Identification and characterization of cells with stem cell properties in normal and malignant muscle cultures

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Identification and characterization of cells with stem cell properties in normal and malignant muscle cultures

Identificatie en karakterisatie van cellen met stamcel eigenschappen in normaal en kwaadaardig spiercel kweek

Thesis

to obtain the degree of Doctor from the
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by

Fanny Gabrielle Sage

born in Montauban, France

Doctoral Committee

Promoter:	*Prof.dr. E.A. Dzierzak*
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Chapter 1

Introduction

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General introduction

The capacity of living beings to regenerate themselves remains an inexhaustible source for investigation and a matter of astonishment. The idea of regeneration brings us back to a long story, impregnated by mythologies as well as science. It has captured the imagination of humanity since millennia.

One of the best examples illustrating this idea resides in the Greek mythology with the Lernaean Hydra. This water snake, part of the 12 labors of Heracles, had nine heads, one of which was immortal. According to the legend, each time one of the heads was severed, two new heads were formed. This hydra myth describes one of the oldest dreams of humanity, the immortality. During centuries, the spontaneous generation theory summarized by Aristotle in Organon, where he described the appearance of moist on various foods, remains the current biological concept. It is only after the 19th century; with the Pasteur swan neck duck bottle experiment, that this view was definitely dismissed after 2 millennia. In the 18th century the regeneration concept was also emerging with the discovery of A. Tremblay and R.A. Réaumur that polyps and Hydra can regenerate theirs arms right after truncation. At the same period Spallanzani, propagated the idea of regeneration capacity to a larger audience by showing the ability of snail to regenerate their head after ablation (Panagiotis A. tsonis and Timothy P. Fox 1998). Since then, a number of studies on lower organisms like zebrafish (Poss et al., 2002) and axolotls (Echeverri et al., 2001, Kragl M et al., 2009), have shown the enormous regeneration potential of these animals, and their ability to completely regenerate, tail, limb, or part of the heart. However, these regeneration studies also highlight a disparity in the regeneration potential between species. Studies in superior vertebrates show that, upon evolution and organism complexity, the capacity of regeneration progressively decreases. Nevertheless some tissues like liver, skin or muscle have shown to maintain some regenerative potential.

It has been established that the regeneration process is based on three different phenomena that might be mutually exclusive or combined depending of the species. These phenomena are the dedifferentiation of differentiated tissue, the transdifferentiation across tissue types and finally the regeneration by stem cells. (Sugimoto et al. 2011). These processes have been extensively studied in plants, but in animals their existence remains to be definitively demonstrated.
The evidence of dedifferentiation in animals comes from studies showing cells starting to proliferate upon loss of some markers and quiescence (Jopling, C et al. 2010). Moreover, it seems that dedifferentiation occurs in primary myoblast when Rb and ARF are transiently inactivated (Pajcini et al., 2010).
The transdifferentiation hypothesis defined by Okada as an irreversible switch from a differentiated cell type to another has been shown in axolotls after in vivo fluorescent labeling of single muscle fibers (Echeverri et al. 2001). This technique has shown that multinucleated muscle fibers are able to fragment in mononuclear cells, which then

rapidly proliferate.
Finally, the presence of a particular cell type with unique properties in various tissues of the adult organism has been proposed. These cells, referred as stem cells, are able to self-renew, and have the capacity to give rise to multiple descendant cell types through a differentiation process (Weissman et al. 2001).
The first experiments identifying stem cells have been assessed in the blood system more than five decades ago by two Canadian researchers, E.A. McCulloch and J.E. Till. They established the gold standard assay of the field, which consists to transplant bone marrow cells into irradiated mice (Till and McCulloch 1961). Since, the existence of stem cells has been established in other tissues like intestine, skin, kidney and skeletal muscle.

Most skeletal muscles are not essential for the survival of the organism. Therefore, this tissue that contains resident stem cell population provides an excellent paradigm to investigate the degeneration and regeneration processes. Its accessibility and remarkable regeneration potential, despite several rounds of injury, make the muscle an excellent model to study stem cell activity.

