UNIVERSITA' DEGLI STUDI DI ROMA " SAPIENZA"

DOTTORATO DI RICERCA IN SCIENZE MORFOGENETICHE E CITOLOGICHE

Endothelial-derived DLL4 and PDGF-BB reprogram committed skeletal myoblasts to a pericyte fate but do not erase their myogenic memory.

Director of studies: Prof. Giulio Cossu

Tesi di DOTTORATO di RICERCA in

Scienze morfogenetiche e citologiche

Ornella Cappellari matricola 1238825

CONSULTAZIONE TESI DI DOTTORATO DI RICERCA

Il sottoscritto Ornella Cappellari matricola 1238825
Nata a Varese (Va) il 17.03.1984

autore della tesi di DOTTORATO dal titolo

"**Endothelial-derived DLL4 and PDGF-BB reprogram committed skeletal myoblasts to a pericyte fate but do not erase their myogenic memory.**"

AUTORIZZA

La consultazione della tesi stessa, fatto divieto di riprodurre, in tutto o in parte, quanto in essa contenuto.

CONTENTS

ABSTRACT

Pericytes are vessel associated mural cells that form the smooth muscle layer of vessels. They are able to contribute to skeletal muscle regeneration as previously demonstrated for mesoangioblasts that indeed represent their in vitro counterpart. Pericytes are a heterogeneous population characterized by different marker like Alkaline Phosphatase, Smooth muscle actin, Neuro glial2 (NG2). Endothelial cells recruit pericytes from the surrounding mesoderm progenitor through a PDGF-PDGFrB loop.

In this work I have conducted experiments whose results showed that functional pericytes may derive from direct reprogramming of already committed embryonic and, less frequently, fetal skeletal myoblasts. When co-transplanted *in vivo* with endothelial cells, Pax3 or Myf5 expressing myoblasts, downregulate myogenic genes, with the notable exception of Myf5, upregulate pericyte markers, adopting a perithelial position and morphology in newly formed vessel networks. The activity of endothelial cells can be replaced by exposure to PDGF-BB and Dll4 but not Dll1 or Jagged 1, while inhibition of Notch signaling via a γ-secretase inhibitor completely restores myogenesis, confirming that the skeletal myogenic program is not irreversibly erased.

Notch activation in MyoD expressing embryonic cells *in vivo* abolishes myogenesis but not Myf5 expression that however cannot activate myogenin and trigger myogenesis because Notch, beside suppressing MyoD transcription, activates Id and Twist factors that bind to and inhibit Myf5 transcriptional activity.

Myf5 expressing cells activate pericyte genes and adopt a perithelial position, a phenomenon that can be rarely observed also in WT embryos.

These data demonstrate that endothelial cells are able to directly reprogram committed skeletal myoblasts to mature pericytes for the formation and stabilization of vessel wall, suggesting that reprogramming occurs as a natural developmental process that leads in a sort of competition between endothelium and muscle.

INTRODUCTION

1.1 Pericytes

1.1.1 Pericyte characteristics and markers

Although Eberth described their presence in 1871 (Eberth, 1871), the discovery of pericytes is commonly assigned to the French scientist Charles-Marie Benjamin Rouget, who two years later described a population of contractile cells surrounding the endothelial cells of small blood vessels (Rouget, 1873). Zimmermann later called these cells ‘‘Rouget cells’’ and also coined the term ‘‘pericytes,’’ alluding to their location in close proximity to the endothelial cells (Zimmermann, 1923). Between 1920 and 1950 numerous other publications described pericytes, some of them, however, questioning the contractility of the cells (reviewed by(Miller and Sims, 1986). Part of these discrepancies probably had experimental reasons, but they may also reflect pericyte heterogeneity and confusion about cell identity. Today, it is clear that different cell types occupy the peri-endothelial compartments, so that their correct identification is still challenging (reviewed by(Krueger and Bechmann, 2010).

Currently mature pericytes are defined as cells embedded within the vascular basement membrane (BM) thanks to the application of electron microscopy (reviewed(Miller and Sims, 1986). As discussed below, this definition is difficult to apply in situations of active angiogenesis. Another commonly accepted defining criterion is their presence in microvessels, i.e., capillaries, postcapillary venules, and terminal arterioles. Also, this definition

has been challenged by observations of subendothelial pericyte-like cells in large vessels (reviewed by(Diaz-Flores et al., 2009).

The periendothelial location of pericytes is frequently confused with the periendothelial location of vascular smooth muscle cells (vSMCs), fibroblasts, macrophages, and even epithelial cells which are cells distinct from pericytes. Although the field has generally adopted the view that pericytes belong to the same lineage and category of cells as vSMCs, it should be remembered that there is no single molecular marker known that can be used to unequivocally identify pericytes and distinguish them from vSMCs or other mesenchymal cells. The multiple markers that are commonly applied are neither specific nor stable in their expression.

PVC can be classified as pericytes, (*peri,* around; *cyte,* cell); alternatively as vascular mural cells or vascular smooth muscle cells (VSMC) depending on their morphology and location.

Pericytes (as revealed by electron microscopy), possess a prominent nucleus and a small content of cytoplasm with several large processes. They are found in blood capillaries, pre-capillary arterioles, post-capillary venules, and collecting venules. In these vessels, endothelial cells and pericytes share the same basal membrane and are closely associated by tight, gap, and adherence junctions. Vascular smooth muscle cells are detected around large arteries and veins and are separated from the vascular basal membrane by a layer of mesenchymal cells and extracellular matrix, the intima. However, it is important to remember that distinction between pericytes and VSMC is not absolute, both cells may represent subtypes of the related cell type (Armulik et

al., 2005; Armulik et al., 2011) or they can derive from the same progenitor. It has also been suggested that pericytes may reside subjacent to the endothelium of large vessels and be the progenitors of VSMC.

Pericytes' distribution is highly variable according to size and to vessel type: they are quite abundant on small venules and arterioles but rather sparse on capillaries; they cover irregularly the veins with large processes and continuously the artery where they form a cell layer. Distribution seems to be dependent also on blood pressure levels, indeed in human and horses have been demonstrated that they are more abundant further down the torso and the legs.

Pericytes show differences also according to the organ in which they are found: they are abundant in CNS (central nervous system), where they interact via tight junction with the endothelial cells to create the blood brain barrier membrane (BBB) (Ballabh et al., 2004), in kidney (called mesangial cells) (Betsholtz et al., 2004) and in the liver (called Itoh cells) (Nakajima et al., 2000).

All these different characteristics and locations make very difficult a universal definition of pericytes; there are several markers that can be used to detect these cells, but the expression patterns can change according to different tissues, or stage of vascular development. Most of these antigens are not specific and can be detected also in other cells types. Moreover not all pericytes express all pericytes marker.

Some of these markers are cytoskeletal proteins such as Desmin, muscle specific class III intermediate filament, and α-smooth muscle actin (SMA). Desmin is normally expressed by differentiated skeletal muscle, cardiac and

smooth muscle cells, while SMA is known to be present on smooth muscle cells or particular kinds of fibroblasts called myofibroblasts (Ronnov-Jessen and Petersen, 1996).

Neuro-glial 2 (NG2) is a chondroitin sulfate proteoglycan expressed on pericytes, in particular during angiogenesis and vasculogenesis. The name is derived from its expression on glial precursors O-2A; it is used to detect immature neural stem cells capable to differentiate into neuron or glia. It can bind the bFGF, PDGF-AA, plasminogen and angiostatin. NG2 knockout mice are viable, and neovascularization after damage is compromised (Ozerdem and Stallcup, 2004) (Rajantie et al., 2004).

PDGFR-β (Platelet-Derived Growth Factor Receptor beta) is localized on pericytes and it has a crucial role in the recruitment of these cells during angiogenesis (Betsholtz, 2004). PDGFR-β deficient mouse embryos were found to lack microvascular pericytes and to develop numerous capillary microaneurysms (Lindahl et al., 1997).

Comparison between gene expression profile of wild type and PDGFRβ knockout mice, made possible to discover a new marker of pericytes: the Regulatory G protein Signalling 5 (RGS-5), a GTPase activating protein G_{i_α} and G_{q_α}, that is almost absent in PDGFRβ knockout mice (Berger et al., 2005).

Annexin A5 (Anx5) is a protein involved in apoptosis, but unexpectedly is also expressed on perivascular cells and it seems to define a novel mesenchymal stem cell population.

Alkaline phosphatase (AP) isoenzymes, have been found to be expressed on pericytes of skeletal muscle (Grim and Carlson, 1990; Safadi et al., 1991) bone and heart (Schultz-Hector, 1993; Schultz-Hector et al., 1993). As described below, we have used principally this protein to define pericyte-derived cells.

PERICYTE MARKER	SYMBOL	CELLS TYPE EXPRESSING MARKER	COMMENTS	REFERENCES
PDGFR-β(**platelet-derived growth factor receptor-beta)**	Pdgfrb	Interstitial mesenchymal cells during development; smooth muscle; in the CNS certain neurons and neuronal progenitors; myofibroblasts; mesenchymal stem cells	Receptor tyrosine kinase; functionally involved in pericyte recruitment during angiogenesis; useful marker for brain pericytes	Lindhal et al., 1997 (Winkler et al., 2010)
NG2 (chondroitin sulfate proteoglycan 4)	Cspg4	Developing cartilage, bone, muscle; early postnatal skin; adult skin stem cells; adipocytes; vSMCs; neuronal progenitors; oligodendrocyte progenitors	Integral membrane chondroitin sulfate proteoglycan; involved in pericyte recruitment to tumor vasculature	(Ozerdem et al., 2001) (Ruiter et al., 1993) (Huang et al., 2010)
CD13 (alanyl (membrane) aminopeptidase)	Anpep	vSMCs, inflamed and tumor endothelium; myeloid cells; epithelial cells in the kidney, gut	Type II membrane zinc-dependent metalloprotease; useful marker for brain pericytes	(Kunz et al., 1994) (Dermietzel and Krause, 1991)
aSMA (alpha-smooth muscle actin)	Acta2	Smooth muscle; myofibroblasts; myoepithlium	Structural protein; quiescent pericytes do not express aSMA (e.g., CNS); expression in pericytes is commonly upregulated in tumors and in inflammation	(Nehls and Drenckhahn, 1993)
Desmin	Des	Skeletal, cardiac, smooth muscle	Structural protein; useful pericyte marker outside skeletal muscle and heart	(Nehls et al., 1992)

Table 1.1 Pericyte Marker

1.1.2 Pericyte function and origin

Pericytes are supposed to play several common roles in all tissues, and in addition, tissue specific functions in organs where they are particularly abundant.

Since both pericytes and VSC express some contracting molecules, such as Smooth-muscle actin (SMA), and exhibit a number of characteristics consistent with contractile activity, it is possible thet they are able to regulate blood flow. Moreover, pericytes are able to induce vasoconstriction and vasodilatation within capillary beds thanks to the their sensibility to endothelin-1 (Rucker et al., 2000).

Importantly, pericytes are involved in vessel stability. Through their long processes pericytes wrap the capillary and communicate with endothelial cells by direct physical contact and paracrine signals. Gap junctions provide direct connections between the cytoplasm of pericytes and endothelial cells and contain cell-adhesion molecules such as N-cadherin and beta-catenin and extra-cellular matrix (ECM) molecules such as fibronectin (Berger et al., 2005). Many pathways have been shown to take advantage from this particular connection. The loop signalling Tie2-Ang1, where Tie2 receptor is expressed on endothelial cells whereas its agonist ligand Ang1 is expressed mainly by perivascular and mural cells, has been shown to be essential for vessel maturation and stabilization (Sato et al., 1995; Suri et al., 1996).

The process of skeletal muscle regeneration does not involve only muscle fibres, since also new vessels must be generated in order to repair damage tissue. Angiogenesis requires sprouting of endothelial cells (ECs) from pre-existing vessels (Lamalice et al., 2007; Sun et al., 2005) and recruitment of perivascular cells (PVC), (Armulik et al., 2005; Armulik et al., 2011) particular cells that surround the endothelial vessels.

The origin of vascular smooth muscle cells is still not completely understood; there may be many different sources of perivascular progenitors. In general models, it is possible to find different recognize origin; from transi-differentiation of other pericytes, from neural crest and also it seem that pericytes precursors are postulated to be recruited from local surrounding mesenchyme to the vascular wall by chemotactic factors secreted by endothelial cells, specifically endothelial tip cells. Tissue culture studies have shown that TGFβ1 cytokine promotes differentiation of mesenchymal precursors into PDGFRβ+ pericyte progenitors. During vessel growth, endothelial cells in the capillary plexus release PDGF-BB attracting in this way pericytes to the structure. PDGF pathway seems not to be essential for vessel development, but only for pericytes recruitment into the newly formed vessels, (Hellstrom et al., 1999) so mainly during vasculogenesis and angiogenesis. At the same time, TGF-β1 induces vessel maturation, inhibiting endothelial cell proliferation and migration (Orlidge and D'Amore, 1987; Sato et al., 1990).

Recently by clonal analysis, it has been shown that at least a fraction of pericytes of the dorsal aorta derived from PAX3+/α-cardiac-actin+ precursors of hypaxial dermomyotome,(Esner et al., 2006) which has led to the suggestion of a somitic origin for a sub-set of perivascular cells. Moreover a novel common vascular precursor has been identified (Flk1+ embryonic stem cells), that can give rise to EC by VEGF induction and to pericytes by PDGF-BB. According with this observation, it has been shown that ECs in chicken dorsal aorta can differentiate into VSMCs in developing vessels .

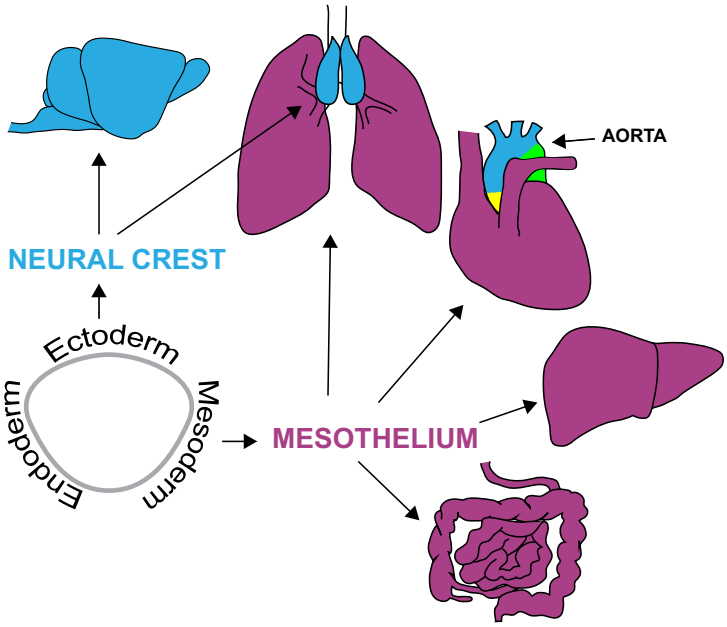

Fig 1.1.2 Pericytes origin

Ectoderm-derived neural crest gives rise to vSMCs and pericytes in the CNS and thymus (light blue). Mural cells in coleomic organs are all mesoderm-and mesothelium-derived (violet). Epicardial mesothelium gives rise to mural cells in heart, lung mesothelium to pericytes in the lung, etc. Note that vSMC coverage around aorta has a multiple developmental origins, indicated by different colors (yellow, secondary heart field; light blue, neural crest; green, somite).

In the adult, vascular smooth muscle cells can be generated from bone marrow derived cells or its stroma (Campbell et al., 2000). In addition fibroblasts can differentiate into myofibroblasts, which in turn differentiate into vascular SMCs in response to mechanical or biochemical cues (Sartore et al., 2001; Tomasek et al., 2002). Thus, the heterogeneity of pericytes is reflected in multiple developmental origins of these cells.

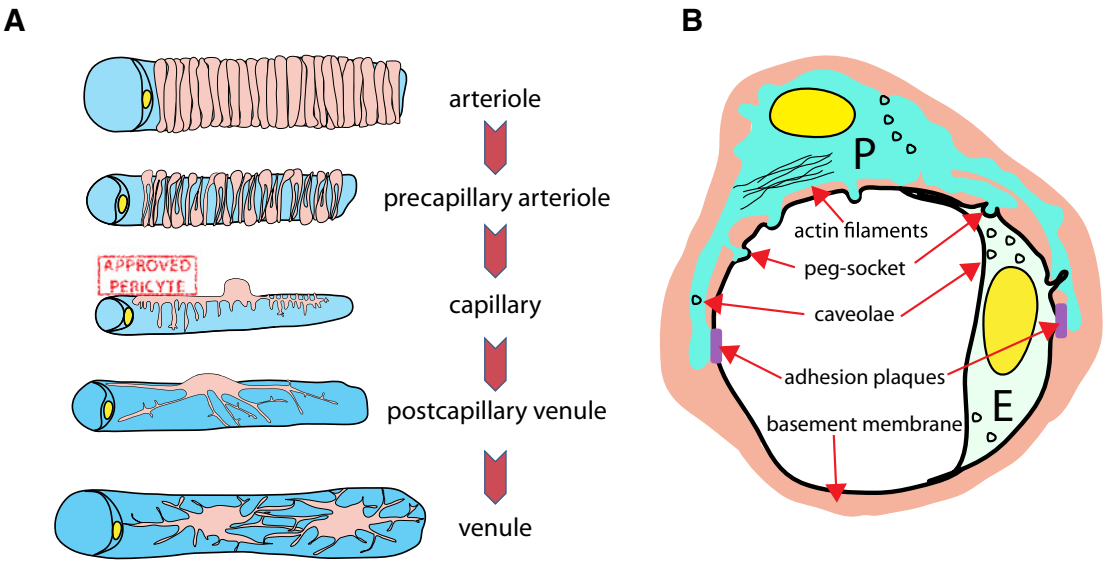

Figure 1.1.3 Pericytes localization

(A) A continuum of mural cell cyto-architecture from arteriole to venule. A single vSMC layer around arterioles and precapillary arterioles encircles the entire abluminal side of the endothelium. vSMCs around arterioles have a flattened, spindle-shaped appearance with few cytoplasmic processes, whereas around precapillary arterioles the cell bodies are distinctly protruding and extend several processes encircling the endothelium. Pericytes investing capillaries have a nearly rounded cell body that gives rise to a few primary processes running on the endothelium in the length of the capillary. The primary processes give rise to secondary perpendicular processes. The tips of secondary processes attach firmly to the endothelium.

(B) Ultrastructural characteristics of pericytes and pericyte-endothelial interactions. Pericytes are rather anonymous in transmission electron microscopy. The mature capillary pericyte (P) has a discoid nucleus that is surrounded by a small amount of cytoplasm containing protein-producing organelles and mitochondria. Despite being separated by the shared BM, pericytes and endothelial cells (E) make numerous direct contacts of different type: schematically depicted are peg-socket contacts and adhesion plaques. Adapted from (Armulik et al., 2011)

1.1.3 Pericyte Plasticity

Recent new experiments suggested that pericytes have the ability to differentiate into different cells of mesodermal lineages.

Canfield and colleagues have shown that pericytes of bovine retina are able to differentiate into osteoblasts, chondrocytes and adipocytes in vitro and in vivo. In these experiments pericytes were inoculated into diffuse chambers that has been implanted into athymic mice; after a few months the tissue contained areas resembling bone (von Tell et al., 2006)or mineralized cartilage with lacunae containing chondrocytes. Moreover cells resembling adipocytes could also be detected. In vitro studies confirmed this plasticity(von Tell et al., 2006).

The pericytes's ability to differentiate into osteoblasts and bone opened the possibility that these phenomena may be implicated in ectopic calcifications that can be detected in arteries of the heart and skeletal muscle . Recently, it has been hypothesised that the skeletal muscle ossification detectable in Fibrodysplasia ossificans progessiva (FOP) may have a vascular origin, and in particular that it can be due to anomalies in the normal pericyte pathway that leads to increase the osteoblastic differentiation (Hegyi et al., 2003).

Another suggestion of pericyte plasticity comes from studies in which Annexin 5 was used to isolate a novel perivascular population that resembles mesenchymal stem cells. Anx5-lacZ+ cells isolated from embryonic brain meninges, by FACS sorting, express several stem cell markers such as Flk1, c-kit, Sca-1, and can differentiate into an adipogenic, chondrogenic and osteogenic phenotype *in vitro*. (Brachvogel et al., 2005)

1.1.4 Akaline phosphatase

Alkaline phosphatases (AP; ortophosphomonoester phosphoidrolase) are a group of membrane-bound glycoproteins that hydrolyze a large range of monophosphate esters at alkaline PH optima.

In human 4 isoforms of AP are know, the placental (PL-AP) the testicular germ-cell (GC-AP); the intestinal (I-AP) and the tissue aspecific AP (mainly present in bone-liver-kidney, TN-AP). I-AP, CG-AP and PL-AP show a very high homology: a minimum of 90% sequence identity at amino acid level, all map on chromosome number 2; (Martin et al., 1987) whereas TN-AP is only 57% identical to I-AP at the aminoacid level. It maps to a distal short arm of chromosome number 1 (Smith et al., 1988) and it is constituted of 12 exons distributed over more that 50 Kb .

In mouse only three isoforms have been identified: the placental AP (PL-AP) and the intestinal AP (I-AP) that are homolog and map on chromosome number 1 and the tissue non-specific AP (TN-AP) on chromosome number 4. The mouse TN-AP gene is constituted of 12 exons, as the human counterpart, and the ATG is localized on exon number 2. (www.ensembl.org)

Physiological function of alkaline phosphatase in still unknown; by histochemical staining it appears at the late 2-4 cell stage in the mouse embryo (Mulnard and Puissant, 1987) (Mulnard J, 1987). The only information about AP gene regulation has been detected in bone where TN-AP levels of expression increase markedly when mineralization is actively occurring (Register and Wuthier, 1984). In *in vitro* assays AP expression is used as indicator of osteoblastic differentiation (Minasi et al., 2002).

Even if TN-AP expression is very strong in tissues as kidney, bone and liver, it is actually diffusely expressed in many tissues of the body. The first evidence of TN-AP expression on capillaries was detected in 1965; (Mizutani and Barrnett, 1965) other studies later on have shown its presence in capillaries and pericytes of rat, mice and guinea pig myocardium. (Schultz-Hector, 1993) Most importantly, AP has been shown to be expressed in intramuscular connective tissue and in capillary-ends of striated muscle in many animal types (such as chick, quail, mouse, rabbit, hamster, guinea pig, dog, monkey) and human. (Grim and Carlson, 1990; Safadi et al., 1991) The AP positive cells can be easily isolated from adult skeletal muscle and cultured *in vitro*, they present a mesenchymal morphology and maintain AP expression along with other typical pericyte markers.(Levy et al., 2001; Lindner et al., 1997)

Moreover recent work demonstrated that resident pericytes in skeletal muscle isolated through AP expression, contributes to muscle growth and to are able to enter the satellite cell compartment during postnatal development.(Dellavalle et al., 2011)

1.2. Myogenesis

1.2.1 The Onset of Myogenesis

PAX family consists of nine transcription factors, which have common paired, homeodomain and octapeptide domains. Each member of this family has a crucial role in organogenesis during embryonic and fetal development. (Chalepakis et al., 1992) PAX proteins regulate a wide variety of cellular processes, including cell proliferation, self-renewal, apoptosis, migration and

differentiation. (Chalepakis et al., 1992)They are further divided into four subfamilies based on their structural and expression domains.(Gruss and Walther, 1992) The Pax3/7 subfamily regulates both myogenesis (Buckingham and Relaix, 2007) and neurogenesis in the neural crest (Koblar et al., 1999).

Pax3 is first expressed in the presomitic mesoderm (Williams and Ordahl, 1994)and is required for survival of the ventro-lateral dermomyotome, which gives rise to the hypaxial and limb musculature. (Teboul et al., 2002) Pax7 is expressed later, in the central dermomyotome, (Jostes et al., 1990) and is thought to be essential only during postnatal myogenesis (Seale et al., 2000). A cell population has been identified that expresses both Pax3 and Pax7, but no additional markers of skeletal muscle such as MyoD or desmin; (Kassar-Duchossoy et al., 2004; Relaix et al., 2006) these cells proliferate in embryonic and fetal muscles of the trunk and limbs throughout development. (Relaix et al., 2006) In the absence of both Pax3 and Pax7, muscle development is arrested and precursor cells do not leave the myotome. (Lagha et al., 2005)However, Pax3 and Pax7 exhibit divergent functions in development: Pax7 can substitute for Pax3 in dorsal neural tube, neural crest cell, somite and muscle formation in the trunk, but not in muscle formation in the limbs.(Relaix et al., 2004) At the same time, Pax3 cannot substitute for Pax7 in postnatal skeletal muscle.(Lagha et al., 2008) Deletion of Pax3 leads to death of progenitor cells in the hypaxial somite, (Buckingham and Relaix, 2007) and Pax3 also is required for the delamination and migration of muscle progenitor cells to sites where skeletal muscle will form, such as the limbs. (Franz et al., 1993)Ectopic Pax3 can drive myogenesis of embryonic carcinoma (Darabi et al., 2008; Franz et al., 1993) cells and embryonic stem cells. In the adult, Pax3 is expressed in a variety of

muscles, but to differing degrees in different muscles. (Teboul et al., 2002)These observations collectively raise important questions about the potential developmental and anatomic diversity of the functions of Pax3.

Similarly to Pax proteins, the four canonical myogenic regulatory factors (MRFs) Myf5, MyoD, myogenin and Mrf4 have also been intensively studied in early development. All these MRFs were initially characterized by virtue of their ability to convert certain non-muscle cell lines, such as fibroblasts, into myoblasts and myotubes. (Weintraub et al., 1991) Studies in targeted mice revealed that the MRFs are essential, to differing extents, for prenatal skeletal muscle development (Weintraub et al., 1991) but are in large part redundant so that a single null allele has a mild phenotype. Pax3 can directly transactivate Myf5 and myogenin expression during embryonic myogenesis, (Bajard et al., 2006)and Pax3 also appears to activate MyoD in some circumstances, (Maroto et al., 1997; Tajbakhsh et al., 1997) although this regulation is probably indirect. (Buckingham and Relaix, 2007) In contrast to developing muscle, Pax3 does not transactivate Myf5 in the adult. (Relaix et al., 2006)

It is widely accepted that all the skeletal muscles in vertebrate body, with the exception of some craniofacial muscles (see below), derive from progenitors present in the somites.

