate through the mesenchyme and eventually acquire a mesenchymal fate, generating skeletal muscle, smooth muscle or other mesodermal lineages.

Furthermore, these hypotheses can explain many of the features of embryonic mesoangioblasts. In vitro culture of dorsal aorta explants could generate the abluminal emergence of haematopoietic/mesenchymal cells that we observe in vivo, which also in culture express endothelial and haematopoietic markers, and are endowed with multipotency. The fact that mesoangioblasts can cross the vessel wall and home to the site of muscle damage [44] could be explained as those cells, being of haematopoietic origin but coming from the mesenchyme,
must be able to enter the vessels to join circulation. Thus, this ability would not be acquired with culture but could be an intrinsic property of mesoangioblasts. Therefore, for all these reasons, we hypothesize that a subset of abluminally emerging haematopoietic cells co-expressing mesenchymal markers could represent the in vivo counterpart of embryonic mesoangioblasts.

**Endothelial derived progenitors participate in muscle regeneration**

We investigated the fate of endothelial derived cells in the adult. We focused our analysis on skeletal muscle. In according with the data obtained in the embryos, we detected a small number of labelled muscle fibers. Interestingly, we found that subsets of pericytes and PICs (PW1+ interstitial progenitors) are labelled; those are two cell types that have been recently demonstrated to be capable of myogenic potency [45, 46]. However, in normal conditions we did not detect any labelled satellite cell at all developmental stages. Although PICs have been shown to be abundant in juvenile mice, we detected labelled PW1+ in skeletal muscle only in adult mice, after P30. The same was found for NG2+ pericytes. This finding could be explained by hypothesizing that embryonic endothelial derived cells could enter the pericyte and PICs lineages only during adult life, after the postnatal development has completed. Damage-regeneration analyses demonstrated that endothelial derived progenitors participate in the muscle regeneration
process, generating muscle fibers and macrophages. However, the entity of the regenerating muscle fibers derived from endothelial progenitors is not high.

Endothelial derived cells in skeletal muscle could possibly function as “alternative” progenitors, to be recruited to the skeletal muscle lineage in case of emergency, when muscle damage is too high to be repaired with canonical muscle progenitors. For these reasons, we set up a multiple muscle damage assay, to induce a stronger challenge for muscle regeneration. Preliminary results indicate that the contribution of endothelial derived progenitors to muscle repair is higher in these conditions.

In conclusion, in this work we have used a genetic lineage tracing approach to label embryonic endothelial progenitors between E8.5 and E9. We have demonstrated that cells derived from those progenitors contribute to skeletal and smooth muscle development, and possibly to other mesodermal lineages, including dermis. Moreover, we found that cells derived by embryonic endothelium are present in the adult muscle, contribute to subsets of myogenic progenitors and participate in muscle regeneration. We isolated embryonic endothelial derived cells from embryo and adult muscle, and showed that both populations are myogenic in vitro and in vivo. Furthermore, we demonstrated that haemogenic endothelium is present in the yolk sac before being active in the body of the
embryo. We identified endothelial derived haematopoietic cells, at least in part originated by endothelial to haematopoietic transition in the yolk sac, which emerge from vessels in the body of the embryo and migrate through the mesenchyme. We also identified a novel population of endothelial derived cells in the embryonic mesenchyme, that express haematopoietic and mesenchymal markers and is probably originated by the process of extravasation that we described, which we hypothesize to represent the in vivo counterpart of embryonic mesoangioblasts.
FIGURE 3
FIGURE 4
FIGURE 5
FIGURE 6

A

Tamoxifen

<table>
<thead>
<tr>
<th>E8.5</th>
<th>P7</th>
<th>P21</th>
<th>P30</th>
<th>P60</th>
<th>+5</th>
<th>+10</th>
<th>+21</th>
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CTX damage (i/M)

Recover muscles

B

VE-Cadherin(PAC)-CreERT²

R26R

R26-NZG

Hoechst, p63

II

Pax7, CD31

merge

Hoechst, EVFP

Hoechst, CD31

merge

Hoechst, EVFP

NG2, CD31

merge

Hoechst, EVFP

PwN, CD31

merge

C

VE-Cadherin(PAC)-CreERT²

CTX+5d

merge

X-gal

merge

CTX+10d

merge

merge

R26-NZG
FIGURE 8
2.5. FIGURE LEGENDS

FIGURE 1

(A): H5V murine heart capillary endothelial cells were transfected with pGAP (a plasmid containing, downstream to upstream, a CMV promoter, a floxed DsRed gene+NLS+polyA, and a eGFP gene), VeCadGFP (a plasmid containing Ve-Cadherin promoter directing the expression of GFP), or VeCadCreERT² (the construct used for transgenic mice production). Where indicated, soluble Cre or 4-hydroxytamoxifen were added. Images show GFP and/or Ds-Red fluorescence.