The skeletal muscle tissue

The muscle fiber

The skeletal muscle is the body's largest tissue, accounting for about 40% of the total body weight (Biressi et al. 2007). More importantly, it plays critical roles in movement, respiration, stabilization of the skeleton, glucose homeostasis, and thermoregulation. The basic structural and functional units composing the adult skeletal muscle tissue are the muscle fibers, also known as myofibers (Figure1). They are multinucleated, elongated, and membrane-bound cells. Each fiber is surrounded by connective tissue called endomysium. Several fibers are associated to each other to form the fasciculi. A layer of connective tissue called perimysium surrounds each fasciculus. The fasces forming the muscles are surrounded by a last connective tissue called epimysium. Finally blood vessels and nerves wander the muscle fibers. Myofibers translate the neural pulse into contraction through the motoneuron innervation. Thus, myofibers form highly specialized syncytia site of the muscular contraction.
Myofibers contain bundles of myofibrils, which have a cylindrical structure of 1-2um in diameter. These myofibrils consist of a long repeated chain of tiny contractile units called sarcomeres. Sarcomeres are not only responsible for the striated appearance of the tissue but they are also the fundamental contractile units of the fiber. Each sarcomere is formed from precisely ordered and parallel partially overlapping network of thin and thick filaments. The thin filaments are composed of actin and associated proteins, and are attached at the plus ends of the sarcomere

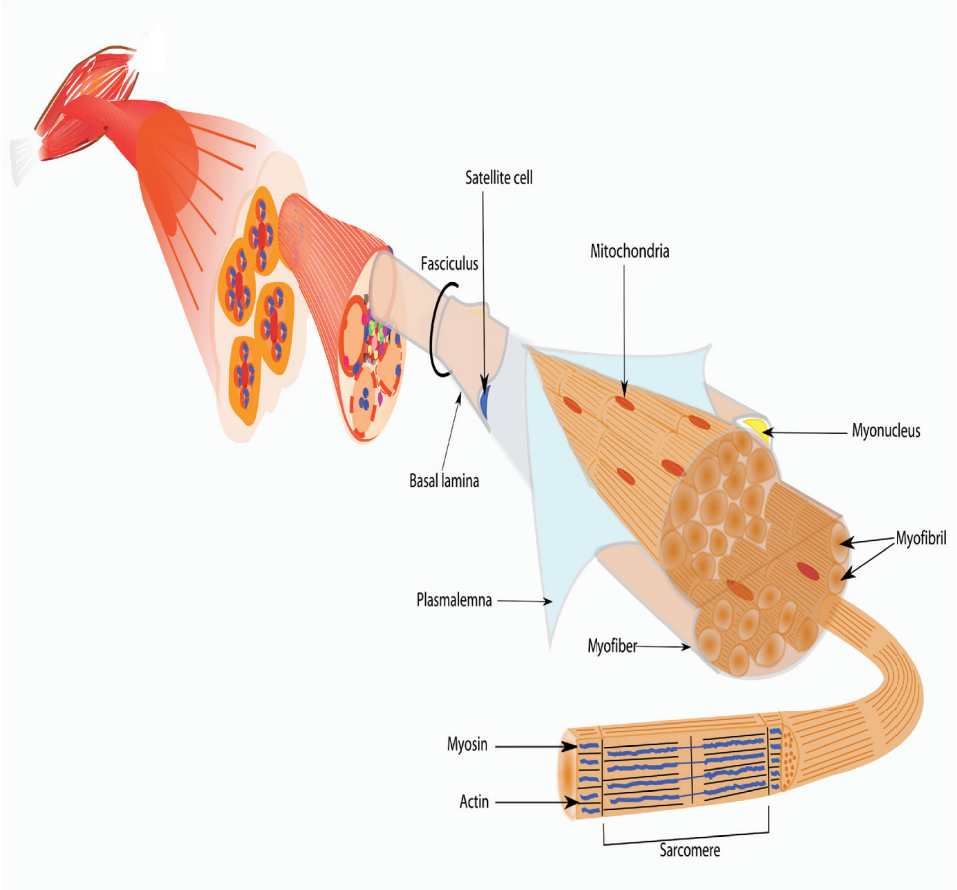

Figure 1: Scheme of the skeletal muscle structure
Satellite cells are located between the basement membrane and the plasmalemma of the myofiber. Represented as well the hierarchical organization of the tissue: muscle, myofiber, myofibril and sarcomere with actin and myosin filaments. (for further details see the text)
Adapted from Tajbakhsh 2009