Somites are transient mesodermal units, which form in a cranio-caudal succession by segmentation of the paraxial mesoderm on both sides of the neural tube. Each newly formed somite rapidly differentiates into a ventral sclerotome and a dorsal dermomyotome from which myogenic precursors originate. Shortly after the onset of somitogenesis (at E 8.75 in the mouse) myogenic progenitor cells differentiate into the differentiated, mononucleated

muscle cells (myocytes) of the “primary” myotome. Primary myotome formation is a multistep process in which precursors translocate from the dermomyotome to a ventrally located domain where they elongate along the axis of the embryo to span the entire somite length. This process has been intensively studied and some aspects are still subject of controversy. (Hollway and Currie, 2005; Kalcheim and Ben-Yair, 2005; Venters et al., 1999)Nevertheless the final pattern is relatively simple, with all muscle cells aligned along the whole cranio-caudal length of the somite, likely as a remnant of muscle differentiation in fish. The role that the myotome exerts during development of higher vertebrates remains unclear. It has been proposed that myotomal cells can act as a kind of scaffold for successive waves of myoblasts. Nevertheless in the Myf5 null mice, where a primary myotome fails to form, myogenesis proceeds in a relatively normal sequence (Braun et al., 1992; Tajbakhsh et al., 1996)suggesting that formation of the primary myotome is not a necessary step for later muscle development in amniotes.

Only a minor fraction of myogenic progenitors terminally differentiate during primary myotome formation. As schematized in figure 1.2.1, skeletal muscle is established in successive distinct, though overlapping steps involving different type of myoblasts (embryonic, foetal myoblasts and satellite cells). The continued growth of muscles that occurs during late embryonic (E10.5-12.5), foetal and post-natal life was recently attributed to a population of muscle progenitors that arise in the central part of the dermomyotome (Ben-Yair and Kalcheim, 2005; Kassar-Duchossoy et al., 2004; Relaix et al., 2005; Schienda et al., 2006)

Around 90% of these skeletal muscle progenitor cells co-express Pax3 and Pax7 until E14.5. These progenitors can differentiate into skeletal muscle fibres during embryogenesis or remain as a reserve cell population within the growing muscle mass during peri and postnatal stages. In Pax3\Pax7 double mutant the generation of primary myotome is unaffected, whereas successive phases of myogenesis are compromised, due to the non myogenic fate adopted by these progenitors. Therefore it has been proposed that all the cells of the myogenic lineage (with the exception of myotomal cells) could derive from Pax3/7 positive population of myogenic progenitors resident in the central part of the dermomyotome.

At around day E 11.5 (in the mouse), during a phase, which is usually referred as primary myogenesis, embryonic myoblasts invade the myotome and fuse into myotubes, likely incorporating the initially mononucleated myocytes of the myotome, even though this has not been formally demonstrated. These are the first multinucleated muscle fibres, that appear in the embryo and are known as primary fibres. (Dunglison et al., 1999; Evans et al., 1994; Zhang and McLennan, 1995) A new wave of myogenesis takes place between 14.5 and 17.5 d.p.c. This phase is called secondary myogenesis and depends upon fusion of foetal myoblasts that give rise to secondary fibres (originally smaller and surrounding primary fibres with a donut like configuration) but also fuse with primary fibres. At the same time a basal lamina begins to form around each fibre and it is only after its formation that satellite cells can be morphologically and different types of mesodermal stem cells (i.e mesoangioblasts) have the potential to participate to muscle regeneration, though their contribution appear

to be minimal under normal circumstance. (Cossu and Biressi, 2005; Ferrari and Mavilio, 2002)

Old work from our and other laboratories identified specific features of embryonic, foetal myoblasts and satellite cells that characterize them as distinct classes of myogenic cells. (Cossu and Biressi, 2005; Cossu and Molinaro, 1987; Miller et al., 1999; Zappelli et al., 1996)Embryonic and foetal myoblasts, presumed to generate primary and secondary fibres respectively, differ for the morphology of the myotubes they generate where different myosin heavy chains isoforms and muscle enzymes are expressed. (Barbieri et al., 1990; Bonner and Hauschka, 1974; Ferrari et al., 1997; Pin and Merrifield, 1993)They also differ for media requirements (White NK, 1975), integrin-extracellular matrix interactions, (Blanco-Bose et al., 2001) resistance to inhibitors of myogenesis such as phorbol esters and TGFβ.(Zammit and Beauchamp, 2001) Specific features have been reported also for satellite cells: i.e morphology, susceptibility to TGFb but resistance to phorbol esters induced block of differentiation, high PDGF binding capacity, (Yablonka-Reuveni and Seifert, 1993) early expression of acetylcholine receptors (Cossu G, 1987) and acetyl-cholinesterase, (Senni et al., 1987) high clonogenic capacity, differential ability to respond to topographical guidance cues and expression of muscle specific genes (Hartley RS, 1991).

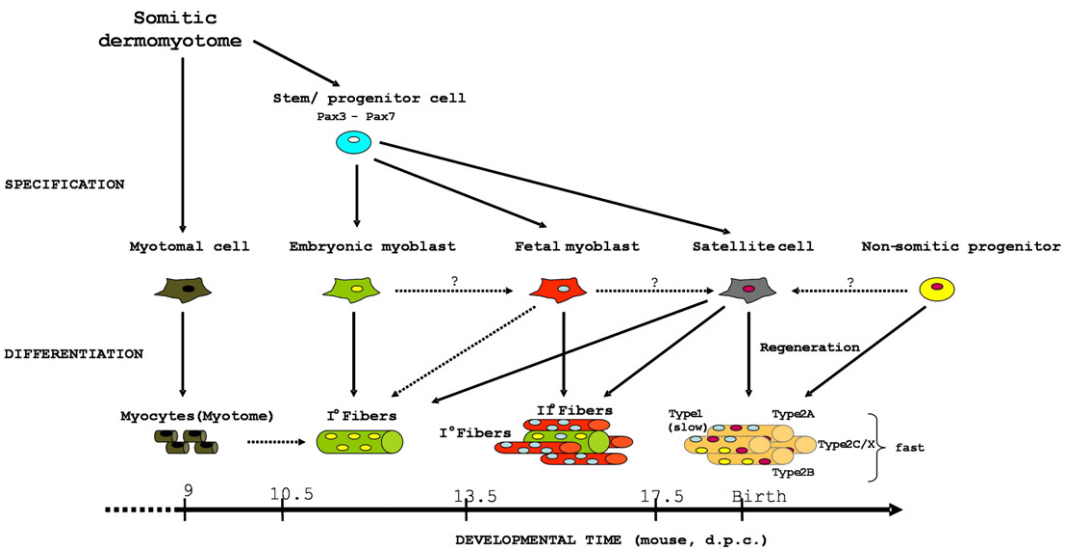

Figure 1.2.1 Proposed lineage sheme for skeletal muscle.

The somatic dermomyotome is the origin of the myotomal cells, which differentiate into the myocytes of early myotome (first terminally differentiated muscle of the embryo). Pax3/Pax7 positive cells identified in the dermomyotome release muscle precursor during development (embryonic fetal myoblast and satellite cells). Embryonic and fetal myoblast give rise to 1° and 2° fibres respectively. Satellite cells appear at the end of the gestation and are responsible of post natal growth and regeneration.

Abbreviations used: d.p.c days post coitum; HSC haematopoietic stem cells; MSC mesodermal stem cells

1.2.2 Asynchronous differentiation during muscle development

Embryonic myogenesis begins in newly formed somites where dorsally located progenitors respond to signals such as Wnts and Shh, emanating from adjacent neural tube, notochord and ectoderm, and activate basic Helix loop Helix transcription factors (Myf5 and MyoD) that commit cells to myogenesis

(for a more detailed description of embryonic myogenesis, several reviews can be consulted.(Cossu and Borello, 1999; Cossu et al., 2000; Pownall et al., 2002) Nevertheless the observation that only a fraction of myogenic progenitors present in the dermomyotome terminally differentiate during somitogenesis and that other myogenic cells differentiate in successive phases, suggest that cells situated in the same environment should understand the same signals in a different way. The Pax3/Pax7 muscle-progenitor cells may represent a mixed population of cells with different developmental potential. Even assuming that all canonical myogenic cells (embryonic and foetal myoblasts and satellite cells) may be specified to myogenesis in the newly formed somite, most of these cells should be kept in a committed but undifferentiated state until subsequent phases of myogenesis. In Drosophila lateral inhibition through Notch and Delta has been shown as the probable mechanism by which adult myogenic progenitors are selected in response to Wnt signalling. (Baylies et al., 1998) Recently a similar mechanism has been shown to operate in adult muscle. (Conboy and Rando, 2002) Thus it appears likely that the same may occur in the mammalian somite. Indeed several Delta and Notch isoforms are expressed in the somites (McGrew and Pourquie, 1998) and Notch inhibits myogenesis, probably through different intra-cellular mechanisms. (Nofziger et al., 1999; Wilson-Rawls et al., 1999) Receptors for growth factors or other signalling molecules such as BMP or TGFβ may be pertinent targets for Notch signalling. However direct evidence for a role of Notch in diversifying cell fate in mammalian somites is still missing. Here in fact we report data that support a possible explanation; in fact it's already know that Notch act as a determinant between smooth and skeletal muscle, but now can think that Notch could also

reprogram an already committed cells .A possible mechanism to ensure that certain myoblasts/progenitors will differentiate in an environment that is permissive for proliferation may be based on the inability of these cells to respond to growth factors and/or to molecules, which inhibit differentiation. Some years ago, a possible mechanism was proposed by which TGFβ might influence the process of primary fibres formation in vivo, by inhibiting differentiation of fetal myoblasts and satellite cells but not of embryonic myoblasts. At late foetal stage intensive foetal myoblast fusion still occurs, leading to a drastic reduction of myogenic mononucleated cells. Since satellite cells do not undergo differentiation at this time, the control of proliferation and differentiation in these cells must be still different, so as to allow the persistence of mononucleated undifferentiated myogenic cells in the post-natal and in the adult muscle. Thus to the generation of the different phases of myogenesis appears to be finely regulated. Nevertheless the molecular mechanisms operating in these cells remain unexplained.

1.3 Fiber diversity in skeletal muscle

1.3.1 Primary and secondary fibres

All fibres are produced by the fusion of myogenic cells, but muscle fibre formation in vertebrates is multiphasic. Both in avian and in rodents, where the process has been studied in detail, an early and a late wave of fibre production can be recognised. During the early phase, fibres, usually referred as primary (or embryonic) fibres, are generated by the fusion of early appearing (embryonic) myoblasts. During primary myogenesis muscles consists of a small numbers of myotubes. Primary myotubes progressively increase in size

becoming larger and with a characteristic round shape in transverse sections. Later-forming fibres are called secondary (or foetal) fibres and are likely generated by the fusion of the late appearing foetal myoblasts. During this process, referred as secondary myogenesis, secondary fibres form on the surface of primary fibres to which they remain attached for a short period. The secondary myotubes subsequently elongate and become independent fibres, which can be distinguished from primary fibres by their relative small size. The innervation of muscles starts while fibres are still forming. Each muscle fibre is initially innervated by multiple axons, all but one of which are subsequently eliminated. After birth, all the muscle fibres that remain contacted by the axon branches of an individual motor neuron are of the same type. The detailed mechanisms whereby nerves become associated with fast or slow muscle fibres are currently unknown, but it is currently assumed that that nerves play a role in generating fibre type diversity while different muscle fibres are predisposed to receive the appropriate axons. Skeletal muscle development is influenced by innervation, in the absence of which primary and secondary fibers still form almost normally but later do not grow in size and eventually some degenerate leading to a reduction of the total number of fibres. Continued denervation leads to eventual degeneration of both primary and secondary fibres.(Evans et al., 1994) As mentioned before for embryonic and foetal myotubes generated in vitro, also primary and secondary fibres differ in the expression of myosin heavy chains (MyHC) isoforms. In mammals primary fibres express embryonic (fast) and I/β(slow)-MyHC and only shortly before the end of primary fibre formation some of them (generally located on the superficial edge of the muscles) express also the perinatal/neonatal (fast)

isoform. On the contrary secondary fibres express all (fast) embryonic and perinatal isoforms from their inception and (with the exception of the soleus muscle) don't express I/β (slow)-MyHC. Thus it can be generalized, that in mammals primary fibres (and embryonic myoblasts) are programmed to express predominantly a slow phenotype, whereas secondary fibres (and foetal myoblasts) more a fast phenotype. (Wigmore and Evans, 2002) Beside myosin isoforms, a few other genes, such as Muscle creatine kinase,(Ferrari et al., 1997) β-Enolase (Feo et al., 1990) and PCθ (Zappelli et al., 1996) have been reported to be differentially expressed between primary and secondary fibres. Moreover alteration of genes which specifically affect primary or secondary myogenesis have been also identified (Cachaco et al., 2003; Fazeli et al., 1996; Kegley et al., 2001)further confirming the different nature of primary and secondary fibres in vivo.

1.3.2 Relationship between developing and mature phenotypes

Embryonic and fetal myoblasts have an intrinsic ability to form fibres expressing different MyHC isoforms, which broadly reflect the isoforms initially formed by primary and secondary fibres. After birth the total number of fibers in a given muscle of a given species remains constant but the total number of nuclei in each fibre increases up to ten folds: most nuclei in mature fibre are derived from satellite cells. It is still unclear whether the characteristics of an adult fibre reflect the features of the numerically dominant satellite cell nuclei or if the myoblasts which first form a fibre may continue to profoundly influence the proprieties of the fibre also when hundreds of satellite cells has been incorporated. The "slow" phenotype of primary fibres and the "fast" phenotype

of secondary fibres suggest a correlation respectively with the adult slow and fast contracting fibres. It should be noted that few new nuclei are added at any given time and thus represent a minority of the total nuclei in a fibre. Thus it may be possible that signals emanating from resident nuclei reprogram the newly added ones. Alternatively the different proprieties of an adult fibre could depend on heterogeneity in the satellite cell population. Some evidence supported this hypothesis. Nevertheless satellite cells do not show a predisposition to fuse with a particular fibre type in vivo suggesting that the different characteristics of the fibres may not simply derive from intrinsic proprieties of satellite cells. Environmental influences (activity, hormones etc.) could also have an important role in the establishment of fibre type diversity as mentioned above. It is conceivable that similar extrinsic signals could act also during fibre generation. Individual fibres have a characteristic proportion and distribution of fibre types, with fast fibres tending to be more superficial than slow fibres. This suggests that the distribution of fibre types is controlled by signals coming from adjacent tissues. Diffusible molecules such as Shh (Blagden et al., 1997)or member of the Wnt gene family, which have been shown to be involved in the initiation of myogenesis in the somites, could be involved. (Cossu and Borello, 1999; Wigmore and Evans, 2002)Moreover motor innervation appears to play an important instructive role in determining fibre type specification. Muscle cells from fetal slow muscle express slow myosin only when co-cultured with neural tube, whereas muscle cells from fetal fast muscle do not express slow myosin even in presence of neural tube, suggesting that the expression of MyHC isoforms during development is probably regulated by both myoblast lineage and innervation.(DiMario and

Stockdale, 1997) In conclusion it is reasonable that the mature characteristics of a fibre could be determined in part by extrinsic and in part by intrinsic factors, although is difficult to ascertain which of the two exert the major role. Even less clear is the degree of influence that the founding nuclei (from embryonic or foetal myoblasts) exhert on the numerically dominant, later incorporated nuclei (from satellite cells) in regulating the adult fibre phenotype.

1.4 Anatomical heterogeneity in skeletal muscle

1.4.1 Epaxial and Hypaxial myogenesis

A complex array of muscles is present in the vertebrate body. Based on their innervation pattern it is possible to distinguish the epaxial muscles, innervated by the dorsal branch of the spinal nerves, and the hypaxial innervated by the ventral branch. Epaxial muscles are located dorsally and correspond to the deep muscles of the back in amniotes, whereas hypaxial muscles are located superficially, laterally and ventrally and include diaphragm, body wall and limb muscles. The two type of muscles are physically separated by a connective tissue sheet. In amniotes all the trunk muscles derive from the somitic dermomyotome. During this process, myogenic progenitors derived from the dermomyotome create in successive phases (see above), in the area between the dermomyotome and the medio-ventrally located sclerotome, a structure called myotome or alternatively undergo a ventral migration towards sites of hypaxial myogenesis such as the limb or the tongue.

Each dermomyotome (and myotome) can be subdivided into a hypaxial (underneath the axis-notochord) and an epaxial portion. The former is located more ventro-laterally and give rise to hypaxial muscles, whereas the latter is

located dorso-ventrally and will origin the epaxial muscles. (Cossu et al., 2000) Work of several laboratories showed that only the precursors of the epaxial are dependent upon signal from axial structures, whereas precursors of the hypaxial muscles do not need neural tube and notochord for the myogenic commitment but rather signals from the dorsal ectoderm (Fig. 2.4.1). In particular it was shown that axial structures, probably trough the release of Shh and Wnts (i.e. Wnt1) preferentially activate a myogenic program by inducing Myf5 expression. On the contrary dorsal ectoderm, by releasing different members of the Wnt family (i.e. Wnt7a), activate the myogenic program through a MyoD mediated pathway. (Cossu and Borello, 1999; Cossu et al., 2000) This view fits with the phenotype observed in Myf5 and MyoD mutant embryos. The former present initially epaxial muscles defects, the latter a delayed myogenesis in the limbs. In both cases the residual myogenic determination genes are able to support an almost normal skeletal muscle development. (Hadchouel et al., 2003; Kablar et al., 1997; Rudnicki et al., 1992) Interestingly distinct cis-regulatory elements controlling the expression of Myf5 in different portions of the myotome have been identified. More recently the transcription factors En1 and Sim1 have been proposed as markers respectively of the epaxial and hypaxial portion of the dermomyotome in the chick.

At the limb level myogenic progenitors delaminate from the ventro-lateral domain of the dermomyotome (hypaxial) and migrate distally to the limb bud were they start to express the myogenic determination genes. Once activated myogenic program, myoblasts differentiate and fuse into discrete clusters, corresponding to the major muscle masses of the dorsal and ventral aspects of the developing limb. Subsequent phases of myogenesis (secondary and post-

natal) will generate the definitive limb muscles. These migrating myogenic progenitor cells express several specific genes, which are essential for their migration and morphogenesis: (Birchmeier and Brohmann, 2000; Francis-West et al., 2003) Pax3 is expressed in the entire dermomyotome, but its expression is up-regulated laterally. (Goulding and Paquette, 1994) In the absence of Pax3 and its downstream target c-met, the limb myogenic progenitors in the lateral dermomyotome are not able to undergo a normal delamination and migration. (Epstein et al., 1996; Relaix et al., 2004) Following inactivation of the Lbx1 gene, which is, like c-met, a target of Pax3, the premyogenic cells delaminate appropriately, but appear unable to migrate. (Gross et al., 2000) A phenotype similar has been observed in animals in which the c-met signalling transducer Gab1 has been mutated. (Sachs et al., 2000) Mice mutant for Mox2 present a down-regulation of the expression of Myf5 and Pax3 and perturbations at the level of subsets of limb muscles. (Mankoo et al., 1999) Interestingly at least a subset of premyogenic cells migrating to the limb express the transcriptional repressor Msx1, which has been shown to block myogenic differentiation. During delamination, migration and differentiation (Bendall et al., 1999) premyogenic limb cells receive signals from the surrounding tissues (i.e. lateral plate mesoderm, apical ectoderm ridge, limb ectoderm and mesenchime). Scatter factor (the ligand of c-met), (Heymann et al., 1996)Fibroblast growth factors (FGFs), Shh, (Amthor et al., 1998)Bone morphogenic proteins (BMPs) (Amthor et al., 1998) and other members of the TGFβ superfamily appear to be involved. (Francis-West et al., 2003)Migratory myogenic progenitors are not present only at the limb level, but also in the lateral domain of the occipital/cervical somites from which migrate to contribute to the muscles of the

tongue and larynx. The progenitors involved in this process are characterized, as the limb premyogenic cells, by the expression of Lbx1. On the contrary hypaxial progenitors located at the interlimb level don't express Lbx1. Instead of undergoing a long range migration, they enter into the ventral part of the underlying myotome and progressively extend ventrally to generate the hypaxial body wall and intercostal muscles. Interestingly, this strategy is adopted in the developing fin bud of the spotted dog fish shark (*Scyliorhinus canicula)*, whereas the teleost zebrafish use the migratory mode similarly to amniotes. (Neyt et al., 2000) These observations suggest that the interlimb-level somites of amniotes and teleosts have conserved the primitive mode of hypaxial muscle formation and that the migratory strategy observed at the limb level was generated before the evolution of the tetrapod limb (i.e. is already present in zebrafish) (Hollway and Currie, 2005; Neyt et al., 2000)

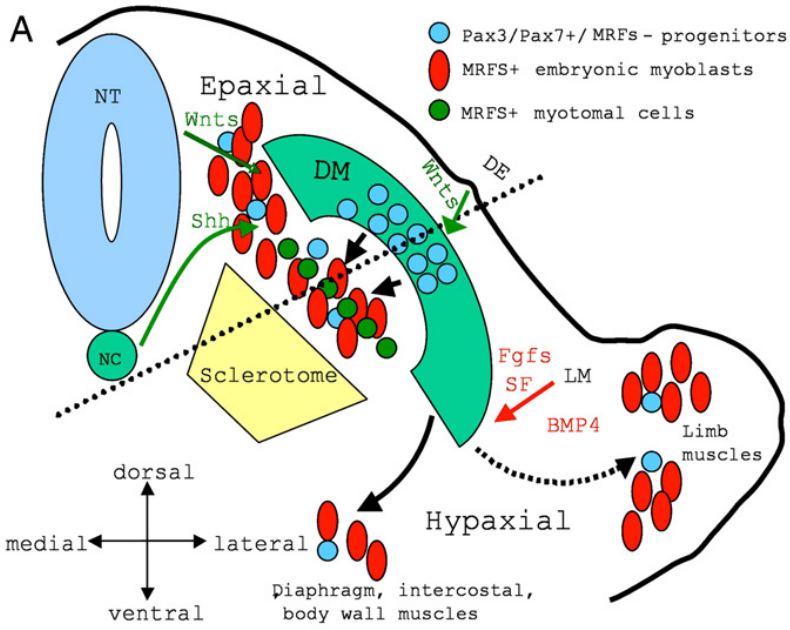

Figure 1.4.1 Epaxial and hypaxial myogenesis.

(A) Model showing the early phases of myogenesis. Myotomal cells and embryonic myoblasts, specified from a Pax3/Pax7+ population of progenitors, invade the area between the dermomyotome and the sclerotome generating the myotome. The dorso-medial domain of the myotome/dermomyotome will give rise to the epaxial (back) muscles, whereas from the ventro-lateral domain the hypaxial muscles will be generated. Surrounding tissues induce myogenesis in the epaxial and hypaxial domains with different mechanisms involving different molecules (see text). At the limb level Pax3+, MRFs− progenitors migrate from the ventro-lateral (hypaxial) domain of the dermomytome to the limbs, where they activate the myogenic program. Different signaling molecules such as FGFs, BMPs and Scatter Factor (SF) are likely to regulate these events.

1.4.2 Rostro-caudal identity

Although morphologically very similar, somites differentiate into distinct mesodermal tissues, depending on their axial level. The identity of the somite is specified by their unique combinatorial expression of HOX genes, which is generally referred as “HOX code”. (Burke et al., 1995; Kessel and Gruss, 1991)Classical transplantation experiments clearly indicated that this segmental identity is able to influence the fate of sclerotomal (vertebrae and ribs) and non-myogenic dermomyotomal (back dermis and scapula) derivatives of the somite.(Ehehalt et al., 2004; Jacob et al., 1975; Kieny et al., 1972) On the contrary, for many years it has been generally accepted that somitic myogenic precursors are completely naive, do not possess positional information and depend exclusively on extrinsic cues for their site-specific fate. This view was based on classical embryological experiments in the chick, in which somites

(Christ et al., 1977)were heterotopically transplanted at the limb level and the generation of graft-derived appendicular muscles could be observed. Although a few reports already suggested that myogenic precursors in the somite could also have a positional identity (Grieshammer et al., 1992; Murakami and Nakamura, 1991) only a recent paper completely changed the traditional view. With an elegant transplantation approach Alvares and coworkers clearly showed that the competence to either generate migratory (Lbx1+) or non-migratory (Lbx1-) muscle precursors depends on intrinsic values of the somite. Moreover they show that limbs contain a potent signalling mechanism (i.e. FGFs) that can override the non-migratory program of flank somites and induce in the myogenic progenitors the expression of the migration marker Lbx1. They also showed that the somitic predisposition toward a particular myogenic program (migratory or not migratory) directly or indirectly depends on Hox genes. These observation indicate that results obtained by heterotopic grafting into the limb area (Christ et al., 1977) can not be generalized for other axial levels and conclusively demonstrate that the axial identity to the somites, conferred by HOX genes, can determine the fate also of skeletal muscle precursors. Nevertheless whether this positional information is intrinsically encoded in myoblasts or in neighbouring cells of the same somite remains still unknown.

1.4.3 Lateral asymmetry in the skeletal muscle

The vertebrate body shows left-right asymmetry along the body axis. This is clearly evident from the organisation and anatomical localisation of different organs, such as heart, stomach, intestines, liver and lungs. (Beddington and Robertson, 1999) Skeletal myogenesis is generally thought to occur

symmetrically. Differences in the strength and size of muscles between the right and left are well known in humans, (Beddington and Robertson, 1999; Chhibber and Singh, 1970)although it is likely that they simply depend on training and lifestyle and not on a developmental process. Nevertheless in a recent work Golding and colleagues observed an asymmetric expression of myosin light chain 3F (MLC3F) and α-skeletal actin during the generation of the primary myotome. (Golding et al., 2004) This asymmetric expression appears to be transient and do not continue during primary myogenesis. Although the significance of this phenomenon remains unclear, it reveals that novel aspects of left-right asymmetry may involve also skeletal muscle development.

