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CHAPTER 4

# NON MUSCLE STEM CELLS AND MUSCLE REGENERATION

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**Abstract:** Skeletal muscle of the vertebrate embryo originates from paraxial mesoderm (somites, somitomers and prechordal cephalic mesoderm) (Christ and Ordahl, 1995) and is formed in discrete steps by different classes of myogenic progenitor cells (Cossu and Biressi, 2005). After myotome formation, embryonic myoblasts give rise to primary fibers in the embryo, while fetal myoblasts give rise to secondary fibers, initially smaller and surrounding primary fibers. Satellite cells appear underneath the newly formed basal lamina that develops around each muscle fiber, and contribute to their post-natal growth and regeneration (Bischoff, 1994). In addition to canonical progenitors, evidence accumulated through the years that cells cultured from tissues that do not derive from paraxial mesoderm and do not contain skeletal muscle such as thymus, brain or kidney may differentiate at low frequency into skeletal muscle. Initially dismissed as a tissue culture artifact, the phenomenon came under closer scrutiny when it was unequivocally demonstrated that the bone marrow of adult normal mice contain cells capable of contributing to skeletal muscle regeneration in vivo (Ferrari et al., 1998). In the following years, different types of non-somitic stem-progenitor cells have been shown to contribute to muscle regeneration. The origin of these different cell types and their possible lineage relationships with other myogenic cells as well as their possible role in muscle regeneration is actively studied in these years and will be the subject of this chapter. Finally, the possible use of different non-canonic myogenic cells in experimental protocols of cell therapy will be briefly outlined.

**Keywords:** Skeletal myogenesis; muscle satellite cells; skeletal myoblasts; mesoangioblasts; muscle regeneration.



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**Abbreviations:** BMP2: Bone morphogenetic protein 2; GFP: green fluorescent protein; HSC: hematopoietic stem cell; MSC: mesoderm stem cell (referred to as non hematopoietic); PKC: protein kinase C; Shh: Sonic hedgehog; SP: side population; TGF β: transforming growth factor β.

## 1. A BRIEF HISTORY OF UNORTHODOX MYOGENESIS AND OF ITS POSSIBLE SIGNIFICANCE IN REGENERATION

Myogenic progenitor cells, termed myoblasts, have been isolated and cultured since the early 60' of the last century. Originally isolated from the muscle anlagen of avian embryos, myoblasts were later cultured from muscles of virtually all vertebrates, both embryonic and adult. Removal or consumption of growth factors (often provided as serum or embryo extracts) induces irreversible withdrawal from the cell cycle and terminal differentiation of myoblasts that fuse into multinucleated myotubes. During further maturation, which occurs only partially in vitro, myotubes complete sarcomerogenesis, assemble a functional excitation-contraction coupling system and contract in response to appropriate stimuli (Okazaki and Holtzer, 1966).

Because they are easily recognized morphologically in living cultures, myotubes were occasionally observed in cultures of cells that were not myogenic nor derived from tissues that in vivo contain skeletal muscle. These observations remained anecdotic and largely unpublished, also because they lacked a rational explanation. "Contamination with myogenic cells during isolation" or "tissue culture artifact" represented the easiest interpretations of these data (Cossu, 1997).

Nevertheless papers accumulated through the years, some of which reporting solid and unquestionable data. Perhaps the most striking example is represented by the thymus that is derived from pharyngeal pouches and does not contain any skeletal muscle fiber. In 1975, it was reported the occurrence of striated muscle fiber differentiation in monolayer cultures of adult thymus reticulum (Wekerle et al., 1975). Later it was reported that in the thymus from adult but not neonatal mice, MyoD or myogenin-positive cells are concentrated in the medullary region but do not differentiate within the normal murine thymic environment. However, myogenesis takes place both in vitro, as demonstrated in the original paper, and in vivo, upon transplantation into regenerating muscle (Grounds et al., 1992).

Another example is represented by the so called "myogenic conversion of fibroblasts" originating from dermis and, to different extent, other mesoderm tissues. The first example of this phenomenon was the correction by fibroblast-myoblast fusion of the genetic defect of the *mdg* mouse mutant muscle fibers (Chaudary et al., 1989; Courbin et al., 1989). Subsequently, several groups reported that genetically labeled dermal fibroblasts could be incorporated into differentiated myotubes both in vitro and in vivo (Gibson et al., 1995; Breton et al., 1995; Salvatori

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et al., 1995). These studies showed evidence for fusion of fibroblasts with myogenic cells. In these cases myogenesis could be activated as it occurs in heterokaryons where the fibroblast nucleus is exposed to muscle transcription factors. Interestingly however, this myogenic potency is not restricted to dermis, but is present in virtually all organs containing a significant mesoderm component, such as smooth and skeletal muscle or kidney and also the central nervous system. At variance with cells from the thymus, these other cells require signals from differentiating myogenic cells, possibly related to a "community effect" (Gurdon et al., 1993; Cossu et al., 1995) present during skeletal muscle histogenesis and possibly regeneration. Moreover, normal murine dermal fibroblasts implanted into the muscles of the mdx mouse participate in new myofiber formation and direct the expression of the protein dystrophin, deficient in these mice (Gibson et al., 1995). Interestingly, the lectin galectin-1, expressed and secreted by the myoblasts, induces the conversion of dermal but not of muscle fibroblasts to skeletal muscle (Goldring et al., 2002).