to Z discs. The capped minus end is overlapping with the thick filaments composed of a specific isoform of myosin. The sarcomere shortening allowing muscle contraction is obtained by the sliding of the myosin filaments past the actin filaments with no change in length of either type of filaments. Furthermore, the myofiber are expressing different myosin heavy chain (MyHC) isotypes. The basic mechanism behind the contraction phenomenon is the same in every fiber type. It results from the sliding of MyHC filaments on actin filaments. The myofiber set off a mechanical contraction in response to neuronal or electrical stimuli, generating the contractile force needed by a particular skeletal muscle to perform its function. On the other hand, the surrounding connective tissue allows the transformation of the contractions into movement. Moreover the fiber-type composition (slow

fibers type I, fast fibers type II or intermediate fibers) determines the muscle's contractile behavior/physiological characteristic. The fiber composition varies from one muscle to another one (for example the soleus is enriched in type I fiber) or from one individual to another one. Overall, it is the proportion of each fiber type that will determine the contractile capacity of a particular muscle.
Regarding the maintenance of integrity and the repair upon damage of the skeletal tissue, adult skeletal muscle also houses several populations of stem cells. These cells guarantee the homeostasis of the tissue through a regeneration process.

The different muscle cell populations

The process upon which the regeneration occurs has been extensively studied, and the presence of an endogenous stem cell population has been demonstrated. This population was coined as satellite cells (SCs) due to their particular anatomical location at the periphery of the fibers, between the sarcolemma and the basal lamina. Since their discovery, half a century ago (Mauro 1961), SCs have been extensively studied. So far, they are characterized as a heterogeneous mix of quiescent and activated stem cells and committed progenitors (Beauchamp et al. 2000, Sherwood et al. 2004, Kuang et al. 2007).
Transplantation studies of freshly isolated muscle cells have demonstrated the high potential of SCs to contribute to repair and regeneration processes of damaged muscle fibers (Sacco et al. 2008). In contrast myoblasts, more committed muscle progenitors, have only a limited engraftment capacity and a poor regeneration potential (Collins et al. 2005, Sacco et al. 2008). Upon damage SCs become activated by breaking the quiescent state. They proliferate and differentiate into myoblasts, the proliferative unit of the muscle tissue. Myoblasts give rise to myocytes that are elongated tubular cells able to fuse together to form myofibers (Figure 3).

Myogenic differentiation

The skeletal muscle tissue organization involves several physiological processes, including myogenesis, formation of the surrounding connective tissues, innervation and vascularization. All these processes are equally important to obtain a fully functional muscle. However, we will only focus on myogenesis, the process responsible for myogenic differentiation and organization.
A considerable progress in understanding muscle differentiation process was achieved by the derivation of muscle cell lines from mouse (e.g. c2c12) (Yaffe and Saxel, 1977) and rat (Yaffe et al. 1968). It then became possible to set up in vitro assays where myoblasts could differentiate into myotubes, multinucleated structures that are the in vitro equivalent of the myofibers.

The complex in vivo microenvironment of the myogenic cells is very difficult to reproduce in vitro. However the in vitro differentiation system represents an interesting reductionist model allowing to study the effect of specific factors in a controlled manner. A large amount of information has been gained from in vitro assays notably

on the genes playing a role in myogenesis and their epistatic relation (d'Albis et al. 1988, Launay et al. 2001, Messner et al. 2000, Zhao et al.2002).

The in vivo study of embryonic and postnatal myogenesis has been heightened with the advancement of molecular biology and the development of mouse models. While embryonic myogenesis involves the generation of muscle tissue, postnatal myogenesis includes muscle homeostasis and regeneration process after muscle damage. Although the final goals in a developing embryo and adult organism are different, the process leading to myogenesis in both shares common features and factors such as signaling pathways and transcription factors (Shi et al. 2006).