1.5 Molecular mechanisms in skeletal muscle determination and differentiation

1.5.1 Myogenic regulatory factors

Skeletal muscle development in vertebrates occurs in several steps: mesoderm-derived precursors became committed to the myogenic lineage, develop into myoblast, which became post-mitotic and differentiate fusing together. This process leads to the generation of terminally differentiated multinucleated myotubes expressing a characteristic set of myofibrillar proteins (i.e. myosin heavy and light chains, tropomyosin, components of the troponin complex, sarcoplasmatic reticulum ATPase, Z line and M line proteins), which confer to the mature fibres the capacity to contract. The muscle regulatory factors (MRFs) are key regulators of the myogenic program. In vertebrates, four MRF genes have been identified: MyoD (Myf3), Myf5, Mfr4 (Myf6/herculin), Myogenin (Myf1). (Braun et al., 1989a; Braun et al., 1989b)MRFs are

specifically expressed in the skeletal muscle lineage. When transfected in fibroblasts and other cellular types MRFs can convert them to a myogenic fate. Moreover they have the capacity to activate their own expression and to crossactivate the other MRFs. They form heterodimers with a ubiquitous class of bHLH transcription factors known as E proteins and bind a consensus DNA sequence called E-box (CANNTG) in the control regions of muscle-specific genes. Myf5 appears in the rostral somites of the mouse around 8 d.p.c., Myogenin at around 8.5 d.p.c., followed by MRF4 (9.5 d.p.c.) and MyoD, which appears at about 10.5 d.p.c. MyoD-/- mice show a delayed differentiation of limb muscle and an enhanced Myf5 expression during the embryonic stage of development. (Kassar-Duchossoy et al., 2004) Nevertheless after birth they appear grossly normal. (Rudnicki et al., 1992)Similarly in adult Myf5 -/- mice no striking muscular defects could be observed, although a defective myotome formation could be clearly noticed at the embryonic stage. Mrf4 -/- mice also present a mild phenotype, showing an increase in the expression of Myogenin. Embryos, in which the expression of MyoD, Myf5 and Mrf4 was simultaneously ablated, completely lack of skeletal muscle in any anatomical location and die at birth because of respiratory failure. (Kassar-Duchossoy et al., 2004) When the expression of both Myf5 and Mrf4 was down-regulated mice showed a severely delayed myotome formation but subsequent muscle development occurred relatively normal. (Braun et al., 1992; Kassar-Duchossoy et al., 2004; Tajbakhsh et al., 1996)Myogenin -/- mice form Myf5 and MyoD expressing muscle progenitors, but are deficient in differentiated muscle and die at birth. In particular secondary myogenesis appear to be affected in Myogenin -/- mice. Similarly in Myf5-/-: MyoD-/- mice secondary, but not trunk primary myogenesis,

appears to be completely abolished. (Kassar-Duchossoy et al., 2004; Valdez et al., 2000) Notably MyoD-/- :MRF4 -/- and MRF4-/-:Myogenin-/- double mutant embryos present a phenotype similar to Myogenin -/-. Also in MyoD-/-: Myogenin-/- and Myf5-/-: Myogenin-/- double mutant mice the muscle deficit at birth seems not to be significantly more severe than in single Myogenin-/- mice. On the contrary MRF4-/-: Myogenin-/-:Myod-/-triple mutant posses normal numbers of progenitors, but fail to form any differentiated muscle fibres either in vivo or in vitro.(Valdez et al., 2000) All together these reports suggest that Myf5, MyoD and Mrf4 can independently begin the myogenic program and thus act as myogenic “determination” genes during primary myogenesis, whereas only Myf5 and MyoD can exert this role during secondary myogenesis. (Kassar-Duchossoy et al., 2004) On the contrary it is likely that Myogenin operate downstream to Myf5, MyoD and Mrf4 (at least during primary) by promoting muscle differentiation, a role that Myf5 appear not to be able to exert alone. (Valdez et al., 2000)Interestingly, introduction of the Myogenin cDNA into the Myf5 locus is unable to fully compensate for the absence of Myf5. Finally, in absence of Myogenin residual muscle fibres are present in vivo and an efficient generation of multinucleated myotubes could be observed in vitro suggesting that MyoD and Mrf4 could partially substitute for the absence of Myogenin.

1.5.2 Regulation of expression and function of MRFs

Pax3 and its paralogue Pax7 have been implicated in the specification of cells that will enter the myogenic program. It has been suggested that embryonic, foetal myoblasts and satellite cells could all derive from Pax3/Pax7 positive population of myogenic progenitors. (Kassar-Duchossoy et al., 2004; Relaix et al., 2005; Schienda et al., 2006)In contrast to MRFs, Pax3 and Pax7 are not

muscle specific. Nevertheless in absence of Pax3, Myf5 and Mrf4 the expression of MyoD and the myogenic program fails to be activated in the trunk (Tajbakhsh S, 1997), indicating that Pax3 (and Myf5) act upstream to MyoD. In an independent study, ectopic expression of Pax3 in embryonic tissues was shown to induce the expression of MyoD and Myf5. (Maroto et al., 1997) Pax3 functions in cooperation with other protein, such as Dach2, Six1, 2, Eya 2 and 4. (Relaix and Buckingham, 1999)

All MRFs bind E-box sequences motif. Therefore, additional transcription factors are required to regulate MRF specificity for particular target genes. One such factor is MEF2. There are four MEF2 family members: Mef2A, Mef2B, Mef2C and Mef2D. They directly interact with MRFs and synergise with the MyoD-E12 heterodimer. (Black et al., 1998; Black and Olson, 1998)Moreover Mef2 genes are targets of the myogenic bHLH proteins and Mef2 proteins appear to control Mrfs expression. Similarly to Mef2 protein Serum response factor and muscle LIM protein appear to cooperate with Mrfs. MyoD has been shown to directly interact with p300/CBP, suggesting that the induction of the myogenic program by MyoD imply chromatin remodelling events. MRFs have been shown to be negatively regulated by several proteins. The HLH proteins Id and mTwist act in a dominant negative manner by sequestering E proteins and preventing their association with MRFs. Moreover the bHLH factor Mist1 has been shown to interact with MyoD and prevent its binding to the DNA. The transcriptional inhibitor Msx1 appears to be able not only to inhibit differentiation, but also to induce the reversal of terminal differentiation. Moreover MRFs activity is potentially negatively regulated by phosphorylation and appear to be tightly coupled to the cell cycle. Thus myogenesis appears to

be a process involving different cellular types whose fate is finely regulated by complex molecular mechanisms involving the interaction between the key regulators MRFs (Groisman et al., 1996) and a set of accessory proteins, which are important for the correct development of the muscles.

1.6 Notch signalling

Notch signaling is evolutionarily conserved and operates in many cell types and at various stages during development. Notch signaling must therefore be able to generate appropriate signaling outputs in a variety of cellular contexts. This need for versatility in Notch signaling is in apparent contrast to the simple molecular design of the core pathway.(Andersson et al., 2011) In mammals, there are four Notch receptors (Notch1-4) and five canonical ligands of the Delta-Serrate-Lag (DSL) type [Jag1 and Jag2 and delta-like 1 (Dll1), Dll3 and Dll4] (reviewed by(D'Souza et al., 2010). This generates a large number of receptor-ligand combinations, which could potentially generate distinct responses. There is, however, little evidence for differences in signaling output between particular receptor-ligand combinations, with the notable exception of Dll3, which is the most structurally divergent ligand and lacks an extracellular Delta and OSM-11-like protein (DOS) domain as well as lysine residues in the intracellular domain (Dunwoodie et al., 1997) Dll3 is incapable of activating Notch receptors in trans (Ladi et al., 2005)and is rarely, if ever, present at the cell surface (Chapman et al., 2011; Geffers et al., 2007)

The Notch ligand Dll4 is essential for vascular formation. (Shutter et al., 2000)When Dll4 is inhibited in developing mouse retinas or in xenograft tumor models such as colon and lung carcinoma, excessive proliferation of

nonfunctional vasculature occurs. Blood vessels become unorganized and display increased sprouting and microvessel density, but decreased perfusion and function. The etiology of this seemingly paradoxical situation of more vessels but less perfusion and the reason for the decreased functionality are poorly understood. The microvessels created by the hyperproliferation of endothelial cells in the absence of Dll4 are immature; they lack coverage by α-smooth muscle actin^{+} (α-SMA^{+}) cells. A correlation between Dll4 expression and blood vessel maturation in bladder cancer has been demonstrated: 98.7% of Dll4^{+} tumor vessels were surrounded by α-SMA^{+} pericytes/vSMCs, while only 64.5% of Dll4^{-} vessels had α-SMA^{+} cell coverage. These data suggest that Dll4 may play a role in the formation of-cell–derived pericytes/vSMCs during vasculogenesis, and also that Dll4 secreted by endothelial tip cells could be the signal to recruit pericytes from the sorrounding mesoderm.

Mature blood vessels are composed of a single layer of endothelial cells, which form the inner tube of the vessel, surrounded by a supportive layer(s) of pericytes/vascular smooth muscle cells (vSMCs). Pericytes/vSMCs provide vessel structural support and contribute to the regulation of blood flow. In addition, pericytes/vSMCs provide growth and survival factors such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF2), and angiopoietin (Ang1) to endothelial cells, while preventing endothelial cell hyperproliferation. Thus, pericytes/vSMCs help to maintain a stable state of functional, nonproliferative, mature vasculature. (Bergers and Song, 2005; Hirschi et al., 1998)Without pericytes/vSMCs, blood vessels are leaky, less stable, and more susceptible to antiangiogenic therapies and regression due to pathologic conditions such as hyperoxia.(Abramovitch et al., 1999)

A functional vascular network is essential for the growth of solid tumors. Two of the processes by which tumors can form vessels are angiogenesis, the sprouting of preexisting blood vessels, and vasculogenesis, the recruitment of bone marrow (BM) cells to the tumor with subsequent formation of a de novo vessel network. During vasculogenesis, BM cells migrate to the tumor, adhere to sites of developing vasculature, and contribute to the endothelial and pericyte/vSMC populations within mature vasculature. One molecular mechanism likely to contribute to the process of recruitment of pericytes by endothelial cells is delta-like ligand 4 (Dll4)–Notch signaling, that is one of the four Notch ligands existing in mammals. All ligands and receptors are membrane bound and signal by cell-to-cell contact. When a ligand binds to a receptor, 2 cleavage events occur to activate receptor signaling. The Notch intracellular domain (NICD) is released by the second cleavage event, which is mediated by the γ-secretase complex. NICD is then translocated to the nucleus, where it forms a transcriptional activating complex that includes recombination signal-binding protein-Jκ (RBP-Jκ) and mastermind-like protein (MAML). The NICD-RBP-Jκ-MAML complex induces transcription of Notch effectors such as the Hes and Hey family members, which are themselves transcription factors that go on to regulate the expression of downstream Notch targets.

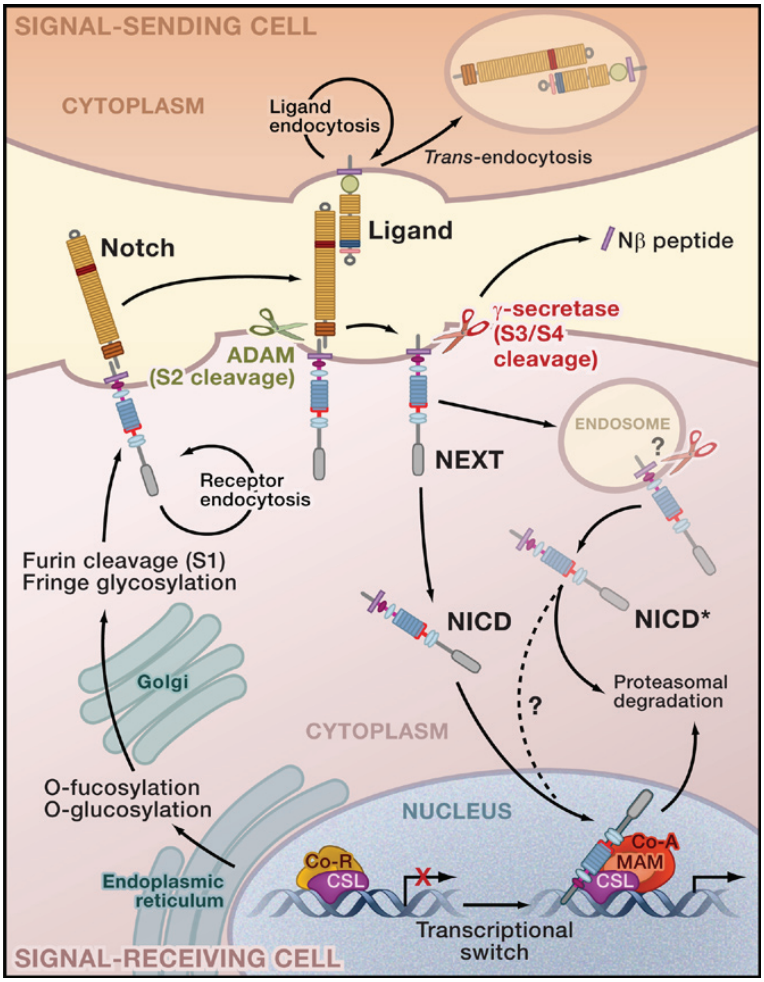

Figure 1.6.1

The newly translated Notch receptor protein is glycosylated by the enzymes O- fut and Rumi, which are essential for the production of a fully functional receptor. Mature receptor is produced after proteolytic cleavage by PC5/furin at site 1 (S1). It is then targeted to the cell surface as a heterodimer that is held together by noncovalent interactions. In cells expressing the glycosyltransferase Fringe, the O-fucose is extended by Fringe enzymatic activity, thereby altering the ability of specific ligands to activate Notch. Notch receptor is activated by binding to a ligand presented by a neighboring cell. Endocytosis and membrane trafficking regulate ligand and receptor availability at the cell surface. Ligand endocytosis is also thought to generate mechanical force to promote a conformational change in the bound Notch receptor. This conformational change exposes site 2 (S2) in Notch for cleavage by ADAM metalloproteases (perhaps after heterodimer dissociation at site 1). γ-Secretase then cleaves the Notch transmembrane domain in NEXT progressively from site 3 (S3) to site 4 (S4) to release the

Notch intracellular domain (NICD) and Nβ peptide. γ-Secretase cleavage can occur at the cell surface or in endosomal compartments, but cleavage at the membrane favors the production of a more stable form of NICD. NICD then enters the nucleus where it associates with the DNA-binding protein CSL (CBF1/RBPjκ/Su(H)/Lag-1). In the absence of NICD, CSL may associate with ubiquitous corepressor (Co-R) proteins and histone deacetylases (HDACs) to repress transcription of some target genes. Upon NICD binding, allosteric changes may occur in CSL that facilitate displacement of transcriptional repressors. The transcriptional coactivator Mastermind (MAM) then recognizes the NICD/CSL interface, and this triprotein complex recruits additional coactivators (Co-A) to activate transcription.

1.7 Reprogramming

1.7.1 Reprogramming to an embryonic-like state or directly to another mature cell

Almost 20 years after MyoD discovery, Takahashi and Yamanaka showed that transduction of embryonic mouse fibroblasts with four transcription factors now known as the Yamanaka factors (Oct4, Klf4, cMyc and Sox2), reprograms somatic cells into an embryonic-like state: “induced Pluripotent Stem cells” or iPS cells, (Takahashi and Yamanaka, 2006)(Fig. 1). This discovery hit the field like an hurricane and the number of papers published yearly on the topic keeps increasing exponentially (for recent reviews see:(Hanna et al., 2010; Wilmut et al., 2011; Yamanaka and Blau, 2010). The possibility of deriving patient-specific ES-like cells that can be indefinitely expanded in vitro, genetically corrected if needed, and then induced to differentiate into the desired cell type, appeared as a real breakthrough over previous reprogramming approaches based on nuclear transfer; it is technically simpler and apparently bypasses the ethical controversies and their political consequences described above. For the sake of records, it should be mentioned that several recent papers have challenged the

complete equivalence of iPS and ES cells (for a recent review see:(Power and Rasko, 2011). Until now, the differences reported do not seem to have a major impact on the possible future clinical use of these cells, with the possible exception of immunogenicity.

Moreover, the argument that the reprogrammed nucleus is anyway “old”, i.e. has the same age of the patient, appears to be at least disingenuous when compared to spared embryo derived ES cells, which are ”non-self”: a reprogrammed cell should be compared to a nuclear-transferred ES cells that has exactly the same age and “selfness”. Only time will tell whether iPS will replace ES cells, but in any case, the value of ES cells for science and medicine will remain immense, since they led to a revolution in biology, and the same iPS cells would have never been discovered without them.

1.7.2 Direct reprogramming

Finally, in the last two years, several laboratories showed that it is possible to directly reprogram an adult fibroblast to a cardiomyocyte or a neuron (and even specific neural subtypes), by forced expression of usually two or three transcription factors. (Ieda et al., 2010; Szabo et al., 2010; Vierbuchen et al., 2010) In all cases, and examples are accumulating at a weekly pace, cells repress their own transcriptional program and activate the new one without transiting through an ES-like state. Although many issues remain to be solved, e.g. frequency of complete terminal differentiation into the desired cell type, these data appear to move the field even further toward their safe use since the tumorigenic risk, associated with ES/ipS cells, would not exist anymore. Obviously, especially in the case of certain human cells, the total number of

cells that can be expanded in vitro would return as a problem due to the limited proliferation potency of human fibroblasts. Why earlier attempts (at the time of MyoD discovery) at directly reprogramming fibroblasts into other types of differentiated cells failed, and more recent, iPS cell-boosted attempts succeed, is probably due to the major advances in our understanding of the transcriptional machinery, that took place in the last twenty years.

1.7.3 Environmental reprogramming

Parallel to these events, and currently outdated by the development of molecular approaches, a flurry of data accumulated during decades of work showing that, following transplantation, embryonic or even adult cells, may change their fate and adopt that of the surrounding cells. Indeed, heterotopic transplantation of a group of cells, naturally fated to give rise to tissue A, into developing tissue B, is a classic assay for fate determination in embryology. If cells maintain the A phenotype, they are considered “determined” or “committed” to fate A, whereas if they turn into tissue B, they are considered “undetermined” and ready to be instructed by signals emanating from the extra-cellular microenvironment. It is conceivable that these signalling molecules may lead to the activation of the same genes that, once transfected into adult fibroblasts in vitro, “reprogram” them to the desired cell fate.

Despite the fact that the mammalian embryo is considered to be “regulative”, it was generally assumed, at least until Yamanaka’s work, that once committed, cells could only progress towards their fixed differentiation pathway or die. Yet, numerous examples of “spontaneous change of fate” exist in the old literature, but they are often linked to post-natal tissue damage and

regeneration. For example, retina regeneration by pigment cells in *amphibians* is a classic case of trans-differentiation (or “spontaneous reprogramming”) that only occurs after tissue damage. On the other hand, a spontaneous trans-differentiation from smooth to skeletal muscle in the mouse oesophagus was reported in 1995 (Patapoutian et al., 1995) but later questioned based upon lineage tracing studies. (Rishniw et al., 2003)

The field was changed in 1998, by a paper showing that the bone marrow of normal adult mice contains cells that can participate in skeletal muscle regeneration and give rise to new muscle fibre nuclei, (Ferrari et al., 1998; Ying et al., 2002) Bone marrow-derived muscle cells were very few (less than 1%) and were easily detected in the host muscle despite their low frequency, because they expressed a muscle-specific nuclear LacZ. Within a few years, the literature was flooded with papers, often in high profile journals (e.g., (Krause et al., 2001; Lagasse et al., 2000; Orlic et al., 2001)showing that many cells of adult tissues, when transplanted in a different regenerating tissue, may give rise to one or more cell types that are typical of that recipient environment. Often, conclusions were based on double fluorescence, where one cell would show a tracer of its origin and an antigen typical of the tissue where it had been transplanted. Nevertheless, these data suggested that it would have been possible to isolate patient’s own cells from an unaffected tissue, expand and, if needed, genetically correct them for transplantation into the affected tissue or organ. It was obvious that the frequency of these events was almost invariably very low, far below the threshold of any possible clinical efficacy. The papers were heavily criticized.

It was proposed that “plasticity” was the consequence of immune staining

or tissue culture artefacts, and explaining that the ones that were confirmed by other independent laboratories were the result of spontaneous cell fusion, where, as described above, one nucleus would impose its transcriptional program to the other. This culminated in three papers published at the same time and practically burying the field of environmental reprogramming and plasticity. (Terada et al., 2002; Wagers et al., 2002; Ying et al., 2002) The fact that spontaneous cell fusion is the natural mechanism through which skeletal muscle forms (Mintz and Baker, 1967) in vertebrates was not considered at that time, even though it did explain, at least in part, the result of bone marrow giving rise to skeletal muscle (Ferrari et al. op.cit.), and later and unexpectedly, to Purkinjie cells. (Weimann et al., 2003)Nevertheless, years went by and "plasticity" remained a synonym for "artefact". However, in recent years, several papers, scientifically unquestionable and in high profile journals, showed that cells can be environmentally reprogrammed to a complete and mature fate. For example, it was recently shown that nerve-derived Shh defines a niche that maintains hair follicle stem cells in their multipotent state, whereas in its absence, cells adopt an epidermal "only" fate. (Brownell et al., 2011) Even more strikingly, clonally expanded epithelial cells of both embryonic and adult rodent thymus were reprogrammed to multipotent hair follicle stem cells in vivo, as they were found able to give rise to all skin lineages, i.e. hair follicle, epidermis and sebaceous glands, upon serial transplantation. Moreover, reprogrammed thymic cells re-isolated from the hair follicle, were able, to a variable extent, to revert to their primitive fate once transplanted back into the thymus. (Bonfanti et al., 2010) Other examples keep accumulating, such as those showing extra-cellular signals enhancing transcription factor-mediated reprogramming to iPS

(Lluis et al., 2008) or to another differentiated cell type .(Aviv et al., 2009) Also, pathological conditions such as atherosclerosis induce smooth muscle cells to differentiate into osteochondrogenic precursors and chondrocytes. (Speer et al., 2009) Finally, it was also shown that physical cues, such as substrate stiffness may, by themselves, direct mesenchymal stem cell fate towards one or another differentiation pathway. (Engler et al., 2006)

1.7.4 What is the difference between "direct reprogramming and environmental reprogramming"?

"Plasticity", defined as the ability of a cell to change its phenotype in response to both intra- or extra- cellular signals has now acquired a broad and ill-defined general meaning. It does not literally correspond to "trans-differentiation", because the latter only refers to already differentiated cells that directly switch to another differentiation program without regressing to an ES-like state. Plasticity instead refers also to a still undifferentiated but "committed" cell, either embryonic or adult, that during its pathway towards the expected terminal differentiation can be diverted towards another type of terminal differentiation. At first sight, it would appear that approaches such as cell fusion, exposure to oocyte extract, and environmental cell reprogramming may be outdated by the most direct transfer of defined transcription factors.

However, in our opinion, environmental reprogramming maintains an important role in stem cell biology for three main reasons. First, the mammalian body is composed of thousands of different cell types and we currently have the recipe to convert "fibroblasts" or direct ES/iPS towards a specific terminally differentiated cell (e.g. a dopaminergic neuron: (Caiazzo et al., 2011) only in a

handful of cases. The “environmental” approaches may still be invaluable to identify extra-cellular signals and downstream transcription factors that are required to obtain a functional beta or alpha cell of pancreatic islets or a cone or a cell of the heart conduction system. Second, evidence is accumulating that fusion may occur in vivo in many tissues, resulting in cell reprogramming and thus contributing to regeneration. (Sanges et al., 2011)Third and more importantly, environmental reprogramming may mimic in vitro, or following experimental transplantation, natural processes that occur in vivo, though likely at low frequency, and may be needed to finely tune the amount of progenitor cells that are distributed among neighbouring developing tissues. In this regard, we showed in the past that, upon transplantation, human pericytes from skeletal muscle are recruited to a skeletal muscle fate rather than following their default pathway, i.e. the formation of the smooth muscle layers surrounding the endothelium of blood vessels. (Dellavalle et al., 2007) We now have evidence that pericytes spontaneously change their fate, contributing to up to 7% of developing skeletal muscle fibres and 20% of their associated satellite cells during unperturbed post-natal development of the mouse. (Dellavalle et al., 2011) This supports the hypothesis that pericytes represent a resident progenitor of post-natal tissues endowed with the potency to generate the differentiated cell types of that specific tissue.(Bianco et al., 2008) The implications of this concept for regenerative medicine can be already appreciated as a phase I/II clinical trial, based upon transplantation of mesoangioblasts (the in vitro counterpart of skeletal muscle pericytes) from HLA-identical donors, is ongoing at San Raffaele Hospital in Milan. It is important that the cells to be transplanted possess, as a natural developmental

option, the ability to give rise to the desired tissue, i.e. skeletal muscle in our case, at a frequency that might be clinically relevant.

In conclusion, plasticity is not an artefact. It is likely a compensatory mechanism by which developing or regenerating tissues adjust their cell number. It is rare but important. It may occur by cell fusion or environmental reprogramming that are mimicked in the laboratory by transfer of nuclei or transcription factors. The years to come will likely provide answers to these intriguing issues that are crucial for the future of regenerative medicine.