Two additional examples are represented by in vitro myogenic differentiation of neuro-ectoderm cells from the developing central nervous system and by BHK (Baby Hamster Kidney) cells.

Spontaneous myogenic differentiation of cells from the brain was documented a number of times (examples quoted in Tajbakhsh et al., 1994) but it was only through insertion of the reporter gene *LacZ* into the *myf-5* locus that it was possible to unequivocally identify Myf-5 expressing cells in the neural tube and to show that these cells co-express neural and muscle markers (Tajbakhsh et al., 1994). Once explanted in cultures, some of *myf-5* expressing cells will differentiate into skeletal myocytes, thus suggesting escape from a community-induced inhibition. A similar situation was observed in a specific areas of the brain of the same mice: Myf-5 expression begins to be detected at embryonic day 8 (E8) in the mesencephalon and coincides with the appearance of the first differentiated neurons; expression in the secondary prosencephalon initiates at E10 and is confined to the ventral domain of prosomere p4, later becoming restricted to the posterior hypothalamus (Tajbakhsh and Buckingham, 1995).

BHK cells are derived from proteolitic digestion of newborn kidney and have been widely used as fibroblasts. More careful analysis revealed that these cells express MyoD and myogenin and can be induced to differentiate into skeletal muscle cells (Mayer and Leinwand, 1997).

All these cases of unorthodox myogenesis are conceptually distinct from trans-differentiation, a phenomenon by which an already differentiated cell can be induced the change the repertoire of gene expressed and to express genes typical of a different tissues. In higher vertebrates, this situation is mainly related to pathology (metaplasia), although trans-differentiation from smooth to skeletal muscle has been demonstrated at the single cell level in the post-natal mammalian esophagus (Patapoutian et al., 1995). Trans-differentiation is not discussed in this chapter.

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## 2. A CURRENT CLASSIFICATION OF NON MUSCLE STEM CELLS POSSIBLY INVOLVED IN MUSCLE REGENERATION

### 2.1 Non Muscle Stem Cells From the Ectoderm and Endoderm

#### *2.1.1 Neural stem cells as a source of myogenic cells*

To date, neural stem cells are the only ectoderm-derived stem cells that have been shown to differentiate into skeletal muscle when co-cultured with skeletal myoblasts (Galli et al., 2000). Both acutely isolated cells and clonally expanded neurospheres of both murine and human origin could be induced to undergo myogenesis in vitro and in vivo, upon injection into regenerating muscle. Interestingly direct contact was shown to be required between myogenic cells and neural stem cells, as only the cells at the border of the neurosphere could be converted to myogenesis. Although the possible practical exploitation of these results is not immediate, they nevertheless represent unequivocal evidence of myogenesis arising from cells of a germ layer different from mesoderm. No evidence of skeletal muscle differentiation has so far been reported for stem cells from ectoderm or endoderm derived epithelia, suggesting that, if attempts have been made, they have been unsuccessful.

### 2.2 Non Muscle Stem Cells from the Hematopoietic System

#### *2.2.1 Total bone marrow as a source of myogenic cells*

The first evidence of in vivo development of skeletal muscle from cells of the hematopoietic system was reported in 1998, thanks to the use of a transgenic mouse expressing a nuclear *lacZ* under the control of muscle-specific regulatory elements (MLC3F-n*lacZ*) only in striated muscle (Kelly et al., 1995). Bone marrow-derived cells from these mice were transplanted into lethally-irradiated mice and, when reconstitution by donor bone marrow had occurred, muscle regeneration was induced by cardiotoxin injection into a leg muscle (*tibialis anterior*). Histochemical analysis unequivocally showed the presence of ß-gal positive nuclei at the center of regenerated fibers, demonstrating for the first time that murine bone marrow contains transplantable progenitors that can be recruited to an injured muscle through the peripheral circulation, and participate to muscle repair by undergoing differentiation into mature muscle fibers (Ferrari et al., 1998). The publication of this report raised new interest in myogenic progenitors and in their possible clinical use. It was reasoned that, although the frequency of the phenomenon was very low, in a chronically regenerating, dystrophic muscle myogenic progenitors would have found a favorable environment and consequently would have contributed significantly to regeneration of dystrophin positive, normal fibers.

#### *2.2.2 SP cells as a source of myogenic cells*

This, however, turned out not to be the case. In the following year the groups of Kunkel and Mulligan showed that *mdx* mice transplanted with the bone marrow side population, or SP (a fraction of total cells that is separated by die exclusion and

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contains stem/progenitor cells able to repopulate the hematopoietic system upon transplantation (Goodell et al., 2005)) from syngeneic C57BL/10 mice develop, within several weeks, a small number of dystrophin-positive fibers containing genetically marked (Y chromosome) donor nuclei (Gussoni et al., 1999) Even after many months from the transplantation, the number of fibers carrying both dystrophin and the Y chromosome never exceeded 1% of the total fibers in the average muscle, thus precluding a direct clinical translation for this protocol. Similar results were later obtained in a slightly different animal model, the *mdx4cv* mutant (Ferrari et al., 2001) Together these data indicate that myogenic differentiation from bone marrow occurs but a frequency discouraging low in order to predict possible clinical benefit.