(B): Genotyping of VeCadCreERT² founders, using ICre2FW (5’-AGATGCCAGGACATCAGGAACCTGAC-3’) and ICre2REV (5’-ATCAGCCACACCAGACACAGAGACTC-3’) primers.

(C): Whole mount X-Gal staining (dark blue) of E12.5 F1 embryos obtained by crossing the different VeCadCreERT² founders and VeCadherin(PAC)-CreERT² mice with R26R reporter line. Representative images of at least 3 independent experiments (different litters) are shown. Cre recombination was induced with 1 Tamoxifen injection at E8.5.

(D): X-Gal staining (dark blue) and immunofluorescence using Hoechst (blue), anti-EYFP (green), anti-CD34 (green or red) and anti-CD31 (red) antibodies on transversal sections of E11.5 / E12.5 VeCadherin-CREERT²; ROSA-YFP or VeCadherin-CREERT²; R26R embryos. Cre recombination was induced with 1 Tamoxifen injection at E8.5.
(E): Immunofluorescence using Hoechst (blue), anti-EYFP (green) and anti-CD31 (purple) antibodies on transversal sections of E9.5 / E10.5 VeCadherin(PAC)-CreERT²; R26R-EYFP embryos. Cre recombination was induced with 1 Tamoxifen injection at E8.5. Scale bars, 50 µM.

FIGURE 2
(A): Immunofluorescence using Hoechst (blue), anti-EYFP (green), anti-MF20 (red) and anti-CD31 (purple) antibodies on transversal sections of E12.5 / E13.5 / E15.5 / E17.5 VeCadCreERT²; R26R-EYFP or VeCadherin(PAC)-CreERT²; R26R-EYFP embryos and fetuses. E17.5 stage shows hindlimb sections. Cre recombination was induced in all cases with 1 Tamoxifen injection at E8.5. Arrows indicate EYFP+ MF20+ CD31- myoblasts/myofibers.
(B): Graph showing the count of EYFP+ MF20+ CD31-myofibers / mm² at different developmental stages, in VeCadCREERT² or VeCadherin(PAC)-CreERT² mice. A minimum of five different fields (at 20x magnification) were counted for each stage.
(C-left panel): X-Gal staining (dark blue) plus immunofluorescence using Hoechst (light blue), anti-MF20 (red) and anti-CD31 (purple) antibodies on transversal sections of VeCadCREERT²; R26R P0 hindlimb. Arrow points to a X-Gal+ MF20+ myofiber.
(C-right panel): Immunofluorescence using Hoechst (light blue), anti-EYFP (green) anti-Laminin (red) and anti-CD31 (purple) antibodies on transversal sections of VeCadherin(PAC)-CreERT2;R26R-EYFP P0 hindlimb. Arrows point to EYFP+CD31- myofibers enclosed in basal lamina.

(D-left panel): X-Gal staining (dark blue) plus immunofluorescence using Hoechst (light blue), anti-MF20 (red) and anti-CD31 (green) antibodies on longitudinal sections of VeCadCREERT2; R26R E17.5 hindlimb. Arrow points to a X-Gal+ MF20+ CD31- myofiber.

(D-right panel): Immunofluorescence using Hoechst (light blue), anti-desmin (red), anti-EYFP (green) and anti-CD31 (purple) antibodies on longitudinal sections of VeCadherin(PAC)-CreERT2;R26R-EYFP E17.5 hindlimbs.

Arrow points to a EYFP+ desmin+ CD31- myofiber.

(E): Immunofluorescence using Hoechst (blue), anti-EYFP (green), anti-MF20 (red) and anti-CD31 (purple) antibodies on transversal sections of E15.5 fetuses. Images show the developing mandible. Arrow points to a EYFP+ MF20+ CD31-cell.