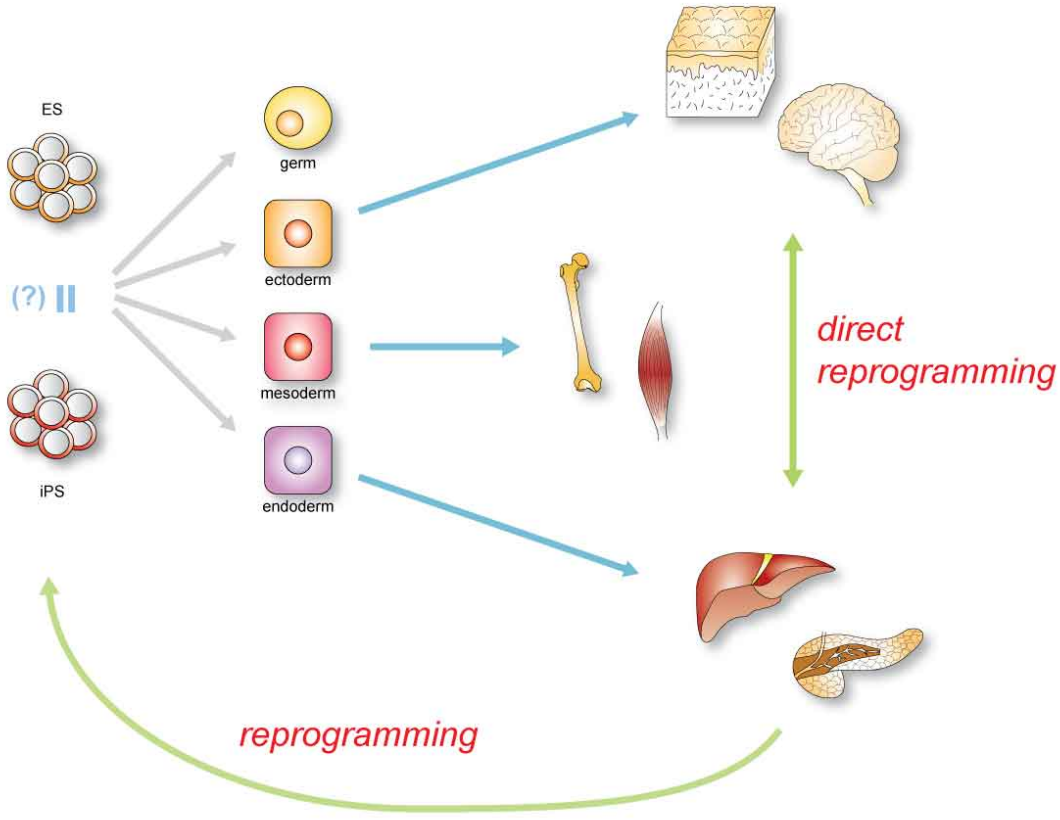

Figure 1.7.1

Changing the model of cell determination and differentiation. Gray arrows indicate the ability of

ES and iPS cells to give rise to germ and somatic layers, that proceed (light blue arrows) towards their various differentiated tissues. Green arrows represent the possibility of reprogramming differentiated cells to a pluripotent state or directly to a different differentiated cell type, independently from the germ layer of origin (trans-differentiation). The debated equivalence of ES and iPS is indicated by the symbol “=” preceded by a question mark.

RATIONALE

Notch signaling is one of the most studied pathway during development. It exerts its function by controlling the balance between proliferation and differentiation. It prevents tumor formation and by the other hand the abundance of his control made the pathway really difficult to understand.

On the contrary, skeletal muscle has some defined “dogma”. In fact, it is known that once a cell (suppose to be myogenic or not) enter myogenic program it is not possible to overcome the commitment; that’s why a myogenic cells has no different choice. In this year, however, seems that some myogenic progenitor, in particular Pax3 expressing cells, has the capability to migrate to t dorsal aorta, (Esner et al., 2006)so to originate vascular smooth muscle cells.

Pericytes are a subset of cells that in this year start to purchase a lot of interests; in fact different works demostrate their capability of trans differentiate in some tissue likely they are resident progenitor of different tissue. Since myoblast and pericytes cooperate during muscle regeneration, this work aim to demostrate that this cooperation is not mutual exclusive: in fact, if pericytes can be recruited by mygenic cells to do muscle, this way pericyte, or better endothelium, can recruit myoblast to build a new vessel. In fact, during embryogenesis myoblast are in the mesenchyme surrounding vessels; so vessel, with proper signaling could recruit cells, this case myoblast, to become pericyte. To investigate this, we choose an *in vitro* model to obtain cells that could maintain embryonic property (cells sorted from $Pax3^{GFP/+}$mouse) and an *in vivo* model to validate the data. Since tip cells, the cells responsible of pericytes recruitment by endothelium, secreted a Notch activator, signaling

used by endothelium is supposed to be Notch signaling; so we crossed two different mice, $MyoD^{iCRE}$ and $ROSA^{NICD}$ to be sure that all myogenic cells activate Notch pathway.

This work could be the first evidence of spontaneous reprogramming, and will demonstrate the capability of myogenic cells to exit the commitment and change their fate.

MATERIAL AND METHODS

2.1 Mutant Animals and Genotyping

The $Pax3^{GFP-/+}$ mice (Relaix et al., 2005), $MyoD^{iCre}$ mice (Kanisicak et al., 2009), $ROSA^{NICD}$ mice (Murtaugh et al. 2003), $ROSA^{EYFP}$ (Srinivas et al., 2001) and their genotyping strategies have been published. Expression of the transgene in the various tissues was evaluated by RT-PCR. All experiments were performed under internal regulations for animal care and handlings (IACUC 355).

2.2 Cell isolation

Embryonic myoblasts were isolated from E11.5 (after removal the neural tube) embryos; $Pax3^{GFP-/+}$ embryos were distinguished from their wild-type littermates by examination under a fluorescence microscope. Embryonic myoblasts were obtained from dissection of somites and limbs of E11.5 embryos. Tissues were 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 $MgSO_4$, 0.3% (W/V) bovine serum albumin (BSA), pH 7.4. Dissociated cells were resuspended in DMEM–high glucose (Gibco), supplemented with 20% fetal calf serum (FCS; BioWhittaker), 20 mM HEPES and 2 mM EDTA and filtered through 40-µm cell strainers (Falcon) before sorting using a Vantage Sorter SE (Becton-Dickinson) at a flow rate of 3000 cells per second. GFP was exited at 488 nm using an argon laser. Specific green fluorescence, forward (FSC) and side (SSC) light scatter were measured. FSC and SSC parameters were used to gate out cell clumps and debris. Cells dissociated from wild-type littermates were used to set the gating to exclude

green autofluorescence. The purity of each sorted sample was evaluated by immediate reanalyzing of approximately 10,000 cells.

2.3 Cell culture

For the differentiation assay, sorted embryonic myoblasts were resuspended in DMEM–high glucose supplemented with 20% horse serum (BioWhittaker) and 20 mM HEPES and spotted onto calf skin collagen (Sigma)- coated dishes, at a density of approximately 80,000 cells/cm_2.

To induce pericytes differentiation embryonic myoblast were plated onto Dll4 (R&D SYSTEMS, 1389-D4) 10ug/mL coated dishes. Medium was composed by DMEM-high glucose supplemented with 20% horse serum and PDGF (SIGMA) 50ug/mL.

HUVECs were cultured in MCDB 131 with endothelial cell supplements as described previously (Lampugnani et al., 2003). For the experiments 1,800 and 42,000 cells/cm2 were seeded to obtain sparse and confluent cultures, respectively. HUVECs were used till passage 4.

2.4 Alkaline phosphatase reaction.

Different kits for AP enzymatic detection were used (see below) to exploit different reaction colors. Sections or cells were fixed with PFA 4% at 4 °C for 10 min and, after 3 washes, incubated with BCIP-NBT kit (Roche, 12296226 and 12329020) in an alkaline buffer (NaCl 100 mM, Tris 100 mM PH 9.5, MgCl2 50 mM, Tween 0.1%) for 20 min in the dark, or with PermaRed/AP (Histo-Line laboratories, K049) for 20 min. Moreover, AP substrate kit from Vector (SK-5100) were used and visualized by confocal microscopy (Leica) at 456 nm, along with other immuofluorescent antibodies.

2.5 Immunofluorescence on Cultured Cell and Tissue Sections

Frozen sections and cultured cells were fixed with PFA 4% at 4 °C for 10 min, washed 3× with Triton 0.2% BSA 1% in PBS, and incubated with the same buffer for 30 min, followed by 10% Donkey serum for 30 extra min. Primary antibody was incubated for 1 h at room temperature or O/N at 4 °C in the same buffer. Samples were washed 3 times and then incubated with secondary antibodies (Alexa Fluor 488-594-546 or 647, Invitrogen) for 1 h at RM, washed 3 times and then mounted and examined under epifluorescence or confocal Leica microscope. When necessary, adjacent sections were stained with H&E. Immunofluorescent staining was carried out with the following antibodies: rabbit policlonal anti-Myf5 1:100 (Santa Cruz biotecnology), Rat policlonal anti-PECAM 1:2 (gift from Elisabetta Dejana), mouse monoclonal anti-SMA 1:300 (SIGMA), Goat policlonal anti-AP 1:100 (R&D SISTEMS) rabbit policlonal anti-NG2 1:300 (Chemicon AB5320), anti-MyHC at 1:2 (MF20 DHSB), anti-GFP 1:300 (Chemicon, AB3080) mouse monoclonal antiBV9 (gift from Elisabetta Dejana). 488,647 or 594-conjugated donkey anti-mouse. Hoechst was used to stain nuclei.

2.6 RT-PCR

The RNA was isolated from cells (sorted fraction from $Pax3^{GFP/+}$ embryos) and embryos/foetus from mice at different developmental stages. One microgram of RNA, extracted with the RNeasy micro kit (QIAGEN) from cells or TRIZOL (Invitrogen) from tissues, was converted into double-stranded cDNA with the cDNA synthesis kit ImProm™-II Reverse Trascription System (Promega),

according to the manufacturer's instructions. The primers used are described in the Supplementary materials and methods.

2.7 Statistical analysis

Data were analyzed using GraphPad Prism 5 and values were expressed as means ± standard error (SEM). Statistical significance was tested using Unpaired and Paired Student t-test or one-way ANOVA analysis of variance followed by Bonferroni multiple comparisons post-test. A probability of less than 5% ($P < 0.05$) was considered to be statistically significant.

2.8 Quantitative Real-Time PCR

RNA was retrotrascribed as described above. Real time quantitative PCR were performed with a real-time PCR machine (Mx3000P; Stratagene). Each cDNA sample was amplified in triplicate using GoTaq® qPCR master mix (Promega). Data are expressed as mRNA levels relative to the house keeping gene Gapdh, or as the percentage of target gene mRNA levels relative to levels detected in their relative controls.

2.9 Angiogenic assay

Matrigel was incubated on ice at 4 degree O/N. Then it was transferred 150 ul of pre-cooled matrigel reduced Growth Factor (BD matrigel™ 356230) to a 48-well plate. All this passages has been performed on ice. Then the matrigel was solidify at 37 degree C for 30 min. Endothelial cells were collected, count and dilute to 4 x 105 cells/ml in cell culture media mixture to the 48-well plate. HUVEC cells were cultured alone as control, then with not treated sorted cells and with treated Dll4 and PDGF-BB sorted cells. Incubate at 37 degree, 5%

CO_2 for 3 to 30 h. Any increase or decrease in the formation of tubes in the test wells was compared to control wells (the one with non-treated cells) and this would indicate that the network is stable thanks to pericytes covering.

2.9.1 Matrigel plug

Matrigel reduced Growth Factor (BD matrigel™ 356230)(500 uL) was injected subcutaneously in the dorsal region of 2.5–3.5 month old nude mice and permitted to solidify. In the plug there was mixed HUVEC cells plus $Pax3^{GFP/+}$ cells treated with Dll4 and PDGF-BB. Mice were observed after 24 h to monitor condition of the wound. Plugs could be recovered for several weeks but typically we sacrifice the mice after 7 and 14 days. After the sacrifice plug was embedded in OCT .

2.10 Protein Extraction and Western Blot

Cells were lysed in boiling Sample Buffer 1 (50 mM Tris-HCl pH 6.8, 2%SDS, 10% glycerol, 100 mM dithiothreitol (DTT)). 30-50 ug of protein were resolved on 8% or 12% SDS-PAGE (according to the different molecular weights) and then transferred onto nitrocellulose. After saturation in 5% milk in tris-buffered saline (TBS)-T (TBS plus 0.02% Tween20), filters were incubated with the following antibodies: monoclonal anti-MyHC, monoclonal anti-Myogenin, rabbit polyclonal anti-Ng2, goat policlonal anti-TNAP (sc-718), rabbit polyclonal anti-PDGFR, mouse monoclonal anti-MyoD antibody policlonal anti-Myf5, mouse monoclonal anti-tubulin (ICN), mouse monoclonal anti-GAPDH. After washing, membranes were incubated with horseradish-peroxidase-conjugated species-specific secondary antibodies (BIO-RAD) followed by enhanced chemiluminescence system (Amersham).

2.12 Supplemental information

- Myf5 (351bp)

forward TGCCATCCGCTACATTGAGAG
reverse CCGGGGTAGCAGGCTGTGAGTTG

- MyoD (396bp)

forward GCCCGCGCTCCAACTGCTCTGAT
reverse CCTACGGTGGTGCGCCCTCTGC

- MyHC (160bp)

forward GGCCAAAATCAAAGAGGTGA
reverse CGTGCTTCTCCTTCTCAACC

- Myogenin (156bp)

forward GACATCCCCCTATTTCTACCA
reverse GTCCCCAGTCCCTTTTCTTC

- MRF4 (235bp)

forward GAGCTGGGCGTGGACCCCTA
reverse CCACGCTGGGGAGTTTGCGT

- NG2 (443bp)

forward ACAAGCGTGGCAACTTTATC
reverse ATAGACCTCTTCTTCATATT

- AP (329bp)

forward GTGGATACACCCCCCGGGGC
reverse GGTCAAGGTTGGCCCCAATGCA

- PDGFbeta (88bp)

forward AAGTTTAAGCACACCCATGACAAG
reverse ATTAAATAACCCTGCCCACACTCT

- RGS5 (110bp)

forward GCTTTGACTTGGCCCAGAAA
reverse CCTGACCAGATGACTACTTGATTAGC

- ID1 (210bp)

forward CGCAAAGTGAGCAAGGTGGAGA
reverse TCAGCGACACAAGATGCGATC

- ID2 (264bp)

forward GCCCAGCATCCCCCAGAA
reverse CCATTTATTTAGCCACAG

- ID3 (225bp)

forward ACAGCTGAGCTCACTCCGGAACT
reverse TCCAGCCTCGAGGCGTTGAGTT

- Twist (197bp)

forward CACGCTGCCCTCGGACAA
reverse GGGACGCGGACATGGACC

- Notch1 (202bp)

forward TGGACGCCGCTGTGAGTCA
reverse TGGGCCCGAGATGCATGTA

- Notch3 (218bp)

forward CCTGGATGCTGGGGCGGACAC
reverse CGGCAT GGCTGGCGATGAGCT

- Gapdh (250bp)

forward TTCACCACCATGGAGAAGGC
reverse GGCATGGACTGTGGTCATGA

- Pax3 (413bp)

forward GAGACTGCCTCCATACGTCC
reverse ACGGTGTTTCGATCACAGAC

- Pax7 (282bp)

forward GTGGGGTCTTCATCAACGGTC
reverse GCAGCGGTCCCGGATTTCCCAG

RESULTS

3.1 Purification of embryonic myoblast from Pax3$^{GFP/+}$ mice

To obtain a pure population of embryonic myoblasts, GFP expressing cells are isolated by FACS sorting from cellular suspension obtained from Pax3$^{GFP/+}$ at E11.5 dpc. Somites and limbs were dissected so, the cells derived from the digestion does not contain contamination of a number of progenitor expressing Pax3, that could give rise a non-myogenic population. During mouse development at 11.5 primary myogenesis is ongoing and almost all myoblast present are thought to be embryonic. Approximately 1-2% of the cells obtained from the somites and limbs express GFP (Fig. 3.1.1 B). It was possible to sort 20-25.000 cells from each E11.5 embryo. When reanalyzed by FACS, sorted populations were 95-98% pure (data not shown). To demonstrate that cells sorted from Pax3$^{GFP/+}$ mice were indeed myogenic, immunofluorescent staining with antibodies specific for myogenic marker Myosin Heavy Chain (MyHC) was performed after 2-3 days of differentiation *in vitro*. Almost all (> 98%) of sorted cells express MyHC. Then it has been analyzed mitogen influence. As already published (Biressi et al., 2007a) embryonic myoblast are not influenced by any kind of mitogen. The same results is obtained from primary myoblast from Pax3$^{GFP/+}$ mouse (Fig. 3.1.1 C), in fact, it is possible to see that the differentiation capability is not at all influenced by TGFβ, BMP4 and bFGF, that from oligonucleated myotubes express MyHC like control cells. So embryonic myoblasts obtained from Pax3$^{GFP/+}$ have the same characteristic of myoblasts obtained from Myf5$^{GFP/+}$.

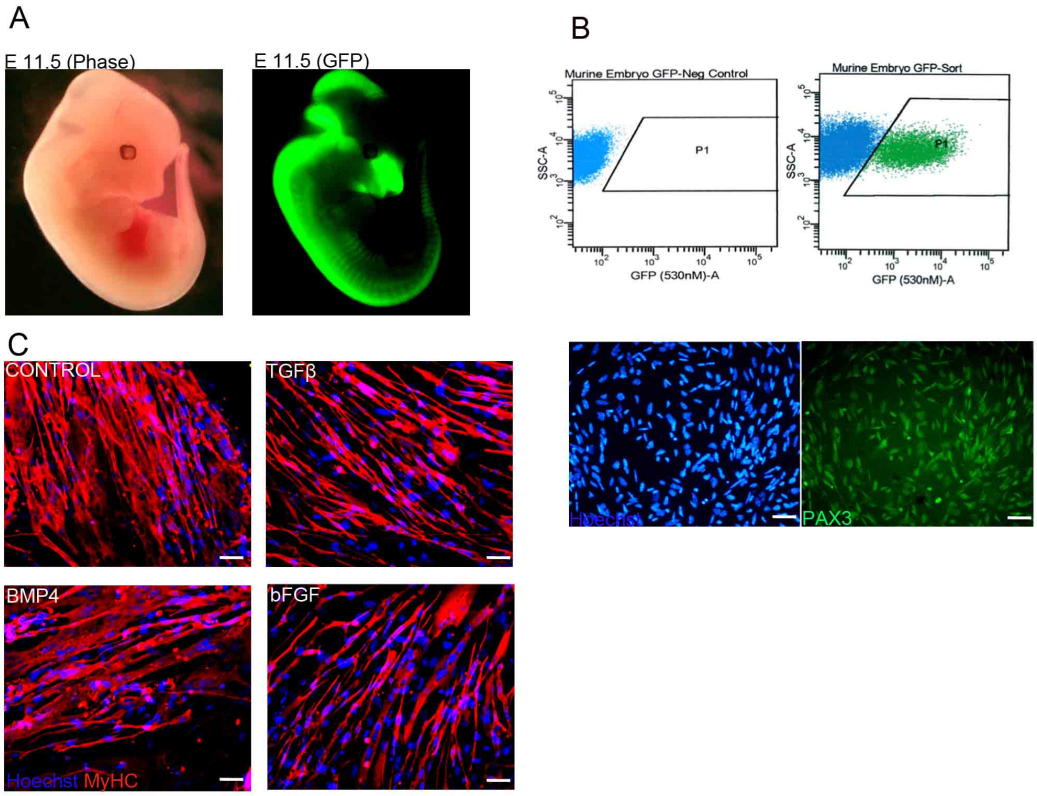

Figure 3.1.1 Embryonic myoblast from Pax3$^{GFP/+}$ mouse isolation

Efficent isolation of embryonic myoblast from cellular digestion obtained from Pax3$^{GFP/+}$ mice at day 11.5 (A,B). GFP expressing cells are separated in two dimensional plot of GFP (abscissa) and intrinsic SSC (ordinate). Sorted cells immediately differentiate into thin myotubes almost 95% positive for myogenic marker (MyHC). Cells immediately after sorting are still Pax3 positive (B bottom panel). Embryonic myoblast insensitivity to mitogen (C) such as BMP4, bFGF and TGFβ. As shown in the panel cells differentiate with the same extent of control cells. Scale bar refers to 100um

3.2 Dll4 and PDGF-BB inhibit skeletal myogenesis and induce pericyte markers in Pax3 expressing skeletal myoblasts.

Embryonic myoblasts, sorted from $Pax3^{GFP/+}$ E11.5 embryos after removal of the neural tube, and a dissection of somites and limbs, were cultured in standard conditions (see materials and methods) that allow rapid and massive myogenic differentiation, with formation of thin oligonucleated, myosin heavy chain (MyHC)+ myotubes (Fig. 3.2.1 A). Since recent works demonstrate that Pax3+ cells migrate to form smooth muscle layer of dorsal aorta we address wheter myogenic progenitor could be able to be reprogram/recruit from endothelium. However, seeding sorted $Pax3^{GFP/+}$ myoblasts on dishes coated with the Notch ligand Dll4 (responsible for pericyte recruitment during vasculogenesis) in the presence PDGF-BB (which receptor is present on embryonic myoblast) inhibited, as expected, skeletal myogenesis. Fig. 3.2.1 B/C shows that Dll4 and PDGF-BB prevented almost completely myogenic differentiation and inducing, within few hours, expression of pericyte makers such as Alkaline Phosphatase (AP), RGS5 and NG2, undetectable in control cells by either qPCR (Fig. 3.2.2 A), immunofluorescence or Western Blot analysis (Fig. 3.2.1 A-C). Among the other pericyte markers, PDGF Receptor B was expressed in myoblasts but its expression increased two fold after exposure to Dll4 and PDGF-BB (Fig. 3.2.2 A central panel). Western Blot did not detect this receptor in myoblasts, possibly because of post-transcriptional regulation (Fig. 3.2.1 C). Both qPCR and Western blot analysis (Fig. 3.2.2 A, 3.2.1 C) revealed an enhanced expression of Myf5 at variance with all the other myogenic markers tested, which have been strongly downregulated. Moreover, if myogenic markers are immediately downregulated, in the first hours of

culture, pericytes marker up-regulation appears at the end of the culture. Exposure to Dll4 and PDGF-BB did not interfere with the low level of apoptosis observed (<5% data not shown) while treated cells continued to proliferate (Fig. 3.2.2 B). Dll4 and PDGF-BB-dependent inhibition of myogenesis was found to be not reversible after subculture in fresh, control medium (data not shown). However, inhibition of Notch signaling using a γ-secretase inhibitor (that blocks proteolytic activation of Notch receptors, preventing nuclear translocation of the intracellular domain in $Pax3^{GFP/+}$, Dll4 and PDGF-BB treated cells, caused rapid and massive myogenic differentiation (Fig. 3.2.1 A/B, bottom line), leading to the formation of multinucleated myotubes. Reversion is also confirmed by qPCR analysis (Fig. 3.2.2 A) So embryonic myoblast commonly insensitive to any kind of mitogen, are sensitive to Notch signaling, and more interesting it appear that after Notch up-regulation, myogenic cells loose their myogenic capacity to acquire a pericyte property.

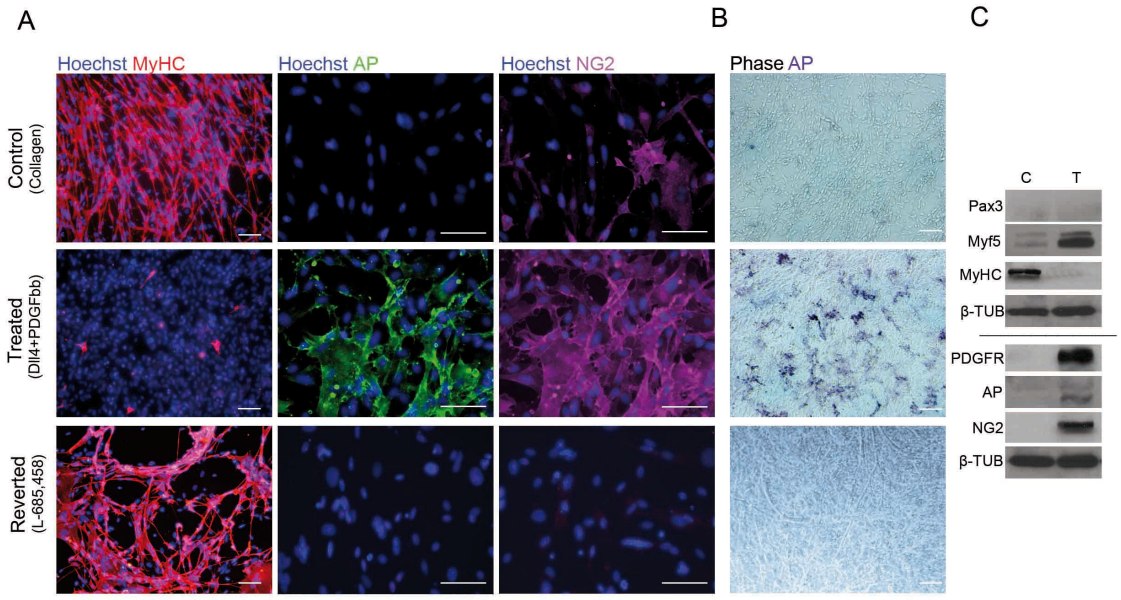

Figure 3.2.1 Characterization of embryonic myoblast treated with Dll4 and PDGF-BB

Immunofluorescence analysis of marker express by Pax$^{GFP/+}$ sorted cells. Cells differentiate immediately after sorting (A upper panel). After exposure to Dll4 and PDGF-BB embryonic myoblast do not differentiate and start to express pericytes markers. (A second and third panel). In (B) it is possible to see morphology of sorted cells and the expression of alkaline phospatase. Cells treated with Notch inhibitor (Y-secretase) restore myogenesis (A bottom panel) and downregulate pericytes markers. (C) Western Blot analysis confirmed the data of immunofluorescence where myogenic markers are downregulate with the exception of Myf5, while pericytes marker are all upregulate. Scale bar refers to 100 um.