Following these initial observations, experiments were conducted to identify the cell type within the heterogeneous bone marrow cells which may give rise to skeletal muscle upon transplantation. When bone marrow was fractionated into CD45 positive and negative fractions, the muscle forming activity was associated with the CD45+ fraction (McKinney-Freeman et al., 2002); retrospective analysis in a Duchenne patient that had undergone bone marrow transplantation confirmed persistence of donor derived skeletal muscle cells over a periods of many years, again at very low frequency (Gussoni et al., 2002). Together these data suggested that a myogenic potential is present in the hematopoietic stem cell itself or in a yet to be identified cell that expresses several markers in common with true HSC. More recent and sophisticated approaches confirmed these first observations but disagreed on the underlying mechanism: it was reported that the progeny of a single cell can both reconstitute the hematopoietic system and contribute to muscle regeneration (Corbel et al., 2003). Integration of bone marrow cells into myofibers was shown to occur spontaneously at low frequency and to increase with muscle damage. It was concluded that classically defined single hematopoietic stem cells can give rise to both blood and muscle. A similar study showed that, although myogenic activity in bone marrow is derived from HSCs and their hematopoietic progeny, contribution to regenerating skeletal muscle does not occur through a myogenic stem cell intermediate. Evidence was presented through a lineage tracing strategy, that myofibers were derived from fusion of mature myeloid cells in response to injury (Camargo et al., 2003).

SP cells are not exclusively present in bone marrow, but rather can be isolated from most tissues (for a review see Challen et al., 2006). It became thus obvious to search for myogenic potency of SP derived form skeletal muscle itself (Asakura et al., 2002). Indeed it was shown that freshly isolated progenitors contained within the adult skeletal muscle side population (SP) can engraft into muscle fibers of dystrophic mice after intravenous or intra-arterial transplantation (Bachrach et al., 2004 and 2006). Engraftment rate was however quite low, ranging from 1% of skeletal muscle fibers expressing donor-derived gene products for intra-venous to 8% for intra-arterial delivery.

### *2.2.3 AC133 cells a source of myogenic cells*

As another example of non-muscle stem cells arising from the hematopoietic system, a subpopulation of circulating cells expressing AC133, a well-characterized marker

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of hematopoietic stem cells, also expresses early myogenic markers (Torrente et al., 2004). It was shown that freshly isolated, circulating AC133+ cells are able to undergo myogenesis when cocultured with myogenic cells or when transplanted in vivo into the muscles of transgenic *scid/mdx* mice (which allow survival of human cells). Injected cells also localized under the basal lamina of host muscle fibers and expressed satellite cell markers such as M-cadherin and Myf5. Furthermore, functional tests of injected muscles revealed a substantial recovery of force after treatment. As these cells can be isolated from the blood, manipulated in vitro, and delivered through the circulation, they represent a possible tool for future cell therapy applications in DMD disease or other muscular dystrophies: current limit of this approach is related to the difficulty of expanding in vitro this rare cell population to numbers that would be suitable to treat systemically a pediatric patient.

## 2.3 Non Muscle Stem Cells from Solid Mesoderm

### *2.3.1 Mesenchymal stem cells*

Mesenchymal stem cells, mainly originate from perycytes, are located in the perivascular district of the bone marrow stroma and are the natural precursors of bone, cartilage and fat, the constituent tissues of the bone (Bianco and Gehron Robey, 2000). Although MSC were reported to give rise to myotubes in culture upon induction with 5-azacytidine (Watanaki et al., 1995) they do not differentiate into muscle under normal conditions (Bianco and Cossu, 1999). When transplanted in sheep fetus *in utero*, human MSC colonized most tissues, including skeletal muscle, although their effective muscle differentiation was not demonstrated (Liechty et al., 2000).

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Recently it was reported that MSC expressing a truncated form of Notch and exposed to certain cytokines were able to differentiate into skeletal muscle in vitro with high efficiency (Dezawa et al., 2005). Induced cells differentiated into muscle fibers upon transplantation into degenerated muscles of mdx-nude mice. The induced population contained Pax7-positive cells that contributed to subsequent regeneration of muscle upon repetitive damage without additional transplantation of cells. These MSCs may represent a more ready supply of myogenic cells than other rare myogenic stem cells found in other tissues, but the underlying molecular mechanism needs to be fully elucidated and the risks related to the expression of an oncogenic protein need to be carefully evaluated.