Scale bars, 50 µM.
FIGURE 3
Immunofluorescence using Hoechst (blue), anti-EYFP (green), anti-Pax3 (red), anti-Myf5 (purple) and anti-CD31 (purple) antibodies on transversal sections of E9.5 / E10.5 / E11.5 VeCadherin(PAC)-CreERT²; R26R-EYFP embryos. Cre recombination was induced in all cases with 1 Tamoxifen injection at E8.5. Scale bars, 50 µM.

FIGURE 4
(A): Immunofluorescence using Hoechst (blue), anti-EYFP (green), anti-SMA (red), anti-NG2 (red) and anti-CD31 (purple) antibodies on transversal sections of E13.5 / E15.5 VeCadherin(PAC)-CreERT²; R26R-EYFP embryos and fetuses. Cre recombination was induced in all cases with 1 Tamoxifen injection at E8.5. Arrows indicate EYFP+ SMA+ CD31- cells.
(B): Immunofluorescence using Hoechst (blue), anti-EYFP (green), anti-Collagen I (red) and anti-CD31 (purple) antibodies on transversal sections of E17.5 VeCadherin(PAC)-CreERT²; R26R-EYFP forelimbs. Arrows indicate EYFP+ Collagen I+ CD31- cells. d, dermis layer; e, epidermis.
(C): Immunofluorescence using Hoechst (blue), anti-EYFP (green) and anti-CD31 (purple) antibodies on transversal sections of E11.5 and E15.5 VeCadherin(PAC)-CreERT²; R26R-EYFP embryos and fetuses. Images show the AGM region in the E11.5 embryo and the fetal hemopoietic organs in the E15.5 fetus.
(D): Immunofluorescence using Hoechst (blue) and anti-EYFP (green) antibodies on transversal sections of a E15.5 VeCadherin(PAC)-CreERT²; R26R-EYFP fetal heart. Inset shows a magnification of the heart cushions (or heart valves). Scale bars, 50 μM (except where indicated).

FIGURE 5
(A): Immunofluorescence using Hoechst (blue), anti-EYFP (green), anti-CD45 (red) or anti-CD41 (red) and anti-CD31 (purple) or anti-SMA (purple) antibodies on transversal sections of E10.5, E11.5 and E12.5 VeCadherin(PAC)-CreERT²; R26R-EYFP embryos. Cre recombination was induced in all cases with 1 Tamoxifen injection at E8.5.
(A-I,I',I''): Image shows a section at the level of the developing limb bud. Arrow indicates a EYFP+ CD45+ CD31 (low) cell emerging from a vessel.
(A-II,II',II''): Image shows the mesenchyme between the neural tube and somites. Arrow indicates a EYFP+ CD45+ CD31 (low) cell emerging from a vessel.
(A-III,III',III''): Arrow indicates a EYFP+ CD41+ SMA- cell emerging from a vessel. NT, neural tube. DA, dorsal aorta.
(A-IV,IV',IV''): Arrow indicate EYFP+ CD45+ CD31(low) cells in the mesenchyme next to the dorsal aorta. Note that a cluster of CD45+ cells (they are EYFP-, probably due to Cre efficiency which is less than 100%) is emerging from the aorta. DA, dorsal aorta.
(A-V,V',V''): Arrow indicate clusters of EYFP+ CD45+ CD31(low) cells emerging from the dorsal aorta. On the right, next to the neural tube, another EYFP+ CD45+ CD31(low) cell is emerging from a vessel.

(A-VI,VI',VI''): Arrow indicates a EYFP+ CD41+ SMA- cell lying in the mesenchyme.

(A-VII): Image shows a section of a E12.5 embryo at the level of the developing limb. Inset at higher magnification shows a region in which many CD45+ cells are present.

(A-VIII, VIII', VIII''): Arrows indicate EYFP+ CD45+ CD31- cells.

(A-IX, IX', IX''): Image shows a section of the embryonic liver which contains several EYFP+ CD41+ cells.

(B): Immunofluorescence using Hoechst (blue), anti-EYFP (green), anti-CD41 (red) and anti-CD31 (purple) antibodies on transversal sections of E9.5 VeCadherin(PAC)-CreERT2; R26R-EYFP yolk sacs. Arrow indicates a EYFP+ CD41+ cell emerging from a yolk sac vessel (top panel) and a similar EYFP+ CD31+ cell (bottom panel).