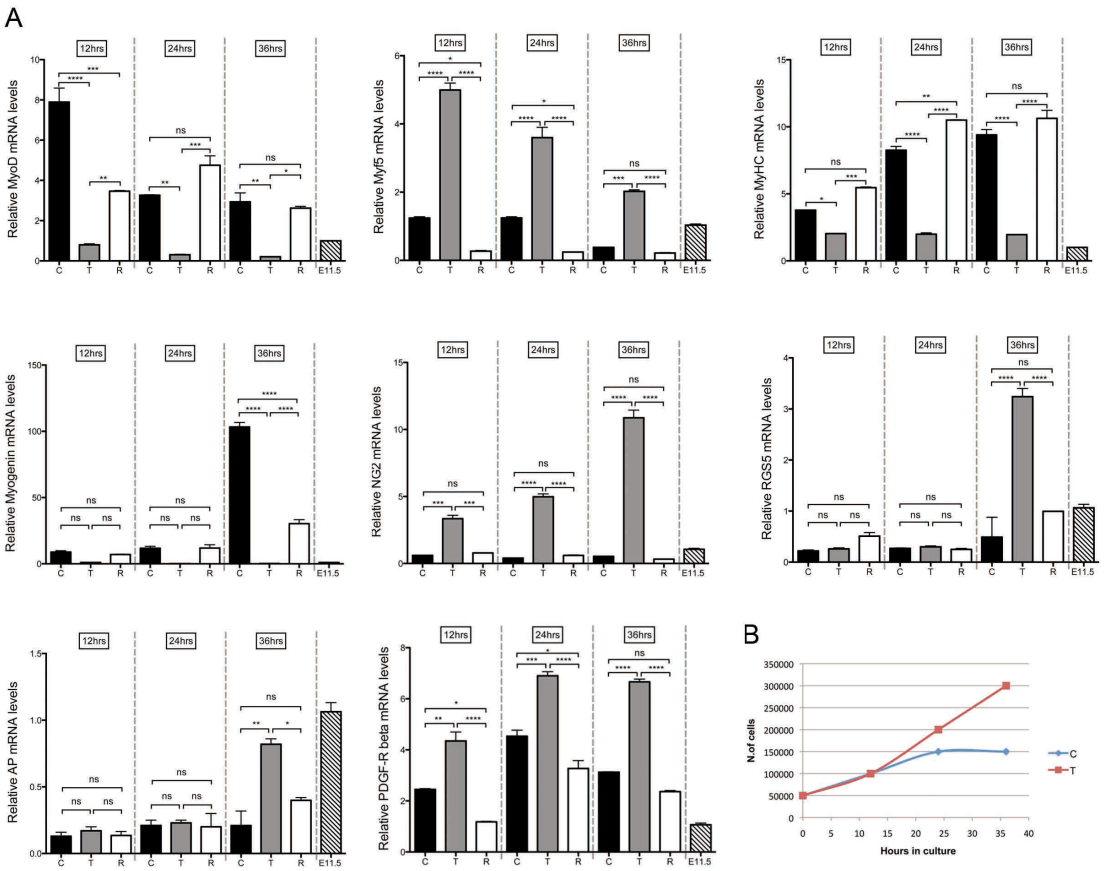

Figure 3.2.2 qPCR analysis of sorted embryonic myoblast

(A) qPCR analysis perform on Pax3$^{GFP/+}$ sorted cells, in control conditions, with Dll4+PDGF-BB and with γ secretase inhibitor (L-685-458). All the analysis were normalized with an embryo at E 11.5. Relative mRNA analyzed was for MyoD, Myf5, Myogenin and MyHC to detect myogenic

differentiatio, while for pericytes NG2, AP, RGS5 and PDGFRβ were tested. mRNA was analyzed in control cells, cells treated with Dll4 and PDGF-BB and cells treated, after Dll4 plus PDGF-BB with γ-secretase inhibitor, that restores myogenesis. Scale bar represent means of n 9 experiments ± s.d was tested using one-way ANOVA analysis of variance followed by Bonferroni multiple comparison post-test. A probability of less than 5% (P < 0.05) was considered to be statistically significant. (B) Growth curve of control cells compared to cells treated with Dll4 and PDGF-BB. Cells, beside normal behavior, go on proliferating and it is possible to culture them despite control one that does not proliferate and terminal differentiate immediately.

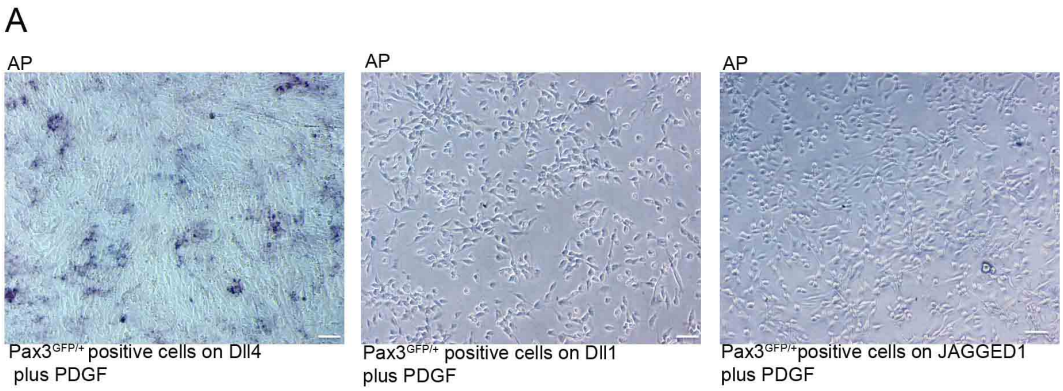

Figure 3.2.3 NOTCH ligands influence

(A) Left panel; Pax3$^{GFP/+}$ sorted cells, plated on Dll4 plus PDGF-BB and stained for alkaline phosphatase (AP) reaction, after 3 days of culture. Middle panel; Pax3$^{GFP/+}$ sorted cells plated on Dll1 plus PDGF-BB and stained for alkaline phosphatase (AP) after 3 days of culture. It is possible to see that no cells express AP. Right panel; Pax3$^{GFP/+}$ sorted cells plated on JAGGED1 plus PDGF-BB and stained for alkaline phosphatase (AP) after 3 days of culture. Also here none of the cells express AP. All cells in this experiment belong from the same sorting. Scale bar refers to 100um.

To then test specificity of Dll4, we repeated the same experiment using other Notch activators such as Dll1 and Jagged1. As expected, Pax3 expressing myoblasts did not differentiate in the presence of Dll1 and Jagged1;

It is in fact known that also Jagged1 is involved in pericytes recruitment.(Liu et al., 2009) .However under these different conditions, treated cells did not activate expression of pericyte markers (Fig. 3.2.3 A), indicating that this effect is specific only for Dll4 and not redundant among the other Notch ligands tested. The only effect that is possible to see is myogenesis inhibition that is expected once Notch is activated.

3.3 Dll4 and PDGF-BB also reprogram Myf5 expressing myogenic cells.

Although Pax3 expression marks early myogenic commitment in the embryo, cells entering myogenic differentiation pathway activate bHLH such as Myf5 and/or Myod (Buckingham and Relaix, 2007). Therefore we investigated whether Dll4 and PDGF-BB could also reprogram Myf5 expressing cells that are more committed than Pax3 to myogenesis. To this aim we FACS-sorted from the $Myf5^{GFP/+}$ mouse embryonic (E11.5) and fetal (E16.5) myoblasts as already publish(Biressi et al., 2007b). Under the same experimental conditions previously employed for Pax3-GFP expressing myoblasts, (Fig. 3.3.1 A-B) Myf5-GFP expressing myoblasts were also inhibited to differentiate, with fetal myoblast much more sensitive to Notch activation, although the frequency of reprogramming was lower than in Pax3-GFP positive myoblasts, with approximately 50% of cells that up-regulated AP (Fig 3.3.1 C bottom panel) and other pericyte markers (data not shown). So this *in vitro* reprogramming Notch driven works, in less extent, also in more committed myogenic cells.

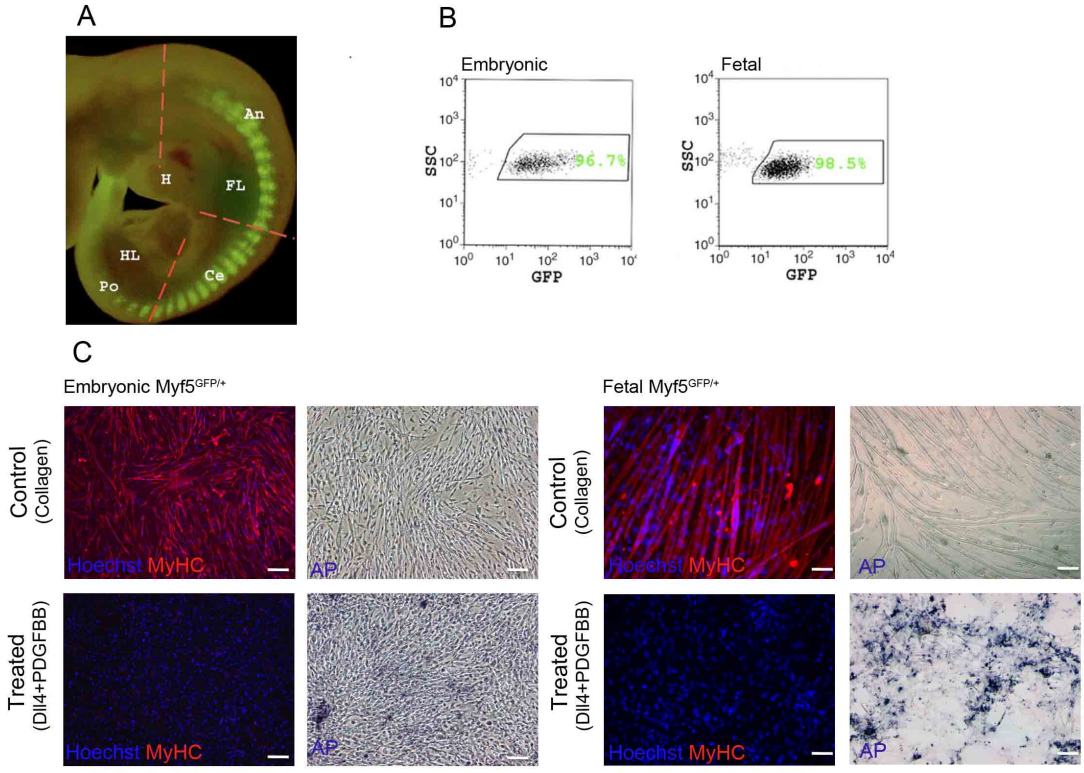

Figure 3.3.1 Embryonic and fetal myoblast sorted from a Myf5$^{GFP/+}$ mouse

Efficent isolation of embryonic and fetal myoblast from cellular digestion obtained form Myf5$^{GFP/+}$ mice at E11.5 (B left panel) and E16.5 (B right panel). (C) Right upper panel: efficient differentiation of embryonic myoblast in phase contrast and stained for MyHC (in red). Left upper panel: efficient differentiation of fetal myoblast in phase contrast and stained with MyHC.(C) Left bottom panel: influence of Notch ligands, Dll4 plus PDGF-BB on embryonic myoblast. As is possible to see, cells does not differentiate and express alkaline phosphatase and do not express MyHC. Right bottom panel: influence of Notch ligands, Dll4 plus PDGF-BB on fetal myoblast. As is possible to see, cells does not differentiate and express alkaline phosphatase, even in a major extent than embryonic one, due to the higher sensitivity of this cells to mitogen, and do not express MyHC. Scale bar refers to 100um.

3.4 Dll4/PDGF-B treated myoblasts associate with endothelial cells to form vessel-like networks *in vitro* and *in vivo*.

We then tested the ability of myoblasts, exposed to Dll4 and PDGF-BB to form a vessel-like structure *in vitro* and *in vivo* (Fig. 3.4.1 A-C). This because to assess that a cells become a pericytes it is important that it express not only pericytes marker, but also to assolve to pericyte function, such as vessel stability and formation. Human Umbilical Vein Endothelial Cells (HUVEC) cultured alone in matrigel, normally formed a network that disappeared after few days (not shown). When coculterd with them, untreated Pax3-GFP positive cells formed thin oligonucleated myotubes in matrigel mainly away from HUVECs, indicating that they readily differentiate into skeletal muscle also under this experimental condition (Fig. 3.4.1 A upper lane). Only when Pax3-GFP positive cells, were previously treated with Dll4 and PDGF-BB, and cultured in matrigel together with unlabeled HUVECs, they closely associated to them and formed a vessel-like network that remained stable for up to two weeks (Fig. 3.4.1 A bottom lane). The same effect was observed when matrigel plug was implanted subcutaneously into nude mice: after two weeks a large vessel network, connected with the host vasculature had developed (Fig. 3.4.1 B). Immunofluorescence analysis confirmed close association of Pax3-GFP positive cells with HUVEC labeled by an anti-BV9 (specific for human endothelial cells) antibody in red (Fig. 3.4.1 C). This association and vessel stability, clearly demonstrate that Pax3-GFP positive cells, previously treated with Dll4 and PDGF-BB, are able to mimic pericyte function and behaviour; however, from the coculture it's possible to appreciate also the morphology and localization of the cells that, closely associated to vessel, stabilize endothelial

network avoiding its disruption. In conclusion, Notch activation in embryonic myoblast drives them not only to the expression of quite all of pericytes marker but also to their functional activity and localization.

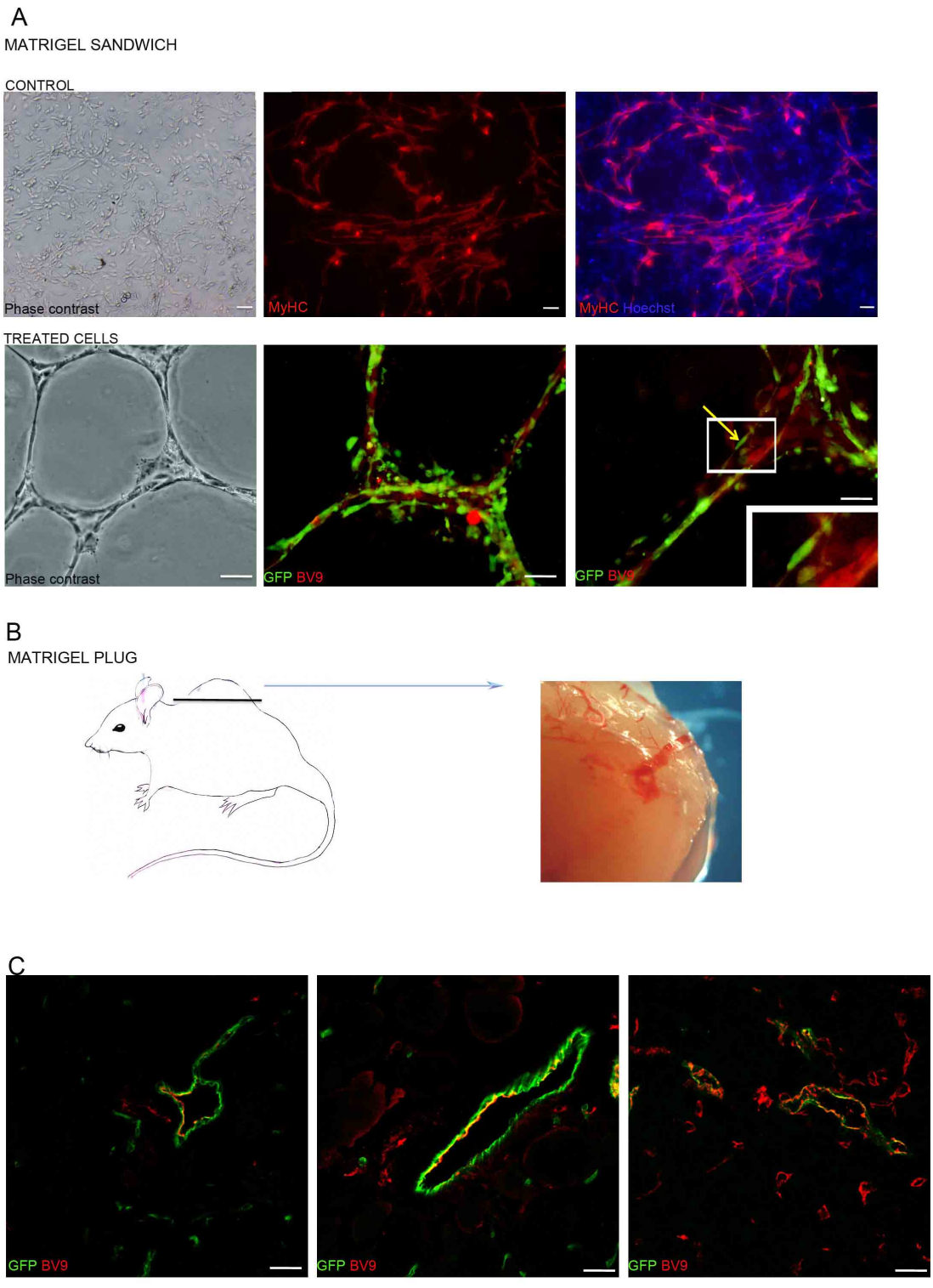

Figure 3.4.1 *In vitro* and *in vivo* angiogenesis assay

Example of a matrigel sandwich (*in vitro*) with HUVEC cells and control cells (A, upper and lower panel respectively). Cells are stained with MyHC antibody (red) and Hoechst (blue). Matrigel sandwich with previously treated cells Dll4 and PDGF-BB mixed with HUVEC cells. Phase contrast shows a stable endothelial network and it is possible to see the vessel tube (B). Cells are stained for GFP (green) and BV9 (red) that mark Human endothelial cells. (B) Scheme of a matrigel plug *in vivo* under the skin of nude mice. Plug is composed of Dll4 plus PDGF-BB treated cells mixed with HUVEC. (C) Confocal analisys of matrigel plug: cells are stained for GFP (green) and BV9 (red). Scale ber refers to 100um.

3.5 Committed skeletal myoblasts can be reprogrammed during *in vivo* development.

3.5.1 E11.5 MyoD^{iCRE}:Rosa^{NICD}

Rosa^{NICD} (Murtaugh and Melton, 2003) transgenic mice express the Notch IntraCellular Domain (NICD) and thus activate the Notch pathway after Cre recombination. We crossed Rosa^{NICD} with MyoD^{iCre} mice (Kanisicak et al., 2009) so all cells that begin to express MyoD will also activate Notch. Although it is well know that Notch inhibits myogenesis, its activation during in vivo skeletal myogenesis had not been previously investigated; more importantly, we wanted to know whether Notch activation would induce a pericyte phenotype also *in vivo*. As expected, E9.5 double transgenic embryos appeared to be normal (data not shown), consistent with the notion that the early myotome is formed under the transcriptional control of Myf5, before MyoD activation (Cossu et al., 1996; Kanisicak et al., 2009). On the other hand at E11.5 however, MyoD^{iCRE}:Rosa^{NICD} embryos appeared to be smaller and showed an edema, more pronounced in the back; hind and forelimbs appeared to be

underdeveloped (Fig. 3.5.1 A). At this stage expression of myogenic markers appeared reduced (though differences were not statistically significant), with the exception of Myf5, whereas pericyte markers showed no variations in expression with the exception of an upregulation of AP that usually start to be express in the embryo around vessel not before E13.5/14.5 (Ugarte et al., unpublished observation) (Fig. 3.5.1 B). Also Western Blot analysis did not show major differences (Fig. 3.5.1 C). At the histological level no major differences were observed (Fig. 3.5.1 D, upper panel) but immunofluorescence analysis revealed reduced MyHC+ cells in the limbs (arrows in Fig. 3.5.1 D, bottom panel), although the myotomes appeared to be similar. Higher magnification immunoflurescence of the trunk mesoderm revealed Myf5+ and SMA (smooth muscle actin) cells in both WT and MyoDiCRE^{ROSANICD} embryos, but widespread expression of AP was only detected in MyoDiCRE^{ROSANICD} embryo and not in WT (Fig. 3.5.1 E upper panel), where it usually appears only around small vessels at E13.5 (Ugarte et al. unpublished observations).

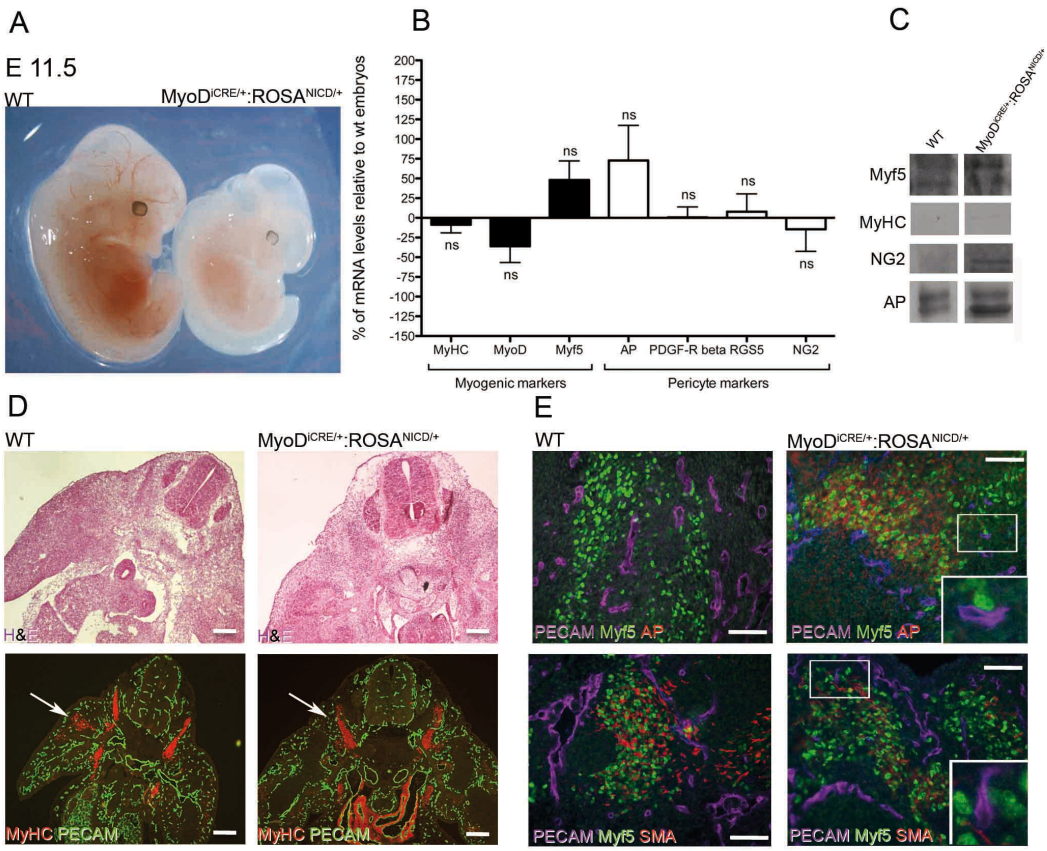

Figure 3.5.1 E 11.5 MyoDiCRE:ROSA^{NICD} analysis

Morphology of MyoDiCRE:ROSA^{NICD} mice and WT at E11.5.qRT analysis on WT and MyoDiCRE:ROSA^{NICD}. Relative mRNA analysis of MyoD Myf5 MyHC and Myogenin to teste myogenesis and NG2, RGS5, AP, PDGFR for pericytes. Western blot analysis of myogenic (Myf5, MyHC) and pericyte (PDGFR, NG2, AP) marker expressed by WT embryo and and MyoDiCRE:ROSA^{NICD}. H&E staining on serial tranverse sections of whole embryo (upper panel part). Immunofluorescence on serial tranverse section of whole embryo stained with MyHC (red) and PECAM (green).Immunofluorescence on serial transverse sections of hindlimb stained with Myf5 (green), AP(red) and PECAM(violet) in the upper part of the panel; in the lower part sections are stained with Myf5 (green) SMA (red) and PECAM (violet). Scale bar refers to 100um.

3.5.2 E13.5 MyoD^{iCRE}:Rosa^{NICD}

At E13.5 a similar phenotype was exacerbated in mutant embryos (Fig. 3.5.2 A); also edema in mutant embryos became bigger while the embryos is still smaller; but now differences in gene and protein expression start to be statistically significant (Fig. 3.5.2 B-E) while the reduction of MyHC positive fibers appeared to be dramatic (Fig. 3.5.2 D, bottom panel). In Fig. 3.5.2 D bottom panel, it is possible to see how the morphology starts to be compromised: MyHc is express only in the heart and there is no development of any muscle. Also the embryos dimension is different. Myf5 expressing cells increase remarkably, this without leading to any terminal differentiation. Confocal microscopy confirmed close association of Myf5 expressing cells with the endothelium and medium and small size vessel, while, in WT embryos, Myf5 expression start to decrease, and SMA positive cells are detactable, anyway, Myf5 positive cells localize far from vessel. Real time analysis of myogenic marker reveal the statistic significant of the decrement in the MyoD^{iCRE}:Rosa^{NICD} despite the WT (Fig 3.5.2 B). Moreover, pericyte markers expression is not significant meaning that to reprogram cells fate in an embryo probably take more developmental stages.