### *2.3.2 Multipotent adult progenitors*

The group of Verfaillie (Reyes et al., 2001) identified a rare cell, within adherent cells cultured from human or rodent bone marrow, which was termed multipotent adult progenitor cell (MAPC). This cell can be expanded for greater than 70 to 150 population doublings (PDs) and differentiates not only into mesenchymal lineage cells but also into endothelium, neuroectoderm, and endoderm. Similar cells can be selected from mouse muscle and brain, suggesting that they may be associated with the microvascular niche of probably many if not all tissues of the mammalian

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body (Jiang et al., 2002a). Furthermore, when injected into a blastocyst, MAP cells colonize all the tissues of the embryo, with a frequency comparable with ES cells (Jiang et al., 2002b). Because of their apparently unlimited lifespan and multipotency, MAP cells appear as obvious candidates for many cell replacement therapies, although complete differentiation into the desired cell type still needs to be optimized. For what concerns skeletal muscle, neither the frequency at which MAP differentiate into skeletal muscle cells after 5-azacytidine treatment, not their ability to rescue dystrophic muscle have been investigated. In addition the ability of MAP to travel through the body using the circulatory route has not been formally demonstrated, although the general features of these cells strongly suggest this to be the case.

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### *2.3.3 Muscle derived stem cells (MDSC)*

Cells that adhere late to the culture dish after proteolytic digestion of adult skeletal muscle were isolated though differential pre-plating and shown to retain their phenotype for more than 30 passages with normal karyotype, ability to differentiate into muscle, neural, and endothelial lineages both in vitro and in vivo. These cells that co-express CD34 and Sca-1 like mesoangioblasts (see below) are clearly different from resident satellite cells and were termed “muscle derived stem cells” (MDSC). Transplantation of MDSC improved the efficiency of muscle regeneration and dystrophin delivery to dystrophic muscle (Qu et al., 2001). The ability to proliferate in vivo for an extended period of time, combined with their strong capacity for self-renewal, their multipotent differentiation, and their immune-privileged behavior, suggested that these cells may be very efficient future cell transplantation experiments.

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More recently it was reported that freshly isolated MDSC are potentially useful for reconstitution therapy of the vascular, muscular, and peripheral nervous systems. These results provide new insights into somatic stem and/or progenitor cells with regard to vasculogenesis, myogenesis, and neurogenesis (Tamaki et al., 2005).

### *2.3.4 Mesoangioblasts*

Searching for the origin of the bone marrow cells that contribute to muscle regeneration (Ferrari et al., 1998) we identified, by clonal analysis, a progenitor cell derived from the embryonic aorta (De Angelis et al., 1999). When expanded on a feeder layer of embryonic fibroblasts, the clonal progeny of a single cell from the mouse dorsal aorta acquires unlimited life-span, expresses angioblastic markers (CD34, Sca1 and Flk1) and maintains multipotency in culture or when transplanted into a chick embryo. We proposed that these newly identified, vessel associated stem cells, the mesoangioblasts, participate in post-embryonic development of the mesoderm and speculated that postnatal mesodermal stem cells may be rooted in a vascular developmental origin (Minasi et al., 2002).

In as much as mesoangioblasts can be expanded indefinitely, are able to circulate and are easily transduced with lentiviral vectors, they appeared as a potential novel strategy for the cell therapy of genetic diseases. Recently we have succeeded in

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isolating mesoangioblast-like cells also from post-natal mouse, dog and human tissues. When injected into the blood circulation, mesoangioblasts accumulate in the first capillary filter they encounter and are able to migrate outside the vessel, but only in the presence of inflammation, as in the case of dystrophic muscle. We thus reasoned that if these cells were injected into an artery, they would accumulate into the capillary filter and from there into the interstitial tissue of downstream muscles. Indeed, intra-arterial delivery of wild type mesoangioblasts in the α-sarcoglycan KO mouse, a model for limb girdle muscular dystrophy, corrects morphologically and functionally the dystrophic phenotype of all the muscles downstream of the injected vessel Furthermore, mesoangioblasts, isolated from α-sarcoglycan null mice and transduced with a lentiviral vector expressing α-sarcoglycan, reconstituted skeletal muscle similarly to wild type cells (Sampaolesi et al., 2003). These data represented the first successful attempt to treat a murine model of muscular dystrophy with a novel class of mesoderm stem cells. In order to move towards clinical experimentation, we have recently isolated canine mesoangioblasts. Indeed, the only animal model specifically reproducing the full spectrum of human pathology is the golden retriever dog model. Affected animals present a single mutation in intron 6, resulting in complete absence of the dystrophin protein, and early and severe muscle degeneration with nearly complete loss of motility and walking ability. Intra-arterial delivery of wild-type canine mesoangioblasts (vessel-associated stem cells) results in an extensive recovery of dystrophin expression, normal muscle morphology and function (confirmed by measurement of contraction force on single fibres). The outcome was a remarkable clinical amelioration and preservation of active motility (Sampaolesi et al., 2006). Overall the data so far accumulated qualify the mesoangioblasts as candidates for future stem cell therapy for Duchenne patients.