(C): Immunofluorescence using Hoechst (blue), anti-EYFP (green), anti-CD45 (red) and anti-SMA (purple) antibodies on transversal sections of E10.5 and E11.5 VeCadherin(PAC)-CreERT2; R26R-EYFP embryos. Cre recombination was induced in all cases with 1 Tamoxifen injection at E8.5. White arrows in the E10.5 and E11.5 panels indicate EYFP+ CD45+ SMA+ cells in the mesenchyme next to the somites (E10.5 panel) or between neural tube and dorsal aorta (E11.5).
Yellow arrow indicates a EYFP+ CD45+ SMA- cell in the E10.5 panel.
Scale bars, 50 µM.

FIGURE 6
(A): Schematic view of the protocol used in the experiments on postnatal mice. VeCadherin(PAC)-CreERT² mice were alternatively crossed with R26R, R26R-EYFP and R26-NZG mice. In all cases, Cre recombination was induced with one single i/P injection of Tamoxifen in the pregnant mother at E8.5. At P7 stage, forelimbs and hindlimbs were collected. At later stages, tibialis anterior, gastrocnemius, quadriceps and triceps brachialis muscles were recovered. In some cases, pectoralis and diaphragm muscles were also collected. Cardiotoxin I/M injection was performed to induce an acute damage and the consequent regeneration response in tibialis anterior, gastrocnemius, quadriceps and triceps brachialis muscles.

(B-I): Whole mount X-Gal staining (dark blue) on P60 VeCadherin(PAC)-CreERT²; R26R tibialis anterior muscle.
(B-I',I''): X-Gal staining (dark blue) on transversal sections of P60 VeCadherin(PAC)-CreERT²; R26R tibialis anterior (I') and pectoralis (I'') muscles. In I'' the muscle was embedded in paraffin, sectioned at a microtome and the section was counterstained with eosin.
(B-II,II',II''): Immunofluorescence using Hoechst (blue), anti-β-gal (green), anti-Pax7 (red) and anti-CD31 (purple) antibodies on transversal sections of P21 VeCadherin(PAC)-CreERT²;
R26R-NZG gastrocnemius muscle. Arrow points to a Pax7+ EYFP- CD31- satellite cell.

(B-III,III',III''): Immunofluorescence using Hoechst (blue), anti-EYFP (green) and anti-CD31 (purple) antibodies on transversal sections of P60 VeCadherin(PAC)-CreERT²; R26R-EYFP pectoralis muscle. An EYFP+ myofiber is present.

(B-IV,IV',IV''): Immunofluorescence using Hoechst (blue), anti-EYFP (green), anti-NG2 (red) and anti-CD31 (purple) antibodies on transversal sections of P60 VeCadherin(PAC)-CreERT²; R26R-EYFP pectoralis muscle. Arrows point to EYFP+ NG2+ CD31- pericytes and interstitial cells.

(B-V,V',V''): Immunofluorescence using Hoechst (blue), anti-EYFP (green), anti-PW1 (red) and anti-CD31 (purple) antibodies on longitudinal sections of P60 VeCadherin(PAC)-CreERT²; R26R-EYFP gastrocnemius muscle. Arrows point to EYFP+ PW1+ CD31- interstitial PICs (PW1+ interstitial cells).

(C-I,I',I'' and II,II',II''): X-Gal staining (dark blue) plus immunofluorescence using Hoechst (light blue), anti-CD31 (red) and anti-Laminin (green) antibodies on transversal sections of CTX-injured P60-P90 VeCadherin(PAC)-CreERT²; R26-NZG gastrocnemius muscle, recovered after 5 or 10 days from damage. Arrows point to regenerating, centronucleated X-Gal+ myofibers.

(C-III,III',III''): Immunofluorescence using Hoechst (light blue), anti-β-Gal (green) and anti-CD68 (red) antibodies on transversal sections of CTX-injured P60-P90 VeCadherin(PAC)-CreERT²; R26-NZG triceps brachialis
muscle, recovered 10 days after damage. Arrows point to β-Gal+ CD68+ macrophages.
Scale bars, 50 µM.