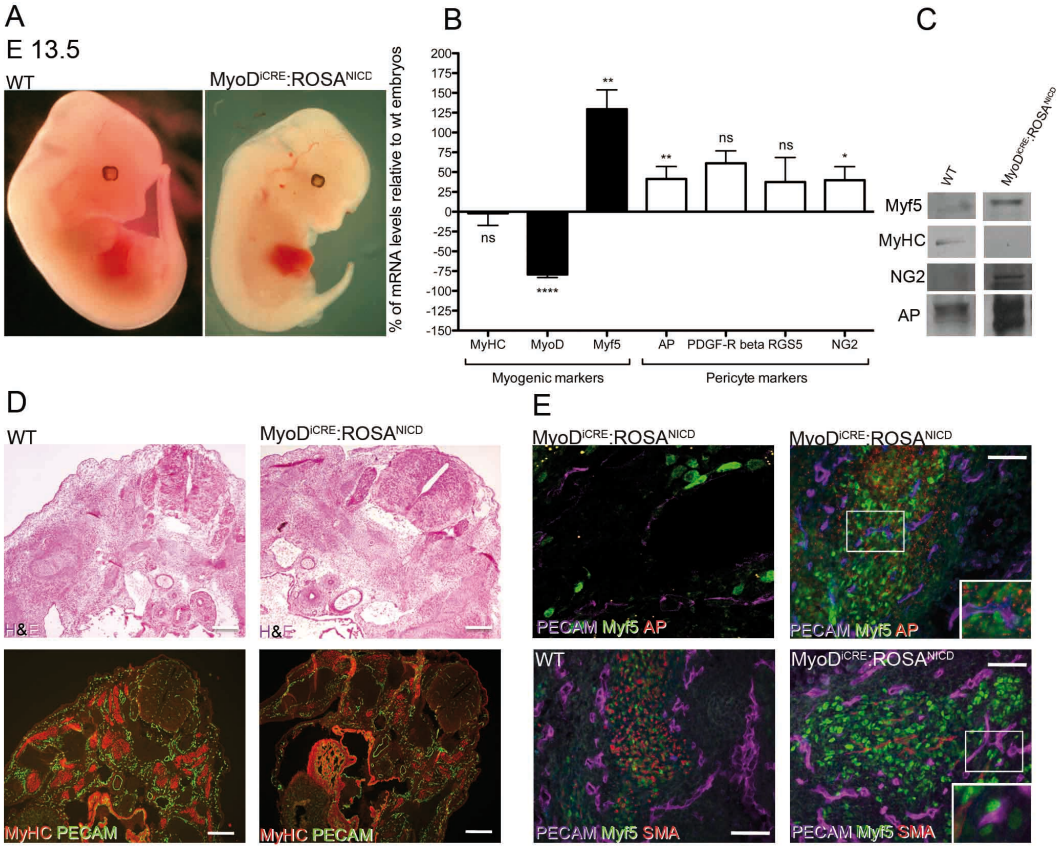

Figure 3.5.2 E 13.5 MyoDiCRE:ROSA^{NICD} analysis

(A) Morphology of MyoDiCRE:ROSA^{NICD} and WT mice at E13.5. (B) qRT analysis on WT and MyoDiCRE:ROSA^{NICD}. Relative mRNA analysis of MyoD Myf5 MyHC and Myogenin to test myogenesis and NG2, RGS5, AP, PDGFR for pericytes. (C) Western blot analysis of myogenic (Myf5, MyHC) and pericyte (PDGFR, NG2, AP) marker expressed by WT embryo and MyoDiCRE:ROSA^{NICD}. (D) H&E staining on serial transverse section of whole embryo (upper panel part). (E) Immunofluorescence on serial transverse section of whole embryo stained with MyHC (red) and PECAM (green).Immunofluorescence on serial transverse sections of hindlimb stained with Myf5 (green), AP(red) and PECAM(violet) in the upper part of the panel; in the lower part sections are stained with Myf5 (green) SMA (red) and PECAM (violet). Scale bar refers to 100um.

3.5.3 E16.5 MyoD^{iCRE}:Rosa^{NICD}

At E16.5, mutant fetuses were severely compromised, with very large edema in the back (Fig. 3.5.3 A), dramatically reduced skeletal muscle differentiation, and dimension of the fetus and development compromise. Looking at the marker of skeletal muscle differentiation is possible to appreciate either by q-PCR (Fig. 3.5.3 B), Western Blot (Fig. 3.5.3 C), histology and immunofluorescence a strong downregulation (Fig. 3.5.3 D). Exactly like *in vitro*, Myf5 expression appeared three-fold increased (Fig. 3.5.3 B) and was clearly detected by immunofluorescence, whereas it appeared fainter in WT embryos where it is known to decline at this stage of development. (Ott et al., 1991)

At E16.5 the development did not proceeded further, lung formation was impaired, and the edema became even more severe (Fig. 3.5.3 A). A similar phenotype was reported for the Myf5/MyoD double mutant embryos (Rudnicki et al., 1993)Here a large percentage of Myf5 expressing cells is associated to vessel as it possible to see in the confocal analysis at higher magnification. Also all pericytes markers analized are strongly up-regulated and, by qPCR, statistically significant (Fig 3.5.3 B). Moreover, by immunoflurescence is clear the AP is express in a large extent in cells that never express it as it's clear in Fig 3.5.3 E right panel. This means that during ongoing of development cells that activate Notch and inhibit myogenesis assume another fate with another localization and marker expression, exactly like *in vitro* results.

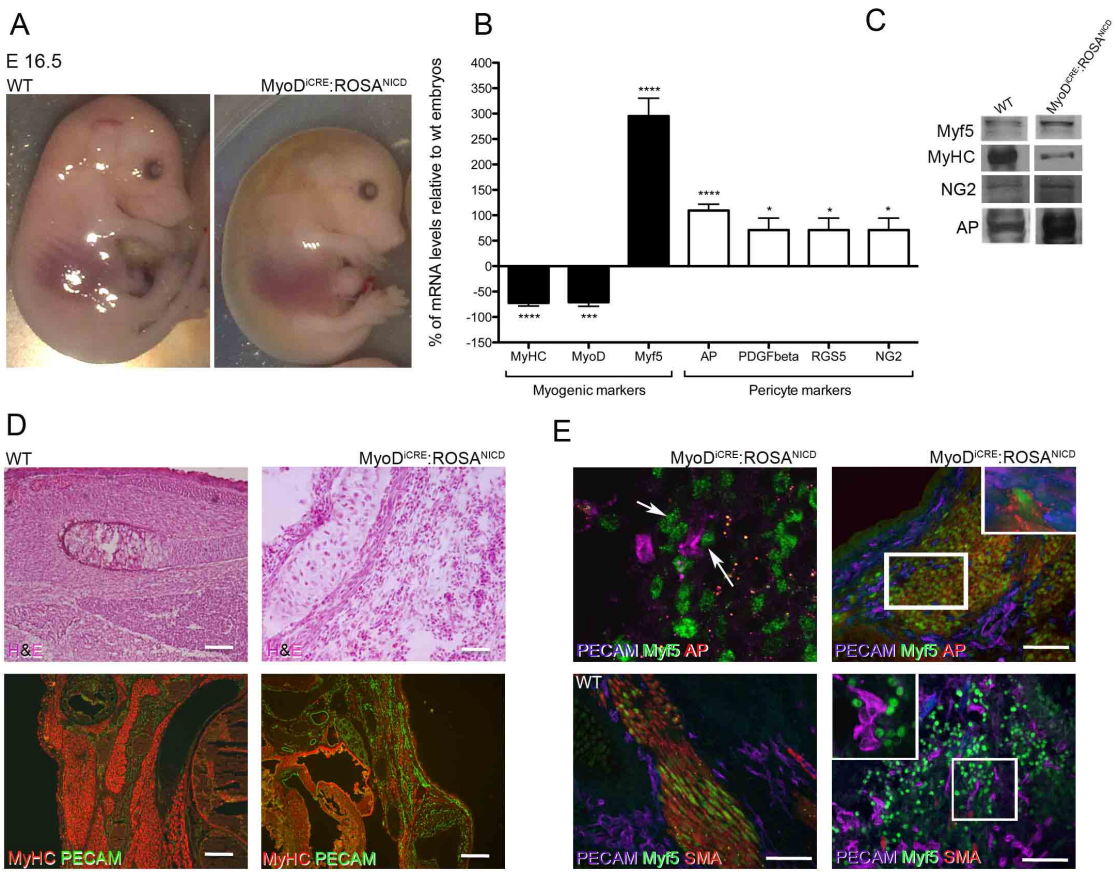

Figure 3.5.3 E 16.5 MyoDiCRE:ROSA^{NICD} analysis

(A) Morphology of MyoDiCRE:ROSA^{NICD} and WT mice at E16.5. (B) qRT analysis on WT and MyoDiCRE:ROSA^{NICD}. Relative mRNA analysis of MyoD Myf5 MyHC and Myogenin to test myogenesis and NG2, RGS5, AP, PDGFR for pericytes. (C) Western blot analysis of myogenic (Myf5, MyHC) and pericyte (PDGFR, NG2, AP) marker expressed by WT embryo and MyoDiCRE:ROSA^{NICD}. (D) H&E staining on serial transverse section of whole embryo (upper panel part). (E) Immunofluorescence on serial transverse section of whole embryo stained with MyHC (red) and PECAM (green).Immunofluorescence on serial transverse sections of hindlimb stained with Myf5 (green), AP(red) and PECAM(violet) in the upper part of the panel; in the lower part sections are stained with Myf5 (green) SMA (red) and PECAM (violet). Scale bar refers to 100um.

3.5.4 E18.5 MyoD^{iCRE}:Rosa^{NICD}

At E18.5 fetus' morphology is completely compromise (Fig. 3.5.4 A). The dimension are completely different from WT there are no muscles formation and the edema is very pronounced in the back. Hind and forelimb are strongly under developed. Looking at the qPCR (Fig 3.5.4 B) is clear, by the significance of the statistical analysis that myogenesis didn't occur, and pericytes marker expression is stronger (Fig 3.5.4 B). In fact, looking at the immunofluorescence is immediately clear the difference: in the MyoD^{iCRE}:Rosa^{NICD} Myf5 expression is three fold increase than the wild type, where, as usual, Myf5 is not express anymore. Western Blot principally confirmed results of qPCR (Fig 3.5.4 C-E). Looking deeper at the morphology is it possible to see that the diaphragm is under developed and also, for this reason lungs are not terminally differentiated; this leads to the impossibility to breathe, and so new born mice died immediately. All these morphological analysis confirm the active role played by Notch during development; in fact Notch is responsible not only for the lineage determination during dermamyotome development (Esner et al., 2006) but also for recruitment of pericytes by tip cells. This data show the evidence that also a committed myogenic cell under suitable stimulus is prone to be reprogram to another fate.

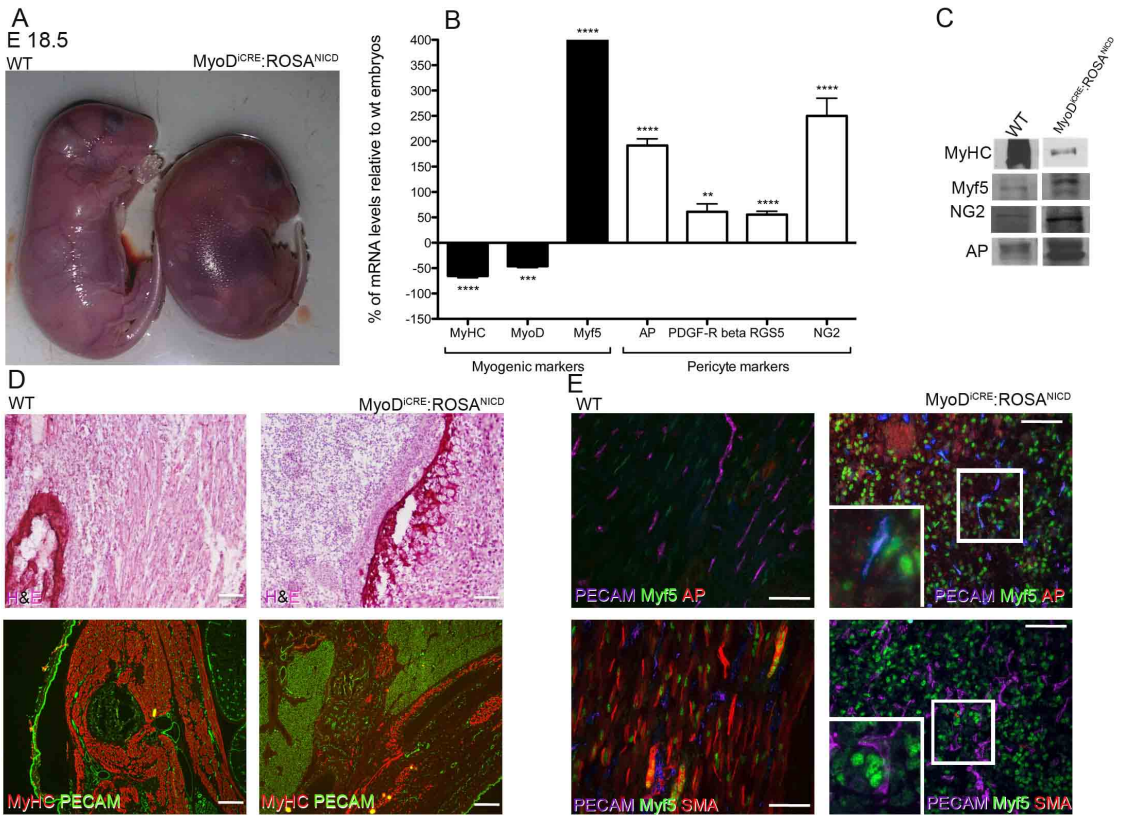

Figure 3.5.4 E 18.5 MyoDiCRE:ROSA^{NICD} analysis

(A) Morphology of MyoDiCRE:ROSA^{NICD} and WT mice at E18.5. (B) qRT analysis on WT and MyoDiCRE:ROSA^{NICD}. Relative mRNA analysis of MyoD Myf5 MyHC and Myogenin to test myogenesis and NG2, RGS5, AP, PDGF for pericytes. (C) Western blot analysis of myogenic (Myf5, MyHC) and pericyte (PDGFR, NG2, AP) marker expressed by WT embryo and MyoDiCRE:ROSA^{NICD}. (D) H&E staining on serial transverse section of whole embryo (upper panel part). (E) Immunofluorescence on serial transverse section of whole embryo stained with MyHC (red) and PECAM (green).Immunofluorescence on serial transverse sections of hindlimb stained with Myf5 (green), AP(red) and PECAM(violet) in the upper part of the panel; in the lower part sections are stained with Myf5 (green) SMA (red) and PECAM (violet). Scale bar refers to 100 um.

3.6 Pericytes localization in WT embryos at E13.5

After mutant embryos and fetus analysis, following step has been to know wheter during normal embryos/fetus development myogenic cells could be reprogram by endothelium upon necessity of vasculogenesis. So in WT embryos we analyzed cells closely adjacent to vessel that are likely to be pericytes. Two different WT mice have been used. The first was a WT (Fig. 6 A-D) where, like a very rare event, happen that some myogenic cells closely associate to vessel and express pericytes maker like SMA and NG2. (Fig 3.6.1 A-D). Then we crossed a $MyoD^{ICRE}$: $ROSA^{EYFP}$ or $MyoD^{ICRE}$: $ROSA^{26R}$ to trace MyoD positive cells localization.

In Fig 3.6.1 E-K it's possible to see cells that expressed MyoD (in green, YFP and in blue X-gal) associate to vessel. In Fig 3.6.1 F we show the correct localization of myogenic cells. In Fig 3.6.1 I also is possible to see that YFP positive cells also express NG2. The last panel (Fig 3.6.1 K) is a confocal analysis revealing the connection of myogenic cells with vessel wall. These data clearly confirm the *in vivo* data illustrated before; the phenomenon we described with a mutation (by activation Notch pathway in already committed myogenic cells) is present also during natural development of WT type embryo meaning that myogenesis and vasculogenesis are two pathways which signaling compete to recruit cells. Once a cell enter myogenic program could have the possibility to be reprogram by a signaling originating from endothelium.

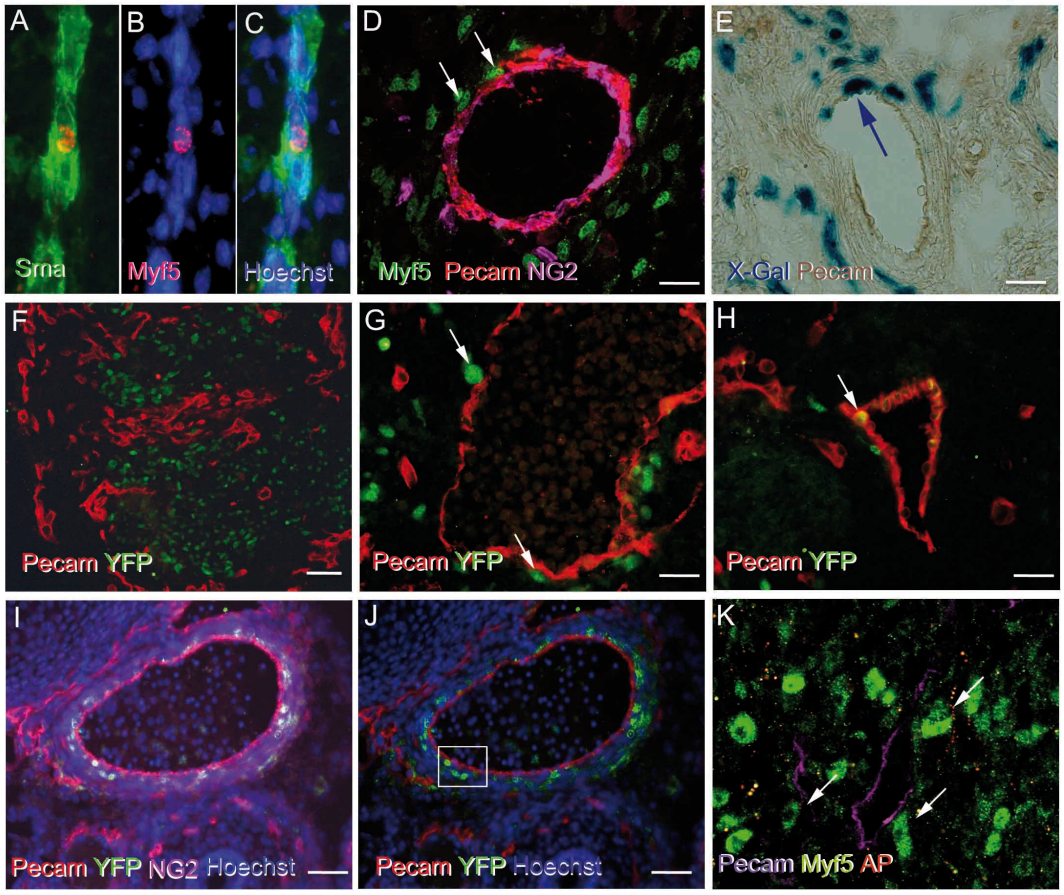

Figure 3.6.1 Marker analysis on WT mouse

Immunofluorescence analysis on serial transverse section of WT mouse embryo at E13.5. Section is stained for SMA (green) Myf5 (red) and Hoechst (blue). Immunofluorescence analysis on serial transverse section of WT mouse embryo at E13.5. Section is stained for SMA (green) Myf5 (red) and Hoechst (blue). Immunofluorescence analysis on serial transverse section of WT mouse embryo at E13.5. Section is stained for SMA (green) Myf5 (red) and Hoechst (blue) Immunofluorescence analysis on serial transverse section of WT mouse embryo at E13.5. Sections represent a trasversal section of a vessel. Section is stained for Myf5 (green) NG2 (violet) and PECAM (red). Immunoistochemistry of a transversal section of a vessel from MyoD^{iCRE}:ROSA^{26R} at E13.5.Lacz staining indicates MyoD positive cells around a vessel stained with PECAM for peroxidase. Immunofuorescence of a transversal section from MyoD^{iCRE}:ROSA^{EYFP} at E13.5. Section is stained for PECAM (red) and YFP (green). Immunofuorescence of a transversal section from MyoDiCRE:ROSA^{EYFP} at E13.5. Section is

stained for PECAM (red) and YFP (green). Immunofuorescence of a transversal section from MyoD^{iCRE}:ROSA^{EYFP} at E13.5. Section is stained for PECAM (red) and YFP (green). Immunofuorescence of a transversal section from MyoD^{iCRE}:ROSA^{EYFP} at E13.5. Section is stained for PECAM (red) YFP (green) NG2 (violet) and Hoechst (blue). Immunofuorescence of a transversal section from MyoD^{iCRE}:ROSA^{EYFP} at E13.5. Section is stained for PECAM (red) YFP (green) and Hoechst (blue). Immunofuorescence of a transversal section from MyoD^{iCRE}:ROSA^{EYFP} at E13.5. Section is analyzed with confocal microscope. It is stained for PECAM (violet) Myf5 (green) and AP (red). Scale bar refers to 100um.

3.7 Notch induced up-regulation of ID3 inhibiting Myf5 ability to activate myogenesis

Dll4 *in vitro* and MyoD^{iCRE}:ROSA^{NICD} *in vivo* up-regulate Notch3 and Notch1 expression that, as expected, leads to a strong down-regulation of MyoD, and MyHC expression. Unexpectedly, Myf5 expression was robustly upregulated, possibly as an attempt to compensate for the absence of MyoD. However Myf5, despite its high level of expression (Fig.3.2.1; Fig 3.5.1; Fig 3.5.2 Fig 3.5.3 Fig 3.5.4), was not sufficient to drive terminal myogenic differentiation. Notch it is known to induce upregulation of Id factors that may interfere better with MyoD but, in its absence, also with Myf5 activity (Langlands et al., 1997). As a consequence we investigated whether Myf5 protein may form a complex with Id3 (the most up-regulated member of the family after Notch activation) (Fig 3.7.1 left panel) and thus become unable to bind Myogenin promoter and trigger myogenesis. To test this possibility immunoprecipitation assays were conducted which revealed binding of the Myf5 and ID3 *in vivo*, thus providing a possible explanation for the inability of Myf5 to activate myogenesis .

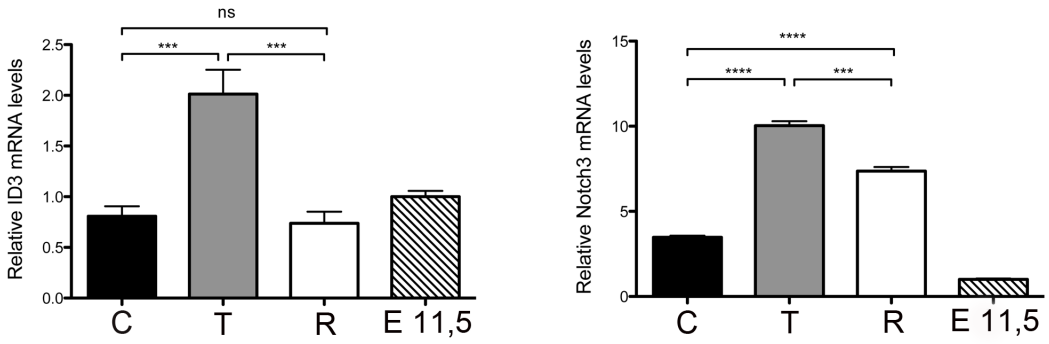

Figure 3.7.1 Expression analysis of Notch3 and Id3

(A) Relative mRNA analysis of ID3 and Notch3 in not treated embryonic myoblasts, embryonic myoblast treated with Dll4 and PDGF-BB and in embryonic myoblast reverted with γ-secretase inhibitor.

3.8 Model of Notch signaling activity in myogenesis

Notch pathway is responsible for lineage determination in dermamyotome.(Esner et al., 2006). Since during development exist a sort of competition between different lineages to recruit cells, Notch signaling is the best candidate for this role. In fact, cells that are attracted to endothelium by tip cells, are likely to be myoblast. The molecule secrete by endothelial tip cells is Dll4, so, in the model, is clear that Notch activation turn off myogenic genes, and activate pericytes ones. Which are these pericytes genes is still not publish. Notch activation also up regulated Id genes (1,2,3) and Twist, which are able to bind MyoD and Myf5. Since MyoD is immediately down regulated after Notch activation, Myf5 binds to Id3 and this way cannot trigger myogenesis (Fig 3.8.1)

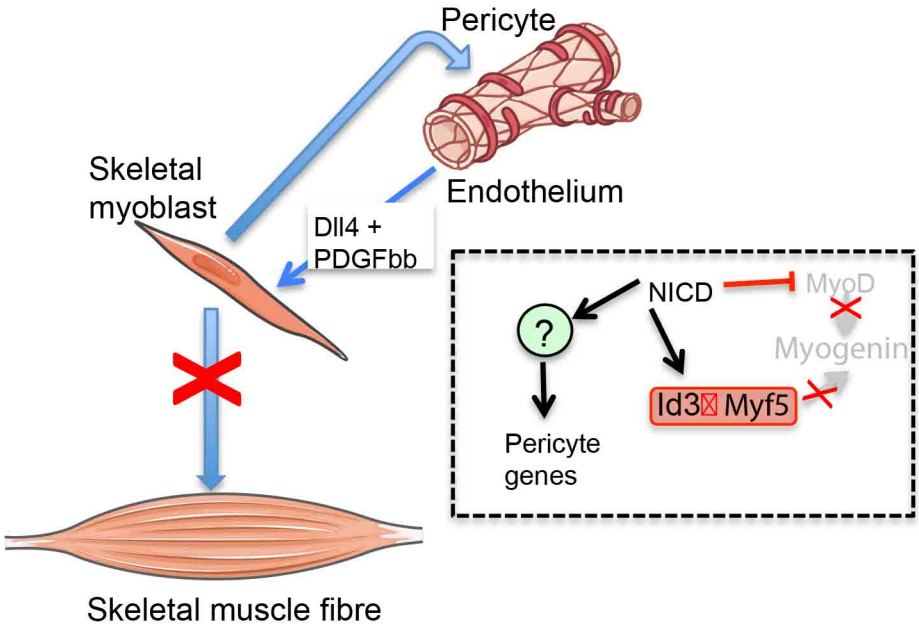

Figure 3.8.1 Model

Possible model of molecular mechanism underneath Notch signaling action to determine cells fate.