### *2.3.5 Endothelial progenitor cells (EPC) and other endothelia*

Initially identified as CD34+, FlK-1+ circulating cells (Asahara et al., 1997), EPC were shown to be transplantable and to participate actively to angiogenesis in a variety of physiological and pathological conditions. *In vitro* expansion of EPC is still problematic and few laboratories have succeeded in optimizing this process. The clear advantage of EPC would be their natural homing to site of angiogenesis that would target them to site of muscle regeneration. It is known that human umbilical cord blood (UCB) contains high numbers of endothelial progenitors cells (EPCs) characterized by co-expression of CD34, CD133, Flk1 and VE-Cadherin (Murohara et al., 2000) and several studies have shown that these CD34+/CD133+ EPCs from the cord or peripheral blood (PB) can give rise to endothelial cells and induce angiogenesis in ischemic tissues (Takahashi et al., 1999; Kocher et al., 2001). Recently, it has been shown that freshly isolated human cord blood CD34+ cells injected into ischemic adductor muscles give rise to endothelial but also to skeletal muscle cells in mice (Pesce et al., 2003). In fact, the treated limbs exhibited enhanced arteriole length density and regenerating muscle fiber density. Under similar experimental conditions, CD34− cells did not enhance the formation of new arterioles and regenerating muscle fibers. These results support the notion that also

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endothelial cells, either resident inside adult skeletal muscle (Tamaki et al., 2002) or isolated from fetal lung and yolk sac (Cusella De Angelis et al., 2003) have the ability to participate to muscle regeneration.

### *2.3.6 Stem cells from adipose tissue*

Several studies have recently reported the isolation of a human multipotent adipose-derived stem (hMADS) cell population from adipose tissue of young donors (Rodriguez et al., 2005). hMADS cells display normal karyotype, have active telomerase, proliferate over 200 population doublings and differentiate into adipocytes, osteoblasts and myoblasts. Flow cytometry analysis indicates that hMADS cells are positive for CD44 and other mesenchymal markers but negative for CD34, c-Kit, Flk-1, CD133. Transplantation of hMADS cells into the mdx mouse, an animal model of Duchenne muscular dystrophy, resulted in substantial expression of human dystrophin in the injected tibialis anterior and the adjacent gastrocnemius muscle (Rodriguez et al., 2005). Surprisingly, long-term engraftment of hMADS cells also takes place in non-immunocompromised animals, which may be due to the very low level of HLA expressed. It remains to be explained if hMADS-derived muscle fibers did not express high level of class I HLA as all muscle fibers do. Still, the easily available tissue source, their strong capacity for expansion ex vivo, their multipotent differentiation and their immune-privileged behavior, suggest that hMADS cells could be an important tool for muscle cell-mediated therapy.

### *2.3.7 Stem cells from sinovium*

Several years ago mesenchymal stem cells wre isolated and characterized from human synovial membrane (SM): it was shown that SM-derived MSCs have a multilineage differentiation potential in vitro (De Bari et al., 2001). The same group demonstrated later their myogenic differentiation in a nude mouse model of skeletal muscle regeneration providing proof of principle of their potential use for muscle repair in the mdx mouse model of Duchenne muscular dystrophy (De Bari et al., 2003). Indeed, when implanted into regenerating nude mouse muscle, hSM-MSCs contributed to myofibers and to long term persisting functional satellite cells. Interestingly no nuclear fusion hybrids were observed between donor human cells and host mouse muscle cells as the myogenic differentiation proceeded through a molecular cascade resembling embryonic muscle development. Moreover, the differentiation was sensitive to environmental cues, since hSM-MSCs injected into the bloodstream engrafted in several tissues, but acquired the muscle phenotype only within skeletal muscle. When administered into dystrophic muscles of immunosuppressed mdx mice, hSM-MSCs restored sarcolemmal expression of dystrophin and ameliorated muscle morphology.

All the examples of stem/progenitor cells that we have described above because of their myogenic potency, differ among themselves for a number of biological features (origin, proliferation and differentiation ability etc.) as well as for expression of myogenic and stem cell markers. These are summarized in Tables 1 and 2 respectively, that suffer of over-simplification but hopefully help the get a general view

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*Table 1.* Features of different myogenic progenitor cells under various experimental conditions

| Cell type | Origin | Proliferation | Systemic delivery | In vitro differentiation | Dystrophin expression in vivo |
|---|---|---|---|---|---|
| Satellite cells | Somite | High | No | Spontaneous | Yes |
| MSC | Vessel wall | High | ND | Induced by Aza-cytidine | Yes |
| EPC | Vessel wall | Low | Yes | Induced by muscle cells | ND |
| MAPC | Vessel wall | High | ND | Induced by Aza-cytidine | ND |
| MDSC | Skeletal muscle | High | ND | ND | Yes |
| MAB | Vessel wall | High | Yes | Induced by muscle cells | Yes |
| ADSC | Adipose tissue | High | ND | Spontaneous | Yes |
| SDSC | Synovium | High | ND | Induced by Aza-cytidine | Yes |
| HSC | Bone marrow | Low | Yes | Induced by muscle cells | Yes |

Main biological features of satellite cells and other stem-progenitor cells endowed with myogenic potency. MSC: mesenchymal stem cells; EPC: endothelial progenitor cells; MAPC: multipotent adult progenitors; MDSC: muscle derived stem cells; MAB: mesoangioblasts; ADSC: adipose derived stem cells; SDSC: Synovium derived stem cells; HSC cells refer to hematopoietic stem cells, independently from the selection method (lineage negative, expression of markers such as c-Kit, CD34, Sca-1, dye exclusion – SP population).

of the current situation. It is likely that the list, admittedly incomplete, may still grow in the future, but it should considered that different source, age and species, different methods of isolation and culture may have led to rediscover several times the same cell types, differences among which may depend on these variables. Time will be needed to reach a clearer and more definitive picture.