FIGURE 7

(A): Schematic view of the protocol used for cell sorting and FACS analysis of embryonic and adult cells. VeCadCREERT² or VeCadherin(PAC)-CreERT² mice were crossed with ROSA-EYFP mice. Cre recombination was induced in all cases with one single I/P injection of Tamoxifen in the pregnant mother at E8.5. For embryonic cells, E13.5 embryos were collected and dissociated to single cells. Alternatively, to isolate adult cells derived from embryonic endothelial progenitors, CTX-injured and not injured muscles were recovered from P60-P90 adult mice, after 10 or 21 days from damage.

(B): Histograms showing the percentage of EYFP+ embryonic or adult cells.

(C): Embryonic sorted EYFP+ cells were cultured for 3 passages. Left image show a representative phase contrast photo of live cells. Right image show an immunofluorescence done using Hoechst (blue) and anti-EYFP antibody (green).

(D): Embryonic and adult sorted EYFP+ cells were cultured for 2-4 passages. When they reached an adequate number, they were exposed to differentiating stimuli.

(D-I): Embryonic cells exposed to VEGF form an endothelial network. Image show a representative phase contrast photo. Adult cells show a less efficient but similar behaviour.
(D-II): Embryonic and adult cells exposed to TGF-β differentiate into smooth muscle actin (SMA) positive cells. Image show a representative immunofluorescence done using Hoechst (blue) and an anti-SMA antibody (red).

(D-III): Embryonic and adult cells exposed to BMP-2 differentiate into bone-like cells expressing alkaline phosphatase. Image shows an alkaline phosphatase reaction (blue).

(D-IV): Embryonic and adult cells cultured in adipogenic inductive medium differentiate into adipocytes. Image shows an Oil Red O staining on cultured cells. Note adipocytes containing red fat droplets in the cytoplasm.

(D-V and VI): Embryonic and adult cells were infected with pRRL.sin.PPT.CMV.nLacZ.pre lentivirus and co-cultured with C2C12 to induce myogenic differentiation. Co-culture assay was stopped after 3 or 5 days. Images show X-gal staining (dark blue), plus immunofluorescence using Hoechst (light blue) and an anti-MF20 antibody (red). Arrows in D-V image point to single-nucleated X-Gal+ MF20+ differentiated myoblasts. D-VI image show large MF20+ myotubes with lots of X-Gal+ nuclei inside them.

(E): Graph showing results of FACS analysis of embryonic EYFP+ cells. The two series (“Total Embryo –Liver” and “Embryonic Liver”) represent EYFP+ cells isolated respectively from embryos deprived of liver and viscera, and from embryonic liver. Results are expressed in percentage of antigen-positive cells on the total of EYFP+ cells.

The two images on the right show phase contrast images of Total Embryo –liver- and Embryonic Liver-derived cells cultured in vitro, after 2 passages.
(F): Graph showing results of FACS analysis of adult EYFP+ cells. The three series represent EYFP+ cells isolated from undamaged muscle (blue), regenerating muscle after 10 days from cardiotoxin damage (red), regenerating muscle after 21 days from cardiotoxin damage (green). Results are expressed as the percentage of antigen-positive cells on the total of EYFP+ cells.

FIGURE 8
(A): Intra-muscular injection of embryonic endothelial derived cells into wild type mice. Embryonic cells were cultured for 3-4 passages and infected with pRRL.sin.PPT.CMV.nLacZ.pre lentivirus. Wild type mice of the same background were injured with intra-muscular injection of CTX [10µM]. Cells were injected 2 days after damage; muscles were collected 6 days later.
(A-I): Whole mount X-Gal staining (dark blue) of a tibialis anterior muscle injected with embryonic cells. Inset at higher magnification show some superficial myofibers with X-Gal positive nuclei inside.
(A-II,II'): X-Gal staining (dark blue) plus immunofluorescence using Hoechst (light blue), anti-Laminin (green) and anti-MF20 (red) antibodies on transversal sections of a tibialis anterior muscle injected with embryonic cells. X-Gal+ nuclei are present in regenerating, centronucleated myofibers, both in central position and in a peripheral, satellite cell-like position.
(B): Intra-muscular injection of embryonic and adult endothelial derived cells into alpha-sarcoglycan (α-SG) knockout mice.
Mice were immunosuppressed and sacrificed 10-12 days after cell injection.
(B-I): X-Gal staining (dark blue) on transversal sections of a tibialis anterior muscle of an α-SG mouse, injected with nLacZ-infected embryonic cells. The representative phase contrast image shows X-Gal positive nuclei, most of which are inside myofibers in a central position.
(B-II): Immunofluorescence using Hoechst (blue) and an anti-α-SG (red) antibody on transversal sections of a TA of an α-SG ko mouse injected with uninfected adult cells. A cluster of α-SG positive, regenerating, centrally nucleated myofibers is shown.
(B-III and IV): Immunofluorescence controls. Immunofluorescence using Hoechst (blue) and anti-α-SG (red) antibody on transversal sections of wild type and uninjected α-SG knockout mice.
(B-V): Immunofluorescence using Hoechst (blue) and an anti-α-SG (red) antibody on transversal sections of a TA of an α-SG ko mouse injected with uninfected embryonic cells. A cluster of α-SG positive, regenerating, centrally nucleated myofibers is shown.
Scale bars, 50 µM.
2.6. REFERENCES