DISCUSSION

Pericytes are still poorly defined cells with multiple embryonic origins (mesenchyme, neural crest in certain anatomical districts and, suggested but not yet demonstrated, trans-differentiation of endothelial cells). They express a number of markers, none of which is unique and, moreover, not all mar;kers are expressed simultaneously in the same cell, making their identification *in vivo* and their prospective isolation difficult task (Armulik et al., 2011). In contrast, myogenic cells have been thoroughly characterized and are unequivocally identified by the expression of a number of unique (MyoD, Myf5, MRF4) or restricted (Pax3, Pax7) transcription factors (Rudnicki et al., 2008); whereas their surface markers are often shared with other cells types, with the possible exception of integrin alpha7. (Burkin and Kaufman, 1999; Conboy and Rando, 2002)

Notch and its ligands are involved in virtually any process during tissue development and regeneration. (Andersson et al., 2011; Dahlqvist et al., 2003) In the case of blood vessels, Dll4 and PDGF-BB secreted from tip cells of growing vessels, respectively bind Notch and PDGF receptor β on surrounding mesoderm cells, in order to recruit them to a pericyte fate and thus building the outer layer of the maturing vessel.(Hellstrom et al., 1999) In developing skeletal muscle, mesoderm tissue contains many skeletal myoblasts at different stages of proliferation, commitment and differentiation. It is thus conceivable that myoblasts, if exposed to recruiting signals from the endothelium, may change their fate and enter the pericyte lineage. Indeed, while embryonic myoblasts are insensitive to most mitogens (Biressi et al., 2007b) they are inhibited only by

Notch ligands. Intriguingly, all the Notch ligands tested, i.e. Dll1, Dll4 and Jagged1 are able to suppress expression of myogenic genes (with the notable exception of Myf5) but only Dll4 activates pericyte genes in committed, Pax3, Myf5 or MyoD expressing myoblasts. Moreover myogenic cells, exposed to Dll4 and PDGF-BB *in vitro* adopt a perithelial position stabilizing vessel-like networks of endothelial cells in matrigel plugs both *in vitro* and *in vivo*. Finally, if NICD (Notch Intracellular Domani) is selectively activated during embryonic development in cells expressing MyoD, these cells silence myogenic genes (again with the exception of Myf5) and express pericyte markers. Whenever they come close to a vessel, they adopt a perithelial position and still maintain a strong Myf5 expression. Under these experimental conditions, we uncover and amplify in myogenic cells a not frequent but detectable event that occurs during normal embryogenesis, where both Myf5 expressing and MyoD^{iCRE} labeled cells are found inside the vessel wall, expressing pericyte markers.

Thus, it appears that endothelial cells can directly reprogram myogenic cells to a pericyte fate and this environmental reprogramming depends upon exposure to Dll4 and PDGF-BB (that however can mimic endothelial cells action), likely as a consequence of physical proximity to vessel during tissue histogenesis. This reprogramming does not erase myogenic identity for two reasons: firstly, *in vitro* γ-secretase inhibitor restores terminal myogenic differentiation; secondly, Myf5 continues to be expressed in vitro and in vivo at levels much higher than WT, even though it fails to drive terminal differentiation. Notch downstream effectors Hairy and Hes1 are known to activate myogenic b-HLH inhibitors such as Twist and Id. We confirm here that indeed all of these genes are up-regulate in myogenic cells exposed to Dll4 (with the exception of

Id2). (Langlands et al., 1997; Reynaud-Deonauth et al., 2002)Among these, Id3, the most strongly upregulated gene, binds to Myf5, thus preventing its ability to bind and activate the Myogenin promoter.

In addition, it should be considered that both endothelial, smooth and skeletal muscle progenitors originate in the dermomyotome during somitogenesis and fate choice at that time is dictated by signaling molecules such as BMP, TGFβ and Notch. (Ben-Yair and Kalcheim, 2008; Esner et al., 2006; Lagha et al., 2009; Vieira et al., 2004) Moreover, Pax3 expressing myogenic precursors are able to migrate ventrally to built the smooth muscle of the aorta and express smooth muscle-specific genes. We now show that signaling molecules act also at later stages of development and are able to drive reprogramming (fate change from skeletal to future smooth muscle) also in committed cells.

Thus, it appears that during embryogenesis cells adopt a specific fate depending upon the timely appropriate exposure to signaling molecules emanating from surrounding cells. However, these fate choices are not irreversibly fixed but are reinforced and stabilized by the microenvironment. Experimental or natural exposure to different signals can still reprogram cells to a different (though embryologically related) fate; transcription factor-dependent in vitro reprogramming may simply reflect the experimental activation, at obviously higher frequency, of intra-cellular pathways also activated by signaling molecules. In the case of developing skeletal muscle, developing fibers and endothelium may compete for surrounding mesoderm cells, whose final fate is irreversibly fixed only at the onset of terminal differentiation.

TABLE OF ILLUSTRATIONS

ACKNOWLEDGEMENTS

I would like to thanks Giulio Cossu for the opportunity to develop this work and for encouraging me in this job. I thank him also for precious supervision and critical reading of the manuscript.

I thank Margaret Buckingham, Sharagim Tajbakhsh, Mariagrazia Lampugnani e Elisabetta Dejana for helping me to develop this work.

A special thanks goes to my sister. For her patience, help, and the constant presence in my life.

I thank my mum, just for all the sustain she gives me every day.

I also thank all Giulio Cossu's lab particularly Francesco Saverio Tedesco for discussion, help and patience. A special thanks goes to Sara Benedetti, Stefania Antonini e Martina Ragazzi, for all the help they gave me in this year, and for the friendship we built during this period. Really thank you.

I thank Laura Perani, Rossana Tonlorenzi for suggestion support and patience they gave me during this period.

I thank Mario Molinaro and Sergio Adamo and all the member of the Phd Program to give me this important opportunity.

BIBLIOGRAPHY

Abramovitch, R., Dafni, H., Smouha, E., Benjamin, L.E., and Neeman, M. (1999). In vivo prediction of vascular susceptibility to vascular susceptibility endothelial growth factor withdrawal: magnetic resonance imaging of C6 rat glioma in nude mice. Cancer Res *59*, 5012-5016.

Amthor, H., Christ, B., Weil, M., and Patel, K. (1998). The importance of timing differentiation during limb muscle development. Curr Biol *8*, 642-652.

Andersson, E.R., Sandberg, R., and Lendahl, U. (2011). Notch signaling: simplicity in design, versatility in function. Development *138*, 3593-3612.

Armulik, A., Abramsson, A., and Betsholtz, C. (2005). Endothelial/pericyte interactions. Circ Res *97*, 512-523.

Armulik, A., Genove, G., and Betsholtz, C. (2011). Pericytes: developmental, physiological, and pathological perspectives, problems, and promises. Dev Cell *21*, 193-215.

Aviv, V., Meivar-Levy, I., Rachmut, I.H., Rubinek, T., Mor, E., and Ferber, S. (2009). Exendin-4 promotes liver cell proliferation and enhances the PDX-1-induced liver to pancreas transdifferentiation process. J Biol Chem *284*, 33509-33520.

Bajard, L., Relaix, F., Lagha, M., Rocancourt, D., Daubas, P., and Buckingham, M.E. (2006). A novel genetic hierarchy functions during hypaxial myogenesis: Pax3 directly activates Myf5 in muscle progenitor cells in the limb. Genes Dev *20*, 2450-2464.

Ballabh, P., Braun, A., and Nedergaard, M. (2004). The blood-brain barrier: an overview: structure, regulation, and clinical implications. Neurobiol Dis *16*, 1-13.

Barbieri, G., De Angelis, L., Feo, S., Cossu, G., and Giallongo, A. (1990). Differential expression of muscle-specific enolase in embryonic and fetal myogenic cells during mouse development. Differentiation *45*, 179-184.

Baylies, M.K., Bate, M., and Ruiz Gomez, M. (1998). Myogenesis: a view from Drosophila. Cell *93*, 921-927.

Beddington, R.S., and Robertson, E.J. (1999). Axis development and early asymmetry in mammals. Cell *96*, 195-209.

Ben-Yair, R., and Kalcheim, C. (2005). Lineage analysis of the avian dermomyotome sheet reveals the existence of single cells with both dermal and muscle progenitor fates. Development *132*, 689-701.

Ben-Yair, R., and Kalcheim, C. (2008). Notch and bone morphogenetic protein differentially act on dermomyotome cells to generate endothelium, smooth, and striated muscle. J Cell Biol *180*, 607-618.

Bendall, A.J., Ding, J., Hu, G., Shen, M.M., and Abate-Shen, C. (1999). Msx1 antagonizes the myogenic activity of Pax3 in migrating limb muscle precursors. Development *126*, 4965-4976.

Berger, M., Bergers, G., Arnold, B., Hammerling, G.J., and Ganss, R. (2005). Regulator of G-protein signaling-5 induction in pericytes coincides with active vessel remodeling during neovascularization. Blood *105*, 1094-1101.

Bergers, G., and Song, S. (2005). The role of pericytes in blood-vessel formation and maintenance. Neuro Oncol *7*, 452-464.

Betsholtz, C. (2004). Insight into the physiological functions of PDGF through genetic studies in mice. Cytokine Growth Factor Rev *15*, 215-228.

Betsholtz, C., Lindblom, P., Bjarnegard, M., Enge, M., Gerhardt, H., and Lindahl, P. (2004). Role of platelet-derived growth factor in mesangium development and vasculopathies: lessons from platelet-derived growth factor and platelet-derived growth factor receptor mutations in mice. Curr Opin Nephrol Hypertens *13*, 45-52.
Bianco, P., Robey, P.G., and Simmons, P.J. (2008). Mesenchymal stem cells: revisiting history, concepts, and assays. Cell Stem Cell *2*, 313-319.

Birchmeier, C., and Brohmann, H. (2000). Genes that control the development of migrating muscle precursor cells. Curr Opin Cell Biol *12*, 725-730.

Biressi, S., Molinaro, M., and Cossu, G. (2007a). Cellular heterogeneity during vertebrate skeletal muscle development. Dev Biol *308*, 281-293.

Biressi, S., Tagliafico, E., Lamorte, G., Monteverde, S., Tenedini, E., Roncaglia, E., Ferrari, S., Cusella-De Angelis, M.G., Tajbakhsh, S., and Cossu, G. (2007b). Intrinsic phenotypic diversity of embryonic and fetal myoblasts is revealed by genome-wide gene expression analysis on purified cells. Dev Biol *304*, 633-651.

Black, B.L., Molkentin, J.D., and Olson, E.N. (1998). Multiple roles for the MyoD basic region in transmission of transcriptional activation signals and interaction with MEF2. Mol Cell Biol *18*, 69-77.

Black, B.L., and Olson, E.N. (1998). Transcriptional control of muscle development by myocyte enhancer factor-2 (MEF2) proteins. Annu Rev Cell Dev Biol *14*, 167-196.

Blagden, C.S., Currie, P.D., Ingham, P.W., and Hughes, S.M. (1997). Notochord induction of zebrafish slow muscle mediated by Sonic hedgehog. Genes Dev *11*, 2163-2175.

Blanco-Bose, W.E., Yao, C.C., Kramer, R.H., and Blau, H.M. (2001). Purification of mouse primary myoblasts based on alpha 7 integrin expression. Exp Cell Res *265*, 212-220.

Bonfanti, P., Claudinot, S., Amici, A.W., Farley, A., Blackburn, C.C., and Barrandon, Y. (2010). Microenvironmental reprogramming of thymic epithelial cells to skin multipotent stem cells. Nature *466*, 978-982.

Bonner, P.H., and Hauschka, S.D. (1974). Clonal analysis of vertebrate myogenesis. I. Early developmental events in the chick limb. Dev Biol *37*, 317-328.

Brachvogel, B., Moch, H., Pausch, F., Schlotzer-Schrehardt, U., Hofmann, C., Hallmann, R., von der Mark, K., Winkler, T., and Poschl, E. (2005). Perivascular cells expressing annexin A5 define a novel mesenchymal stem cell-like population with the capacity to differentiate into multiple mesenchymal lineages. Development *132*, 2657-2668.

Braun, T., Bober, E., Buschhausen-Denker, G., Kohtz, S., Grzeschik, K.H., and Arnold, H.H. (1989a). Differential expression of myogenic determination genes in muscle cells: possible autoactivation by the Myf gene products. Embo J *8*, 3617-3625.

Braun, T., Rudnicki, M.A., Arnold, H.H., and Jaenisch, R. (1992). Targeted inactivation of the muscle regulatory gene Myf-5 results in abnormal rib development and perinatal death. Cell *71*, 369-382.

Braun, T., Tannich, E., Buschhausen-Denker, G., and Arnold, H.H. (1989b). Promoter upstream elements of the chicken cardiac myosin light-chain 2-A gene interact with trans-acting regulatory factors for muscle-specific transcription. Mol Cell Biol *9*, 2513-2525.

Brownell, I., Guevara, E., Bai, C.B., Loomis, C.A., and Joyner, A.L. (2011). Nerve-derived sonic hedgehog defines a niche for hair follicle stem cells capable of becoming epidermal stem cells. Cell Stem Cell *8*, 552-565.

Buckingham, M., and Relaix, F. (2007). The role of Pax genes in the development of tissues and organs: Pax3 and Pax7 regulate muscle progenitor cell functions. Annu Rev Cell Dev Biol *23*, 645-673.

Burke, A.C., Nelson, C.E., Morgan, B.A., and Tabin, C. (1995). Hox genes and the evolution of vertebrate axial morphology. Development *121*, 333-346.

Burkin, D.J., and Kaufman, S.J. (1999). The alpha7beta1 integrin in muscle development and disease. Cell Tissue Res *296*, 183-190.

Cachaco, A.S., Chuva de Sousa Lopes, S.M., Kuikman, I., Bajanca, F., Abe, K., Baudoin, C., Sonnenberg, A., Mummery, C.L., and Thorsteinsdottir, S. (2003). Knock-in of integrin beta 1D affects primary but not secondary myogenesis in mice. Development *130*, 1659-1671.

Caiazzo, M., Dell'Anno, M.T., Dvoretskova, E., Lazarevic, D., Taverna, S., Leo, D., Sotnikova, T.D., Menegon, A., Roncaglia, P., Colciago, G., *et al.* (2011). Direct generation of functional dopaminergic neurons from mouse and human fibroblasts. Nature *476*, 224-227.

Campbell, J.H., Efendy, J.L., Han, C.L., and Campbell, G.R. (2000). Blood vessels from bone marrow. Ann N Y Acad Sci *902*, 224-229.

Chalepakis, G., Tremblay, P., and Gruss, P. (1992). Pax genes, mutants and molecular function. J Cell Sci Suppl *16*, 61-67.

Chapman, G., Sparrow, D.B., Kremmer, E., and Dunwoodie, S.L. (2011). Notch inhibition by the ligand DELTA-LIKE 3 defines the mechanism of abnormal vertebral segmentation in spondylocostal dysostosis. Hum Mol Genet *20*, 905-916.
Chhibber, S.R., and Singh, I. (1970). Asymmetry in muscle weight and one-sided dominance in the human lower limbs. J Anat *106*, 553-556.

Christ, B., Jacob, H.J., and Jacob, M. (1977). Experimental analysis of the origin of the wing musculature in avian embryos. Anat Embryol (Berl) *150*, 171-186.

Conboy, I.M., and Rando, T.A. (2002). The regulation of Notch signaling controls satellite cell activation and cell fate determination in postnatal myogenesis. Dev Cell *3*, 397-409.

Cossu, G., and Biressi, S. (2005). Satellite cells, myoblasts and other occasional myogenic progenitors: possible origin, phenotypic features and role in muscle regeneration. Semin Cell Dev Biol *16*, 623-631.

Cossu, G., and Borello, U. (1999). Wnt signaling and the activation of myogenesis in mammals. Embo J *18*, 6867-6872.

Cossu, G., De Angelis, L., Borello, U., Berarducci, B., Buffa, V., Sonnino, C., Coletta, M., Vivarelli, E., Bouche, M., Lattanzi, L., *et al.* (2000). Determination, diversification and multipotency of mammalian myogenic cells. Int J Dev Biol *44*, 699-706.

Cossu G, E.F., Grassi F, Wanke E. (1987). Acetylcholine receptor channels are present in undifferentiated satellite cells but not in embryonic myoblasts in culture. Dev Biol *123*, 43-50.

Cossu, G., Kelly, R., Tajbakhsh, S., Di Donna, S., Vivarelli, E., and Buckingham, M. (1996). Activation of different myogenic pathways: myf-5 is induced by the neural tube and MyoD by the dorsal ectoderm in mouse paraxial mesoderm. Development *122*, 429-437.

Cossu, G., and Molinaro, M. (1987). Cell heterogeneity in the myogenic lineage. Curr Top Dev Biol *23*, 185-208.

D'Souza, B., Meloty-Kapella, L., and Weinmaster, G. (2010). Canonical and non-canonical Notch ligands. Curr Top Dev Biol *92*, 73-129.

Dahlqvist, C., Blokzijl, A., Chapman, G., Falk, A., Dannaeus, K., Ibanez, C.F., and Lendahl, U. (2003). Functional Notch signaling is required for BMP4-induced inhibition of myogenic differentiation. Development *130*, 6089-6099.

Darabi, R., Gehlbach, K., Bachoo, R.M., Kamath, S., Osawa, M., Kamm, K.E., Kyba, M., and Perlingeiro, R.C. (2008). Functional skeletal muscle regeneration from differentiating embryonic stem cells. Nat Med *14*, 134-143.

Dellavalle, A., Maroli, G., Covarello, D., Azzoni, E., Innocenzi, A., Perani, L., Antonini, S., Sambasivan, R., Brunelli, S., Tajbakhsh, S*., et al.* (2011). Pericytes resident in postnatal skeletal muscle differentiate into muscle fibres and generate satellite cells. Nat Commun *2*, 499.

Dellavalle, A., Sampaolesi, M., Tonlorenzi, R., Tagliafico, E., Sacchetti, B., Perani, L., Innocenzi, A., Galvez, B.G., Messina, G., Morosetti, R*., et al.* (2007). Pericytes of human skeletal muscle are myogenic precursors distinct from satellite cells. Nat Cell Biol *9*, 255-267.

Dermietzel, R., and Krause, D. (1991). Molecular anatomy of the blood-brain barrier as defined by immunocytochemistry. Int Rev Cytol *127*, 57-109.

Diaz-Flores, L., Gutierrez, R., Madrid, J.F., Varela, H., Valladares, F., Acosta, E., Martin-Vasallo, P., and Diaz-Flores, L., Jr. (2009). Pericytes. Morphofunction, interactions and pathology in a quiescent and activated mesenchymal cell niche. Histol Histopathol *24*, 909-969.

DiMario, J.X., and Stockdale, F.E. (1997). Both myoblast lineage and innervation determine fiber type and are required for expression of the slow myosin heavy chain 2 gene. Dev Biol *188*, 167-180.

Dunglison, G.F., Scotting, P.J., and Wigmore, P.M. (1999). Rat embryonic myoblasts are restricted to forming primary fibres while later myogenic populations are pluripotent. Mech Dev *87*, 11-19.

Dunwoodie, S.L., Henrique, D., Harrison, S.M., and Beddington, R.S. (1997). Mouse Dll3: a novel divergent Delta gene which may complement the function of other Delta homologues during early pattern formation in the mouse embryo. Development *124*, 3065-3076.

Ehehalt, F., Wang, B., Christ, B., Patel, K., and Huang, R. (2004). Intrinsic cartilage-forming potential of dermomyotomal cells requires ectodermal signals for the development of the scapula blade. Anat Embryol (Berl) *208*, 431-437.

Engler, A.J., Sen, S., Sweeney, H.L., and Discher, D.E. (2006). Matrix elasticity directs stem cell lineage specification. Cell *126*, 677-689.

Epstein, J.A., Shapiro, D.N., Cheng, J., Lam, P.Y., and Maas, R.L. (1996). Pax3 modulates expression of the c-Met receptor during limb muscle development. Proc Natl Acad Sci U S A *93*, 4213-4218.

Esner, M., Meilhac, S.M., Relaix, F., Nicolas, J.F., Cossu, G., and Buckingham, M.E. (2006). Smooth muscle of the dorsal aorta shares a common clonal origin with skeletal muscle of the myotome. Development *133*, 737-749.

Evans, D., Baillie, H., Caswell, A., and Wigmore, P. (1994). During fetal muscle development, clones of cells contribute to both primary and secondary fibers. Dev Biol *162*, 348-353.

Fazeli, S., Wells, D.J., Hobbs, C., and Walsh, F.S. (1996). Altered secondary myogenesis in transgenic animals expressing the neural cell adhesion molecule under the control of a skeletal muscle alpha-actin promoter. J Cell Biol *135*, 241-251.

Feo, S., Oliva, D., Barbieri, G., Xu, W.M., Fried, M., and Giallongo, A. (1990). The gene for the muscle-specific enolase is on the short arm of human chromosome 17. Genomics *6*, 192-194.

Ferrari, G., Cusella-De Angelis, G., Coletta, M., Paolucci, E., Stornaiuolo, A., Cossu, G., and Mavilio, F. (1998). Muscle regeneration by bone marrow-derived myogenic progenitors. Science *279*, 1528-1530.

Ferrari, G., and Mavilio, F. (2002). Myogenic stem cells from the bone marrow: a therapeutic alternative for muscular dystrophy? Neuromuscul Disord *12 Suppl 1*, S7-10.

Ferrari, S., Molinari, S., Melchionna, R., Cusella-De Angelis, M.G., Battini, R., De Angelis, L., Kelly, R., and Cossu, G. (1997). Absence of MEF2 binding to the A/T-rich element in the muscle creatine kinase (MCK) enhancer correlates with lack of early expression of the MCK gene in embryonic mammalian muscle. Cell Growth Differ *8*, 23-34.

Francis-West, P.H., Antoni, L., and Anakwe, K. (2003). Regulation of myogenic differentiation in the developing limb bud. J Anat *202*, 69-81.

Franz, T., Kothary, R., Surani, M.A., Halata, Z., and Grim, M. (1993). The Splotch mutation interferes with muscle development in the limbs. Anat Embryol (Berl) *187*, 153-160.

Geffers, I., Serth, K., Chapman, G., Jaekel, R., Schuster-Gossler, K., Cordes, R., Sparrow, D.B., Kremmer, E., Dunwoodie, S.L., Klein, T.*, et al.* (2007). Divergent functions and distinct localization of the Notch ligands DLL1 and DLL3 in vivo. J Cell Biol *178*, 465-476.

Golding, J.P., Partridge, T.A., Beauchamp, J.R., King, T., Brown, N.A., Gassmann, M., and Zammit, P.S. (2004). Mouse myotomes pairs exhibit left-right asymmetric expression of MLC3F and alpha-skeletal actin. Dev Dyn *231*, 795-800.

Goulding, M., and Paquette, A. (1994). Pax genes and neural tube defects in the mouse. Ciba Found Symp *181*, 103-113; discussion 113-107.

Grieshammer, U., Sassoon, D., and Rosenthal, N. (1992). A transgene target for positional regulators marks early rostrocaudal specification of myogenic lineages. Cell *69*, 79-93.

Grim, M., and Carlson, B.M. (1990). Alkaline phosphatase and dipeptidylpeptidase IV staining of tissue components of skeletal muscle: a comparative study. J Histochem Cytochem *38*, 1907-1912.

Groisman, R., Masutani, H., Leibovitch, M.P., Robin, P., Soudant, I., Trouche, D., and Harel-Bellan, A. (1996). Physical interaction between the mitogen-responsive serum response factor and myogenic basic-helix-loop-helix proteins. J Biol Chem *271*, 5258-5264.

Gross, M.K., Moran-Rivard, L., Velasquez, T., Nakatsu, M.N., Jagla, K., and Goulding, M. (2000). Lbx1 is required for muscle precursor migration along a lateral pathway into the limb. Development *127*, 413-424.

Gruss, P., and Walther, C. (1992). Pax in development. Cell *69*, 719-722.
Hadchouel, J., Carvajal, J.J., Daubas, P., Bajard, L., Chang, T., Rocancourt, D., Cox, D., Summerbell, D., Tajbakhsh, S., Rigby, P.W., *et al.* (2003). Analysis of a key regulatory region upstream of the Myf5 gene reveals multiple phases of myogenesis, orchestrated at each site by a combination of elements dispersed throughout the locus. Development *130*, 3415-3426.

Hanna, J.H., Saha, K., and Jaenisch, R. (2010). Pluripotency and cellular reprogramming: facts, hypotheses, unresolved issues. Cell *143*, 508-525.

Hartley RS, B.E., Yablonka-Reuveni Z. (1991). Myoblasts from fetal and adult skeletal muscle regulate myosin expression differently. Dev Biol *148*, 249-260.

Hegyi, L., Gannon, F.H., Glaser, D.L., Shore, E.M., Kaplan, F.S., and Shanahan, C.M. (2003). Stromal cells of fibrodysplasia ossificans progressiva lesions express smooth muscle lineage markers and the osteogenic transcription factor Runx2/Cbfa-1: clues to a vascular origin of heterotopic ossification? J Pathol *201*, 141-148.

Hellstrom, M., Kalen, M., Lindahl, P., Abramsson, A., and Betsholtz, C. (1999). Role of PDGF-B and PDGFR-beta in recruitment of vascular smooth muscle cells and pericytes during embryonic blood vessel formation in the mouse. Development *126*, 3047-3055.

Heymann, S., Koudrova, M., Arnold, H., Koster, M., and Braun, T. (1996). Regulation and function of SF/HGF during migration of limb muscle precursor cells in chicken. Dev Biol *180*, 566-578.

Hirschi, K.K., Rohovsky, S.A., and D'Amore, P.A. (1998). PDGF, TGF-beta, and heterotypic cell-cell interactions mediate endothelial cell-induced recruitment of 10T1/2 cells and their differentiation to a smooth muscle fate. J Cell Biol *141*, 805-814.

Hollway, G., and Currie, P. (2005). Vertebrate myotome development. Birth Defects Res C Embryo Today *75*, 172-179.

Huang, F.J., You, W.K., Bonaldo, P., Seyfried, T.N., Pasquale, E.B., and Stallcup, W.B. (2010). Pericyte deficiencies lead to aberrant tumor vascularizaton in the brain of the NG2 null mouse. Dev Biol *344*, 1035-1046.