*Table 2.* Expression of myogenic and stem cell markers in satellite cells and other stemprogenitor cells endowed with myogenic potency

| Cell Type | MRF | Pax3/7 | Sca-1 | CD45 | CD34 | CD31 |
|---|---|---|---|---|---|---|
| Satellite cells | Yes | Low/High | Yes | No | Yes | Yes |
| MSC | No | No | Yes | No | No | Yes |
| MAPC | No | No | Yes | No | No | ND |
| MDSC | No | No | Yes | No | Yes | Yes |
| MAB | No | High/No | Yes | No | Yes | Yes |
| ADSC | No | ND | Yes | No | No | Yes |
| SDSC | No | ND | Yes | No | No | Yes |
| HSC | No | No | Yes | Yes | Yes | Yes |

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## 3. THE POSSIBLE DEVELOPMENTAL ORIGIN OF NON MUSCLE STEM CELLS

At first sight the origin of non muscle-derived stem cells, able to make muscle, appears to be mainly restricted to the hemo-vascular system (hematopoietic, endothelial, pericytes) that derives from the splanchno pleura. Cells associated with developing vessels would be evenly distributed to developing tissues with fetal angiogenesis and thus allocated to the local pool of progenitors for further tissue growth or regeneration. Non muscle stem cells with similar myogenic potency are also present in the neural tissue, but it is possible that they ingress the nervous system with fetal angiogenesis. Although this has never been demonstrated, the reported association of neural stem cells (or possibly a subset of them) with the vasculature (Palmer et al., 2000) would be compatible with this hypothesis. Although all the above mentioned embryonic tissues are unrelated to somites and paraxial medoserm, the situation may be more complex.

### 3.1 Clonal Studies in Mouse and Chick Embryos

Canonic skeletal myogenic progenitors originate from the dorsal somite but several other cell types such as dermis fibroblasts, endothelial cells and smooth muscle also originate in part from the dermomyotome (Christ and Ordahl, 1995). Therefore detecting myogenesis arising from an endothelial or a smooth vascular progenitor would not necessarily imply that it is non somitic in origin.

An unbiased search for a skeletal myogenic progenitor outside the somite in the developing mouse embryo identified the dorsal aorta as a source of skeletal myogenic clones that could not be derived from other anlagen such as the heart, the ectoderm or the gut (De Angelis et al., 1999). Virtually all the cells of the clones derived from the dorsal aorta co-express early endothelial and myogenic markers such as VE-cadherin and MyoD as well as smooth alpha actin. Few years later, an elegant study identified a common progenitor that gives rise to endothelium and skeletal muscle. A library of replication-defective retroviral vectors was used to infect cells in the somite, from which both myogenic and endothelial progenitors migrate to the limb. Single cell PCR confirmed the clonal origin of differentiated cells that shared integration of the same proviral sequence: surprisingly, approximately one third of myogenic and endothelial cells were found to derive from a common somitic precursor.

In this context, a recent report clearly indicated a common clonal origin for cells in the myotome and in the dorsal aorta. A genetic approach that permits retrospective clonal analysis (Bonnerot and Nicolas, 1993) is based on a laacZ reporter that contains a duplication of the lacZ coding sequence under the control of regulatory sequences directing expression to the tissues of interest. In the embryo, a rare intragenic recombination event will remove the duplication to give lacZ, which encodes a functional β-galactosidase (β-gal) protein when the gene is expressed. A common progenitor cell that has undergone such a recombination event will give

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rise to β-gal+ cells that are clonally related. When the α-cardiac actin gene was targeted with a nlaacZ reporter it became possible to examine, in addition to the heart (Meilhac et al., 2003). also embryonic skeletal muscle and the dorsal aorta where this gene is also transiently expressed (Sassoon et al., 1988). This retrospective clonal analysis showed that cells in the dorsal aorta and in the myotome have a common clonal origin. Moreover, based on the long half life of the GFP protein, it was possible to follow the fate of Pax3GFP/+ progenitors in the paraxial mesoderm that appear to migrate from the somite to the dorsal aorta. Most of the clones contained smooth muscle cells, but occasional labeled endothelial cells were present in the clones, in keeping with the existence of a common vascular progenitor.

Thus the relationship among somitic and non somitic vascular progenitors may be complex: cells from the somite may migrate to the dorsal aorta and eventually be distributed to developing tissues with vessels branching from the aorta. If some of these branches reach developing skeletal muscle, these somitic derived vascular progenitors may be recruited to a myogenic fate by signals emanating from developing muscle fibers. Moreover, although experimentally not tested, somitic vascular progenitors may easily associate with inter-somitic arteries and thus be distributed to developing tissues with the same mechanism proposed for the dorsal aorta.