CHAPTER III
3. FINAL DISCUSSION

3.1. SUMMARY

Mesoangioblasts and other vessel associated stem cells are attracting clinical interest because of their potential uses in regenerative medicine. Despite of this, their native identity and physiological role are still unknown. Since unraveling the identity and gaining more insight into the basic biology of those stem cells are obligatory steps towards the development of pharmacological targets to improve regenerative potential, we decided to address those issues by using a genetic lineage tracing approach. Our strategy was based on labeling VE-Cadherin+ endothelial progenitors in a short time window during embryogenesis, to follow their fate during embryonic and adult development.

We have demonstrated that embryonic endothelial derived progenitors contribute to skeletal and smooth muscle development, and to other mesodermal lineages, including dermis. We also found that cells derived by embryonic endothelium persist in the adult muscle, contributing to subsets of myogenic progenitors and macrophages and participating in muscle regeneration.

Isolation of embryonic endothelial derived cells from embryo and adult muscle showed that both populations are myogenic in vitro and in vivo, and are able to differentiate into multiple mesodermal lineages.
Moreover, we demonstrated that haemogenic endothelium is present in the yolk sac before being active in the body of the embryo. We identified endothelial derived haematopoietic cells, at least in part originated from haemogenic endothelium in the yolk sac, which emerge from vessels in the body of the embryo and migrate through the mesenchyme.

We also identified a novel population of endothelial-derived cells in the embryonic mesenchyme, expressing haematopoietic and mesenchymal markers, which we hypothesize to represent the in vivo counterpart of embryonic mesoangioblasts.
3.2. CONCLUSIONS: TRANSLATIONAL CONSIDERATIONS AND FUTURE PERSPECTIVES

The last 10 years have seen a growing interest in regenerative medicine, due to its potential to treat chronic and degenerative diseases, such as atherosclerosis, diabetes, ischemia, aging, and many others, including muscular dystrophy. Those diseases are gaining more and more clinical relevance because of the lengthening of the individual lifespan due to improved medical treatments. However, patients suffering from such illnesses are currently faced with a relatively short list of options, which include long term drug therapy (which may allow a disease to be managed, but rarely cures it), organ transplant, or the use of medical devices like pacemakers.

Regenerative medicine aims to restore the function of diseased or damaged tissues or organs by a variety of approaches, from cell-based therapies through gene therapy and tissue engineering, although much of the recent interest in regenerative medicine arises from developments in stem cell research.

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. Regardless of the potential success of engrafted stem cells, such therapies will be very costly and will require tailoring for each patient, given the current state of the art. Approaches
aimed at mobilizing endogenous stem cells become more plausible in light of a major shift in the field of stem cell biology, which has provided increasing evidence that pluripotent stem cells with regenerative potential are present in adult tissues, contrary to the generally accepted view just 5-10 years ago. While many tissues possess limited regenerative potential, the capacity for regeneration declines with age and chronic disease. In addition, stem cell requirement in response to injury or disease often produces inappropriate re-patterning of the tissue, culminating in scar tissue formation (fibrosis), inadequate revascularization, or chronic inflammatory disorders. Other drawbacks to the approach of using engrafted stem cells are the requirement for immune suppression (if allogenic cells are used) and the requirement for cell culture, which can alter the stem cell capacity and sometimes can not be applied because some cell populations are refractory to be cultured.