Ieda, M., Fu, J.D., Delgado-Olguin, P., Vedantham, V., Hayashi, Y., Bruneau, B.G., and Srivastava, D. (2010). Direct reprogramming of fibroblasts into functional cardiomyocytes by defined factors. Cell *142*, 375-386.

Jacob, M., Christ, B., and Jacob, H.J. (1975). [Regional determination of the paraxial mesoderm in young chick embryos]. Verh Anat Ges *69*, 263-269.

Jostes, B., Walther, C., and Gruss, P. (1990). The murine paired box gene, Pax7, is expressed specifically during the development of the nervous and muscular system. Mech Dev *33*, 27-37.

Kablar, B., Krastel, K., Ying, C., Asakura, A., Tapscott, S.J., and Rudnicki, M.A. (1997). MyoD and Myf-5 differentially regulate the development of limb versus trunk skeletal muscle. Development *124*, 4729-4738.

Kalcheim, C., and Ben-Yair, R. (2005). Cell rearrangements during development of the somite and its derivatives. Curr Opin Genet Dev *15*, 371-380.

Kanisicak, O., Mendez, J.J., Yamamoto, S., Yamamoto, M., and Goldhamer, D.J. (2009). Progenitors of skeletal muscle satellite cells express the muscle determination gene, MyoD. Dev Biol *332*, 131-141.

Kassar-Duchossoy, L., Gayraud-Morel, B., Gomes, D., Rocancourt, D., Buckingham, M., Shinin, V., and Tajbakhsh, S. (2004). Mrf4 determines skeletal muscle identity in Myf5:Myod double-mutant mice. Nature *431*, 466-471.

Kegley, K.M., Gephart, J., Warren, G.L., and Pavlath, G.K. (2001). Altered primary myogenesis in NFATC3(-/-) mice leads to decreased muscle size in the adult. Dev Biol *232*, 115-126.

Kessel, M., and Gruss, P. (1991). Homeotic transformations of murine vertebrae and concomitant alteration of Hox codes induced by retinoic acid. Cell *67*, 89-104.

Kieny, M., Mauger, A., and Sengel, P. (1972). Early regionalization of somitic mesoderm as studied by the development of axial skeleton of the chick embryo. Dev Biol *28*, 142-161.

Koblar, S.A., Murphy, M., Barrett, G.L., Underhill, A., Gros, P., and Bartlett, P.F. (1999). Pax-3 regulates neurogenesis in neural crest-derived precursor cells. J Neurosci Res *56*, 518-530.

Krause, D.S., Theise, N.D., Collector, M.I., Henegariu, O., Hwang, S., Gardner, R., Neutzel, S., and Sharkis, S.J. (2001). Multi-organ, multi-lineage engraftment by a single bone marrow-derived stem cell. Cell *105*, 369-377.

Krueger, M., and Bechmann, I. (2010). CNS pericytes: concepts, misconceptions, and a way out. Glia *58*, 1-10.

Kunz, J., Krause, D., Kremer, M., and Dermietzel, R. (1994). The 140-kDa protein of blood-brain barrier-associated pericytes is identical to aminopeptidase N. J Neurochem *62*, 2375-2386.

Ladi, E., Nichols, J.T., Ge, W., Miyamoto, A., Yao, C., Yang, L.T., Boulter, J., Sun, Y.E., Kintner, C., and Weinmaster, G. (2005). The divergent DSL ligand Dll3 does not activate Notch signaling but cell autonomously attenuates signaling induced by other DSL ligands. J Cell Biol *170*, 983-992.

Lagasse, E., Connors, H., Al-Dhalimy, M., Reitsma, M., Dohse, M., Osborne, L., Wang, X., Finegold, M., Weissman, I.L., and Grompe, M. (2000). Purified hematopoietic stem cells can differentiate into hepatocytes in vivo. Nat Med *6*, 1229-1234.

Lagha, M., Brunelli, S., Messina, G., Cumano, A., Kume, T., Relaix, F., and Buckingham, M.E. (2009). Pax3:Foxc2 reciprocal repression in the somite modulates muscular versus vascular cell fate choice in multipotent progenitors. Dev Cell *17*, 892-899.

Lagha, M., Kormish, J.D., Rocancourt, D., Manceau, M., Epstein, J.A., Zaret, K.S., Relaix, F., and Buckingham, M.E. (2008). Pax3 regulation of FGF signaling affects the progression of embryonic progenitor cells into the myogenic program. Genes Dev *22*, 1828-1837.

Lagha, M., Rocancourt, D., and Relaix, F. (2005). [Pax3/Pax7-dependent population of skeletal muscle progenitor cells]. Med Sci (Paris) *21*, 801-803.

Lamalice, L., Le Boeuf, F., and Huot, J. (2007). Endothelial cell migration during angiogenesis. Circ Res *100*, 782-794.

Langlands, K., Yin, X., Anand, G., and Prochownik, E.V. (1997). Differential interactions of Id proteins with basic-helix-loop-helix transcription factors. J Biol Chem *272*, 19785-19793.

Levy, M.M., Joyner, C.J., Virdi, A.S., Reed, A., Triffitt, J.T., Simpson, A.H., Kenwright, J., Stein, H., and Francis, M.J. (2001). Osteoprogenitor cells of mature human skeletal muscle tissue: an in vitro study. Bone *29*, 317-322.

Lindahl, P., Johansson, B.R., Leveen, P., and Betsholtz, C. (1997). Pericyte loss and microaneurysm formation in PDGF-B-deficient mice. Science *277*, 242-245.

Lindner, H., Holler, E., Ertl, B., Multhoff, G., Schreglmann, M., Klauke, I., Schultz-Hector, S., and Eissner, G. (1997). Peripheral blood mononuclear cells induce programmed cell death in human endothelial cells and may prevent repair: role of cytokines. Blood *89*, 1931-1938.

Liu, H., Kennard, S., and Lilly, B. (2009). NOTCH3 expression is induced in mural cells through an autoregulatory loop that requires endothelial-expressed JAGGED1. Circ Res *104*, 466-475.

Lluis, F., Pedone, E., Pepe, S., and Cosma, M.P. (2008). Periodic activation of Wnt/beta-catenin signaling enhances somatic cell reprogramming mediated by cell fusion. Cell Stem Cell *3*, 493-507.

Mankoo, B.S., Collins, N.S., Ashby, P., Grigorieva, E., Pevny, L.H., Candia, A., Wright, C.V., Rigby, P.W., and Pachnis, V. (1999). Mox2 is a component of the genetic hierarchy controlling limb muscle development. Nature *400*, 69-73.

Maroto, M., Reshef, R., Munsterberg, A.E., Koester, S., Goulding, M., and Lassar, A.B. (1997). Ectopic Pax-3 activates MyoD and Myf-5 expression in embryonic mesoderm and neural tissue. Cell *89*, 139-148.

Martin, D., Tucker, D.F., Gorman, P., Sheer, D., Spurr, N.K., and Trowsdale, J. (1987). The human placental alkaline phosphatase gene and related sequences map to chromosome 2 band q37. Ann Hum Genet *51*, 145-152.

McGrew, M.J., and Pourquie, O. (1998). Somitogenesis: segmenting a vertebrate. Curr Opin Genet Dev *8*, 487-493.

Miller, F.N., and Sims, D.E. (1986). Contractile elements in the regulation of macromolecular permeability. Fed Proc *45*, 84-88.

Miller, J.B., Schaefer, L., and Dominov, J.A. (1999). Seeking muscle stem cells. Curr Top Dev Biol *43*, 191-219.

Minasi, M.G., Riminucci, M., De Angelis, L., Borello, U., Berarducci, B., Innocenzi, A., Caprioli, A., Sirabella, D., Baiocchi, M., De Maria, R.*, et al.* (2002). The meso-angioblast: a multipotent, self-renewing cell that originates from the dorsal aorta and differentiates into most mesodermal tissues. Development *129*, 2773-2783.

Mintz, B., and Baker, W.W. (1967). Normal mammalian muscle differentiation and gene control of isocitrate dehydrogenase synthesis. Proc Natl Acad Sci U S A *58*, 592-598.

Mizutani, A., and Barrnett, R.J. (1965). Fine structural demonstration of phosphatase activity at pH 9. Nature *206*, 1001-1003.

Mulnard J, H.R. (1987). Ultrastructural localization of non-specific alkaline phosphatase during cleavage and blastocyst formation in the mouse. J Embryo exp Morph *44*, 121-131.

Mulnard, J., and Puissant, F. (1987). [The value of centrifugation for the study of cytoplasmic changes related to the maturation and fertilization of mammalian oocytes]. Bull Mem Acad R Med Belg *142*, 507-516.

Murakami, G., and Nakamura, H. (1991). Somites and the pattern formation of trunk muscles: a study in quail-chick chimera. Arch Histol Cytol *54*, 249-258.

Murtaugh, L.C., and Melton, D.A. (2003). Genes, signals, and lineages in pancreas development. Annu Rev Cell Dev Biol *19*, 71-89.

Nakajima, N., Nagahiro, S., Sano, T., Satomi, J., and Satoh, K. (2000). Phenotypic modulation of smooth muscle cells in human cerebral aneurysmal walls. Acta Neuropathol *100*, 475-480.

Nehls, V., Denzer, K., and Drenckhahn, D. (1992). Pericyte involvement in capillary sprouting during angiogenesis in situ. Cell Tissue Res *270*, 469-474.

Nehls, V., and Drenckhahn, D. (1993). The versatility of microvascular pericytes: from mesenchyme to smooth muscle? Histochemistry *99*, 1-12.

Neyt, C., Jagla, K., Thisse, C., Thisse, B., Haines, L., and Currie, P.D. (2000). Evolutionary origins of vertebrate appendicular muscle. Nature *408*, 82-86.

Nofziger, D., Miyamoto, A., Lyons, K.M., and Weinmaster, G. (1999). Notch signaling imposes two distinct blocks in the differentiation of C2C12 myoblasts. Development *126*, 1689-1702.

Orlic, D., Kajstura, J., Chimenti, S., Jakoniuk, I., Anderson, S.M., Li, B., Pickel, J., McKay, R., Nadal-Ginard, B., Bodine, D.M., *et al.* (2001). Bone marrow cells regenerate infarcted myocardium. Nature *410*, 701-705.

Orlidge, A., and D'Amore, P.A. (1987). Inhibition of capillary endothelial cell growth by pericytes and smooth muscle cells. J Cell Biol *105*, 1455-1462.

Ott, M.O., Bober, E., Lyons, G., Arnold, H., and Buckingham, M. (1991). Early expression of the myogenic regulatory gene, myf-5, in precursor cells of skeletal muscle in the mouse embryo. Development *111*, 1097-1107.

Ozerdem, U., Grako, K.A., Dahlin-Huppe, K., Monosov, E., and Stallcup, W.B. (2001). NG2 proteoglycan is expressed exclusively by mural cells during vascular morphogenesis. Dev Dyn *222*, 218-227.

Ozerdem, U., and Stallcup, W.B. (2004). Pathological angiogenesis is reduced by targeting pericytes via the NG2 proteoglycan. Angiogenesis *7*, 269-276.

Patapoutian, A., Wold, B.J., and Wagner, R.A. (1995). Evidence for developmentally programmed transdifferentiation in mouse esophageal muscle. Science *270*, 1818-1821.

Pin, C.L., and Merrifield, P.A. (1993). Embryonic and fetal rat myoblasts express different phenotypes following differentiation in vitro. Dev Genet *14*, 356-368.

Power, C., and Rasko, J.E. (2011). Promises and challenges of stem cell research for regenerative medicine. Ann Intern Med *155*, 706-713.

Pownall, M.E., Gustafsson, M.K., and Emerson, C.P., Jr. (2002). Myogenic regulatory factors and the specification of muscle progenitors in vertebrate embryos. Annu Rev Cell Dev Biol *18*, 747-783.

Rajantie, I., Ilmonen, M., Alminaite, A., Ozerdem, U., Alitalo, K., and Salven, P. (2004). Adult bone marrow-derived cells recruited during angiogenesis comprise precursors for periendothelial vascular mural cells. Blood *104*, 2084-2086.

Register, T.C., and Wuthier, R.E. (1984). Effect of vanadate, a potent alkaline phosphatase inhibitor, on 45Ca and 32Pi uptake by matrix vesicle-enriched fractions from chicken epiphyseal cartilage. J Biol Chem *259*, 3511-3518.

Relaix, F., and Buckingham, M. (1999). From insect eye to vertebrate muscle: redeployment of a regulatory network. Genes Dev *13*, 3171-3178.
Relaix, F., Montarras, D., Zaffran, S., Gayraud-Morel, B., Rocancourt, D., Tajbakhsh, S., Mansouri, A., Cumano, A., and Buckingham, M. (2006). Pax3 and Pax7 have distinct and overlapping functions in adult muscle progenitor cells. J Cell Biol *172*, 91-102.

Relaix, F., Rocancourt, D., Mansouri, A., and Buckingham, M. (2004). Divergent functions of murine Pax3 and Pax7 in limb muscle development. Genes Dev *18*, 1088-1105.

Relaix, F., Rocancourt, D., Mansouri, A., and Buckingham, M. (2005). A Pax3/Pax7-dependent population of skeletal muscle progenitor cells. Nature *435*, 948-953.

Reynaud-Deonauth, S., Zhang, H., Afouda, A., Taillefert, S., Beatus, P., Kloc, M., Etkin, L.D., Fischer-Lougheed, J., and Spohr, G. (2002). Notch signaling is involved in the regulation of Id3 gene transcription during Xenopus embryogenesis. Differentiation *69*, 198-208.

Rishniw, M., Xin, H.B., Deng, K.Y., and Kotlikoff, M.I. (2003). Skeletal myogenesis in the mouse esophagus does not occur through transdifferentiation. Genesis *36*, 81-82.

Ronnov-Jessen, L., and Petersen, O.W. (1996). ADP-ribosylation of actins in fibroblasts and myofibroblasts by botulinum C2 toxin: influence on microfilament morphology and migratory behavior. Electrophoresis *17*, 1776-1780.

Rucker, H.K., Wynder, H.J., and Thomas, W.E. (2000). Cellular mechanisms of CNS pericytes. Brain Res Bull *51*, 363-369.

Rudnicki, M.A., Braun, T., Hinuma, S., and Jaenisch, R. (1992). Inactivation of MyoD in mice leads to up-regulation of the myogenic HLH gene Myf-5 and results in apparently normal muscle development. Cell *71*, 383-390.

Rudnicki, M.A., Le Grand, F., McKinnell, I., and Kuang, S. (2008). The molecular regulation of muscle stem cell function. Cold Spring Harb Symp Quant Biol *73*, 323-331.

Rudnicki, M.A., Schnegelsberg, P.N., Stead, R.H., Braun, T., Arnold, H.H., and Jaenisch, R. (1993). MyoD or Myf-5 is required for the formation of skeletal muscle. Cell *75*, 1351-1359.

Ruiter, D.J., Schlingemann, R.O., Westphal, J.R., Denijn, M., Rietveld, F.J., and De Waal, R.M. (1993). Angiogenesis in wound healing and tumor metastasis. Behring Inst Mitt, 258-272.

Sachs, M., Brohmann, H., Zechner, D., Muller, T., Hulsken, J., Walther, I., Schaeper, U., Birchmeier, C., and Birchmeier, W. (2000). Essential role of Gab1 for signaling by the c-Met receptor in vivo. J Cell Biol *150*, 1375-1384.

Safadi, A., Livne, E., Silbermann, M., and Reznick, A.Z. (1991). Activity of alkaline phosphatase in rat skeletal muscle localized along the sarcolemma and endothelial cell membranes. J Histochem Cytochem *39*, 199-203.

Sanges, D., Lluis, F., and Cosma, M.P. (2011). Cell-fusion-mediated reprogramming: pluripotency or transdifferentiation? Implications for regenerative medicine. Adv Exp Med Biol *713*, 137-159.

Sartore, S., Chiavegato, A., Faggin, E., Franch, R., Puato, M., Ausoni, S., and Pauletto, P. (2001). Contribution of adventitial fibroblasts to neointima formation and vascular remodeling: from innocent bystander to active participant. Circ Res *89*, 1111-1121.

Sato, T.N., Tozawa, Y., Deutsch, U., Wolburg-Buchholz, K., Fujiwara, Y., Gendron-Maguire, M., Gridley, T., Wolburg, H., Risau, W., and Qin, Y. (1995). Distinct roles of the receptor tyrosine kinases Tie-1 and Tie-2 in blood vessel formation. Nature *376*, 70-74.

Sato, Y., Abe, M., and Takaki, R. (1990). Platelet factor 4 blocks the binding of basic fibroblast growth factor to the receptor and inhibits the spontaneous migration of vascular endothelial cells. Biochem Biophys Res Commun *172*, 595-600.

Schienda, J., Engleka, K.A., Jun, S., Hansen, M.S., Epstein, J.A., Tabin, C.J., Kunkel, L.M., and Kardon, G. (2006). Somitic origin of limb muscle satellite and side population cells. Proc Natl Acad Sci U S A *103*, 945-950.

Schultz-Hector, S. (1993). Experimental studies on the pathogenesis of damage in the heart. Recent Results Cancer Res *130*, 145-156.

Schultz-Hector, S., Balz, K., Bohm, M., Ikehara, Y., and Rieke, L. (1993). Cellular localization of endothelial alkaline phosphatase reaction product and enzyme protein in the myocardium. J Histochem Cytochem *41*, 1813-1821.

Seale, P., Sabourin, L.A., Girgis-Gabardo, A., Mansouri, A., Gruss, P., and Rudnicki, M.A. (2000). Pax7 is required for the specification of myogenic satellite cells. Cell *102*, 777-786.

Senni, M.I., Castrignano, F., Poiana, G., Cossu, G., Scarsella, G., and Biagioni, S. (1987). Expression of adult fast pattern of acetylcholinesterase molecular forms by mouse satellite cells in culture. Differentiation *36*, 194-198.

Shutter, J.R., Scully, S., Fan, W., Richards, W.G., Kitajewski, J., Deblandre, G.A., Kintner, C.R., and Stark, K.L. (2000). Dll4, a novel Notch ligand expressed in arterial endothelium. Genes Dev *14*, 1313-1318.

Smith, M., Weiss, M.J., Griffin, C.A., Murray, J.C., Buetow, K.H., Emanuel, B.S., Henthorn, P.S., and Harris, H. (1988). Regional assignment of the gene for human liver/bone/kidney alkaline phosphatase to chromosome 1p36.1-p34. Genomics *2*, 139-143.

Speer, M.Y., Yang, H.Y., Brabb, T., Leaf, E., Look, A., Lin, W.L., Frutkin, A., Dichek, D., and Giachelli, C.M. (2009). Smooth muscle cells give rise to osteochondrogenic precursors and chondrocytes in calcifying arteries. Circ Res *104*, 733-741.

Srinivas, S., Watanabe, T., Lin, C.S., William, C.M., Tanabe, Y., Jessell, T.M., and Costantini, F. (2001). Cre reporter strains produced by targeted insertion of EYFP and ECFP into the ROSA26 locus. BMC Dev Biol *1*, 4.

Sun, Z., Martinez-Lemus, L.A., Trache, A., Trzeciakowski, J.P., Davis, G.E., Pohl, U., and Meininger, G.A. (2005). Mechanical properties of the interaction between fibronectin and alpha5beta1-integrin on vascular smooth muscle cells studied using atomic force microscopy. Am J Physiol Heart Circ Physiol *289*, H2526-2535.

Suri, C., Jones, P.F., Patan, S., Bartunkova, S., Maisonpierre, P.C., Davis, S., Sato, T.N., and Yancopoulos, G.D. (1996). Requisite role of angiopoietin-1, a ligand for the TIE2 receptor, during embryonic angiogenesis. Cell *87*, 1171-1180.

Szabo, E., Rampalli, S., Risueno, R.M., Schnerch, A., Mitchell, R., Fiebig-Comyn, A., Levadoux-Martin, M., and Bhatia, M. (2010). Direct conversion of human fibroblasts to multilineage blood progenitors. Nature *468*, 521-526.

Tajbakhsh, S., Bober, E., Babinet, C., Pournin, S., Arnold, H., and Buckingham, M. (1996). Gene targeting the myf-5 locus with nlacZ reveals expression of this myogenic factor in mature skeletal muscle fibres as well as early embryonic muscle. Dev Dyn *206*, 291-300.

Tajbakhsh S, R.D., Cossu G, Buckingham M. (1997). Redefining the genetic hierarchies controlling skeletal myogenesis: Pax-3 and Myf-5 act upstream of MyoD. Cell *89*, 127-138.

Tajbakhsh, S., Rocancourt, D., Cossu, G., and Buckingham, M. (1997). Redefining the genetic hierarchies controlling skeletal myogenesis: Pax-3 and Myf-5 act upstream of MyoD. Cell *89*, 127-138.

Takahashi, K., and Yamanaka, S. (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell *126*, 663-676.

Teboul, L., Hadchouel, J., Daubas, P., Summerbell, D., Buckingham, M., and Rigby, P.W. (2002). The early epaxial enhancer is essential for the initial expression of the skeletal muscle determination gene Myf5 but not for subsequent, multiple phases of somitic myogenesis. Development *129*, 4571-4580.

Terada, N., Hamazaki, T., Oka, M., Hoki, M., Mastalerz, D.M., Nakano, Y., Meyer, E.M., Morel, L., Petersen, B.E., and Scott, E.W. (2002). Bone marrow cells adopt the phenotype of other cells by spontaneous cell fusion. Nature *416*, 542-545.

Tomasek, J.J., Gabbiani, G., Hinz, B., Chaponnier, C., and Brown, R.A. (2002). Myofibroblasts and mechano-regulation of connective tissue remodelling. Nat Rev Mol Cell Biol *3*, 349-363.

Valdez, M.R., Richardson, J.A., Klein, W.H., and Olson, E.N. (2000). Failure of Myf5 to support myogenic differentiation without myogenin, MyoD, and MRF4. Dev Biol *219*, 287-298.

Venters, S.J., Thorsteinsdottir, S., and Duxson, M.J. (1999). Early development of the myotome in the mouse. Dev Dyn *216*, 219-232.

Vieira, A.R., Castillo Taucher, S., Aravena, T., Astete, C., Sanz, P., Tastets, M.E., Monasterio, L., and Murray, J.C. (2004). [Mutational analysis of the muscle segment homeobox gene 1 (MSX1) in Chilean patients with cleft lip/palate]. Rev Med Chil *132*, 816-822.

Vierbuchen, T., Ostermeier, A., Pang, Z.P., Kokubu, Y., Sudhof, T.C., and Wernig, M. (2010). Direct conversion of fibroblasts to functional neurons by defined factors. Nature *463*, 1035-1041.

von Tell, D., Armulik, A., and Betsholtz, C. (2006). Pericytes and vascular stability. Exp Cell Res *312*, 623-629.

Wagers, A.J., Christensen, J.L., and Weissman, I.L. (2002). Cell fate determination from stem cells. Gene Ther *9*, 606-612.

Weimann, J.M., Johansson, C.B., Trejo, A., and Blau, H.M. (2003). Stable reprogrammed heterokaryons form spontaneously in Purkinje neurons after bone marrow transplant. Nat Cell Biol *5*, 959-966.

Weintraub, H., Dwarki, V.J., Verma, I., Davis, R., Hollenberg, S., Snider, L., Lassar, A., and Tapscott, S.J. (1991). Muscle-specific transcriptional activation by MyoD. Genes Dev *5*, 1377-1386.

White NK, B.P., Nelson DR, Hauschka SD. (1975). Clonal analysis of vertebrate myogenesis. IV. Medium-dependent classification of colony-forming cells. Dev Biol *44*, 346-361.

Wigmore, P.M., and Evans, D.J. (2002). Molecular and cellular mechanisms involved in the generation of fiber diversity during myogenesis. Int Rev Cytol *216*, 175-232.

Williams, B.A., and Ordahl, C.P. (1994). Pax-3 expression in segmental mesoderm marks early stages in myogenic cell specification. Development *120*, 785-796.

Wilmut, I., Sullivan, G., and Chambers, I. (2011). The evolving biology of cell reprogramming. Philos Trans R Soc Lond B Biol Sci *366*, 2183-2197.

Wilson-Rawls, J., Molkentin, J.D., Black, B.L., and Olson, E.N. (1999). Activated notch inhibits myogenic activity of the MADS-Box transcription factor myocyte enhancer factor 2C. Mol Cell Biol *19*, 2853-2862.

Winkler, E.A., Bell, R.D., and Zlokovic, B.V. (2010). Pericyte-specific expression of PDGF beta receptor in mouse models with normal and deficient PDGF beta receptor signaling. Mol Neurodegener *5*, 32.

Yablonka-Reuveni, Z., and Seifert, R.A. (1993). Proliferation of chicken myoblasts is regulated by specific isoforms of platelet-derived growth factor: evidence for differences between myoblasts from mid and late stages of embryogenesis. Dev Biol *156*, 307-318.

Yamanaka, S., and Blau, H.M. (2010). Nuclear reprogramming to a pluripotent state by three approaches. Nature *465*, 704-712.

Ying, Q.L., Nichols, J., Evans, E.P., and Smith, A.G. (2002). Changing potency by spontaneous fusion. Nature *416*, 545-548.

Zammit, P., and Beauchamp, J. (2001). The skeletal muscle satellite cell: stem cell or son of stem cell? Differentiation *68*, 193-204.

Zappelli, F., Willems, D., Osada, S., Ohno, S., Wetsel, W.C., Molinaro, M., Cossu, G., and Bouche, M. (1996). The inhibition of differentiation caused by TGFbeta in fetal myoblasts is dependent upon selective expression of PKCtheta: a possible

molecular basis for myoblast diversification during limb histogenesis. Dev Biol *180*, 156-164.

Zhang, M., and McLennan, I.S. (1995). During secondary myotube formation, primary myotubes preferentially absorb new nuclei at their ends. Dev Dyn *204*, 168-177.