Therefore all the studies showing origin of myogenic cells from non somitic tissue, should be interpreted with the caveat that cells in vascular system may ultimately derive from somites through the developmental events described above. Since the vascular tree grows into virtually any tissue (excluding cartilage and epidermis) and it may be carrying along somite derived progenitors, a somitic origin for myogenic cells found in other tissues cannot be excluded. Indeed, to formally demonstrate a non somitic origin of at least some of these progenitors, we dissected the lateral mesoderm from mouse embryos at the stage of 3–5 somites, before a vascular connection between somites and lateral mesoderm is established. The embryos expressed the n-LacZ reporter gene under the transcriptional control of the Myosin light chain 1/3 fast promoter/enhancer, restricting transgene expression to striated muscle. As expected no transgene expression was observed in the lateral mesoderm explants, cultured in isolation or on a feeder layer of fibroblasts. However, when the same explants were co-cultured with differentiating C2C12 myogenic cells, many LacZ expressing nuclei were detected inside multinucleated myotubes, indicating that truly non somitic cells have at least the option of fusing in vitro into differentiated myotubes and trans-activate a skeletal muscle promoter (Fig. 1).

### 3.2 Studies on the Origin of Satellite Cells and of Non-muscle Stem Cells

Together these studies strongly argue in favor of a complex lineage relationship among early endothelial, smooth and skeletal myogenic progenitors, but the exact underlying mechanism remains elusive. Since most of these studies were limited to early post-somitic stages, none sheds light on the origin of later progenitors or

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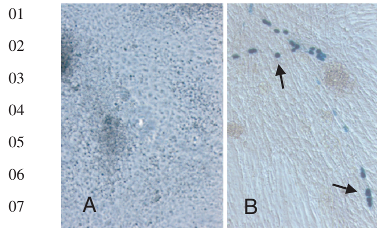


*Figure 1.* Skeletal myogenic differentiation in cells isolated from 3–5 somite stage mouse embryo lateral mesoderm. Lateral mesoderm was dissected from MLC1/3F-nLacZ embryos and cultured either on a feeder layer of 10T1/2 fibroblasts (A) or C2C12 myogenic cells (B). After 5 days, cultures were stained with X-Gal. β-gal positive nuclei are shown by arrows

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post-natal stem cells. Recently however three studies agreed that also post-natal satellite cells and, in one case, muscle SP, are somite derived. A cell population that expresses the transcription factors Pax3 and Pax7 but no skeletal-muscle-specific markers was recently identified in the mouse. These cells are maintained as a proliferating population in embryonic and fetal muscles of the trunk and limbs throughout development and later adopt a satellite cell position characteristic of progenitor cells in postnatal muscle (Relaix et al., 2005).

In another study, electroporation of GFP in chick somites and quail-chick grafting experiments showed that the dorsal compartment of the somite, the dermomyotome, is the origin of a population of muscle progenitors that contribute to the growth of trunk muscles during embryonic and fetal life, including satellite cells (Gros et al., 2005). Finally it was shown, through different approaches (replication-defective retroviruses, quail/chick chimeras, and mouse Pax3-Cre lines) that the majority of limb muscle satellite cells arise from cells expressing Pax3 specifically in the hypaxial somite; moreover they show that a significant number of limb muscle SP cells are derived from the hypaxial somite (Schienda et al., 2006).

As for the origin of the other stem cells described above, not much is known at the moment. We can assume, based on previous embryological studies, that hematopoietic stem cells, pericytes, endothelial progenitors, mesoangioblasts, MAPs and mesenchymal stem cells are all associated to the hemo-vascualar system, which is derived, but not entirely (see above) from the ventral lateral mesoderm or splanchnopleura. Unfortunately, expression of a given repertoire of surface antigens may be useful to prospectively isolate these cells form adult or fetal tissues, but is not informative on their origin since the same cell lineage may change gene expression during development. Indeed, genetic labeling by the cre-lox system has been used so far to demonstrate that endothelial cells in the adult may derive from a common myeloid progenitors. In general these studies are limited by paucity of truly specific promoters, which are also expressed early during development, to allow tracing the developmental origin of a given stem/progenitor cell. In the past we used VE-Cadherin/cre and Tie2/cre mice crossed to floxed Rosa 26 mice aiming to detect β-gal+ cells, originating from the endothelium, inside smooth, skeletal or cardiac

muscle. The results of these experiments showed that rare (less than 1%) smooth muscle cells are derived from founders that once expressed either VE-Cadherin or Tie2. However the frequency of cardiac or skeletal muscle derived from endothelial founders was extremely low (less than 0.01%) indicating that virtually no skeletal muscle is derived from an endothelial cell, at least at a stage when it already expressed VE-Cadherin or Tie2 (Berarducci et al. unpublished results). It remains possible that some muscle cells are derived from a more immature endothelial progenitor or angioblast but, by the time the cells has activated differentiated gene products such as VE-Cadherin its fate is restricted to mature endothelium and possibly rare smooth muscle cell. Here again, absence of a well characterized, truly "angioblast" specific promoter, prevents this kind of approach to be extended to a more immature and possibly still multipotent progenitor.