Vascular associated multipotent progenitors hold promise for several diseases ranging from muscular dystrophy to degenerative vascular diseases, including atherosclerosis, vascular damage in diabetes and peripheral ischemic vascular disease [1-7].

Muscle degeneration provokes a decrease in vascularization due to fibrosis, which exacerbates muscle damage and impedes regeneration. There is increasing evidence that multiple cell types in situ communicate with each other to correctly drive regeneration and that cells from the immune
system regulate stem cell function, directly participating in the regenerative process. It is therefore of fundamental importance to gain more insight into the basic biological process of the tissue environment in which endogenous stem cells are activated, as well as to identify novel precursor populations with pluripotent capacity. Molecules that can be used to therapeutically enhance and directly target these cells will lead to a more effective approach to muscle and vascular regenerative medicine.

The present study identifies a novel population of vessel-associated progenitor cells and define their developmental origin and \textit{in vivo} fate. In particular, we have demonstrated that endogenous endothelial derived progenitors contribute to smooth and skeletal muscle development, and participate in muscle regeneration.

This knowledge will be applied to search for strategies to mobilize endogenous vessel-associated stem/progenitor cells and improve their contribution to skeletal or smooth muscle, or to vasculogenesis, with the ultimate goal to treat chronic and degenerative diseases.

Approaches to better characterize the biology of vessel associated progenitor cells are already ongoing in our laboratory.

The present study focused on following the fate of embryonic vessel-associated progenitors; in parallel, we are also analyzing perinatal and adult vascular progenitors’ fate and their contribution to muscle regeneration.
Taking advantage of transgenic and knockout mice models that are already available in the lab, we are studying the effect of specific genes involved in myogenesis and muscle regeneration, such as Pax7 [8] and Necdin [9], on the in vivo fate of vessel associated progenitors.

Moreover, our laboratory is interested in how the immunological response to muscle damage influences muscle repair. Our group has demonstrated that polarized macrophages possess the ability of attracting mesoangioblasts through the secretion of molecules involved in cell migration, like HMGB-1 and MMP-9 [10]. In particular, we are currently analyzing the relationship between macrophages and the recruitment and differentiation of endogenous vessel-associated progenitors.

Previous work from E. Clementi’s laboratory, in collaboration with G.Cossu’s group and us, has shown that a combinatorial therapy including non steroidal anti-inflammatory drugs and nitric oxide (NO), either combined in the same molecule or separately administered, is effective in functionally ameliorating muscular dystrophy in two murine models, without any secondary effect [11, 12]. This treatment can efficiently slow down the disease and acts by reducing inflammation, preventing muscle damage and preserving the number and function of satellite cells. In addition, this treatment enhances the therapeutic efficacy of mesoangioblasts, increasing their ability to migrate to skeletal muscle and to engraft muscle fibers. Moreover, an ex vivo treatment of mesoangioblasts with NO donors generally improves their muscle repair ability,
increasing their homing to skeletal muscle and reducing apoptosis [13]. At the moment, we are starting to evaluate the effect of nitric oxide donors in the mobilization of endogenous vessel associated progenitors. With the tools currently in our hands we can label different populations of cells depending on the time of Cre recombination induction, in order to evaluate separately the effect of NO in those populations. In addition, we will cross the VE-Cadherin lineage tracer mouse with models of muscular dystrophy, to obtain a tool that will allow us to follow the fate of endogenous vessel associated progenitors in a chronic damage context, and to analyze the effect of nitric oxide in endogenous stem cell mobilization. Translation of these preclinical data into clinical practice will be fast and facilitated by the fact that NO donors and NSAIDs are already approved for use in paediatric patients.

Furthermore, we want to gain more insight into the process of endothelial to haematopoietic transition in the yolk sac, and into the abluminal emergence of haematopoietic cells from the vessels of the embryo, to assess whether those cells undergo extravasation or abluminal endothelial to haematopoietic transition. More analyses will also be needed to understand the identity of the haematopoietic / mesenchymal cell populations derived by embryonic endothelium.

We hope that by understanding the role of the endothelium in embryonic hematopoiesis, translational applications can be made to allow for optimal hematopoietic stem cell growth and expansion, with the ultimate goal to improve their use in patient
transfusions and transplants.
3.3. REFERENCES


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