### 3.3 The Possible Lineage Relationship of Mesoderm Stem Cells with Satellite Cells

Mesoangioblasts are derived from the vessel wall and so are mesenchymal stem cells, EPC and multipotent adult progenitors: thus the vascular niche in the bone marrow and possibly in all mesoderm is a site where different types of multipotent (and potentially myogenic cells) are found in the adult. Furthermore, hematopoietic stem cells (HSC), which also show myogenic potency, are present in the same anatomical site, within the bone marrow and other hematopoietic tissues.

A question relevant to muscle regeneration is whether there is any lineage relationship between one or more types of mesoderm stem cells and muscle satellite cells. In other words it is possible that any of these cells may leave the vessel wall, enter the interstitial space, then cross the basal lamina of the muscle fiber and eventually adopt a satellite cell position, possibly expressing satellite cell specific genes. Evidence for this event has been claimed of the basis of co-expression of a satellite cell markers (M-Cadherin, CD34, Pax7) and a donor cell marker (GFP, LacZ etc.) in a cell located underneath the basal lamina but outside the sarcolemma, after either intra-muscular or intra-arterial injection or bone marrow transplantation. An example is shown in Fig. 2. Even though this event has been found to be

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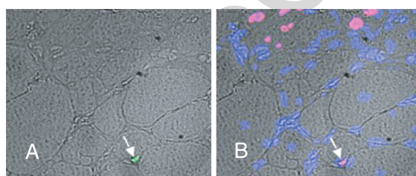


*Figure 2.* Human mesoangioblasts give rise to satellite cells after intra-arterial transplantation. Human cells identified and express satellite cell markers. A Myf5 (green) expressing cell (arrow), located at the periphery of a small fiber, also express Lamin A/C. Human nuclei appear violet (arrowhead), after co-staining with DAPI. Fluorescence is superimposed on the phase contrast image of the tissue. Bar = 20 μm

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rare when analyzed in vivo, a real possibility exists that it may occur constantly during late fetal and post-natal muscle growth, so that it may feed a significant proportion of cells into the satellite cell compartment and thus contribute indirectly to regenerating fibers. Obviously experiments carried out in a short period of time would miss the alternative origin of satellite cells that may have been derived from other mesoderm stem cells before the time of analysis. Importantly, in all these experiments a damage to skeletal muscle and often a depletion of the resident pool of myogenic cells are required to provide a selective advantage to donor cells. This means that it will be very difficult to know what is the turn-over of satellite cells and what part of this turn-over may be carried out by non resident progenitors cells in the healthy muscle of a normal mammal or in the course of a primary myopathy. The argument raised above of the somitic origin of most satellite cells does not contrast this possibility because of the somitic origin of endothelial and smooth muscle cells described above.

## 4. PERSPECTIVES FOR CELL THERAPY

The scenario described above is complex and likely will be expanded, refined and possibly modified by the rapidly accumulating data from the many laboratories involved in this area of research.

Nevertheless, a quest for a therapy for muscular dystrophy and other primary muscle diseases, raises the additional need to choose among these myogenic progenitors those which may best fit the requirements for a successful restoration of muscle morphology and function (Cossu and Sampaolesi, 2004).

To this aim selection of the appropriate cell type should meet the following criteria: (a) accessible source (e.g. blood, bone marrow, fat aspirate, muscle or skin biopsy); (b) ability to grow as a relatively homogeneous population in vitro for extended periods without loss of differentiation potency (since it appears currently unlikely that cells may be acutely isolated in numbers sufficient for therapeutic purposes); (c) susceptibility to in vitro transduction with vectors encoding therapeutic genes (these vectors should themselves meet criteria of efficiency, safety and long term expression); (d) ability to reach the sites of muscle degeneration/regeneration through a systemic route and in response to cytokines released by dystrophic muscle; (e) ability to differentiate in situ into new muscle fibers with high efficiency and to give rise to physiologically normal muscle cells.

Satellite cells that were considered as the first and most obvious candidate for the cell therapy of muscular dystrophy are not able to cross the vessel wall when delivered systemically and need to be locally injected into skeletal muscle at a distance of few mm from each other, since they cannot migrate extensively in the muscle. This fact alone limits the potential application of satellite cells, at least with current technology. Moreover, most of the injected cells die within the first day and this explains the failure of the first trials with satellite cell derived myoblasts in the early 90'.

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Advantages and disadvantages of the other types of non muscle stem cells vary and are summarized in Table 1. Some are difficult to expand in vitro, others show inefficient myogenic differentiation while for others the ability to negotiate the vessel wall when systemically delivered has not been experimentally tested. Right now mesoangioblasts are the cell type for which most parameters have been tested in vitro and more importantly in vivo, first in a mouse model of muscular dystrophy (Sampaolesi et al., 2003) and more recently in the Golden Retriever dystrophic dog (Sampaolesi et al., 2006).

Hopefully in a few years time, phase I clinical trials with stem cells may start and set the stage for one more, and at least in part successful attack to defeat these genetic diseases.

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