Myogenesis

Embryonic myogenesis

During embryonic development, the skeletal muscle is formed after a multistep process including the spatio-temporally regulated activation of specific genes (described in the next section) and the generation of different precursor cell types (Biressi et al. 2007, Tajbakhsh et al. 2000). This process takes place between embryonic day (E)8.5/9 and E18.5. A maturation process will occur during several weeks after birth.

All myogenic progenitors in the body derive from the paraxial mesoderm of the somites (Buckingham 2006), which are the segmental structures formed along the anterior-posterior axis of the embryo. The dorsal part of the somites forms the dermomyotome (Figure 2) that will then give rise to different tissues including the skeletal muscle (as in trunk and limbs, with the exception of some head muscles).
The first skeletal muscle originates from the lateral lips of the dermomyotome called myotome. During this process the central part of the dermomyotome disintegrates and muscle progenitors intercalate into the primary myotome (Gros et al. 2005). Later on, the epaxial parts of the dermomyotome and the myotome form the dorsal muscles whereas the lateral trunk and limb muscles derive from hypaxial domains (Parker et al. 2003). The progenitors from the hypaxial domain are delaminating and migrating under the control of c-met expression (Dietrich et al. 1999). C-met expression pattern is tightly regulated by Pax3 (Epstein et al. 1996). Some muscles (from extremities or diaphragm) do not derive from the primary myotome but from a specific population of Pax3 expressing progenitors (Tajbakhsh et al. 2000). The emergence and migration of these progenitors is tightly regulated by chemokines (CXCR4/SDF1) (Vasyutina et al. 2005) and growth factors (SF/HGF) (Dietrich et al. 1999).

The activation of myogenic differentiation program, in the muscle progenitors, in a specific location, is controlled by the environment through different signaling path-

ways such as Wnt (Gros et al. 2009), BMP, Shh (Marcelle et al. 1997) and Notch (Rios et al.2011).

Regarding the relevance of the early muscle progenitor development, several studies using a lineage tracing approach have demonstrated that the adult SC population comes from embryonic muscle progenitors (Gros et al.2005, Kuang et al. 2007, Lepper et al. 2009). It appears thus relevant to study embryonic progenitors in order to get a better understanding of the adult SCs.

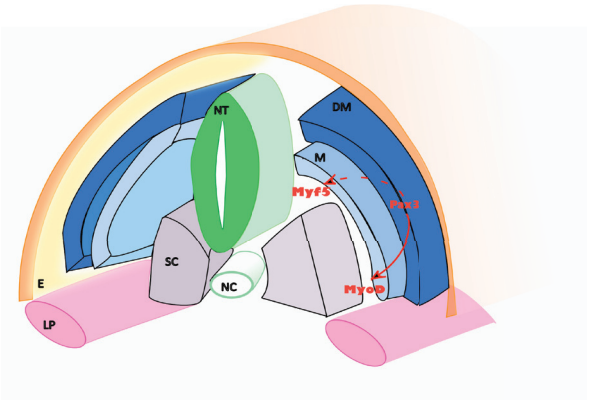

Figure 2: Early myogenesis in embryo

Representation of the mesodermal somitic cells (containing Pax3 progenitors) located in the dorsal part of the somite dermomyotome (DM). Committed myoblast migrate laterally to form the myotome (M). The epaxial part of the dermomyotome and myotome will give rise to the dorsal muscles (with a population of Myf5 progenitors). The hypaxial domain of the myotome and dermomyotome will give rise to the lateral trunk and limb muscles (MyoD progenitor cell population). MyoD functions downstream of pax3/7 (represented by the continue arrow) in the genetic hierarchy, whereas Myf5 can act in parallel of the Pax genes in function of the context (represented by the dotted line arrow). E, ectoderm; LP, lateral plate; SC, sclerotome; NC, notochord; NT, neural tube.
Adapted from Shi et al 2006

Regulators of myogenesis

In mice, several regulatory molecules have been identified as key players of the myogenic fate. In this respect, a number of transcription factors have been shown to be essential for the commitment and/or maintenance of the myogenic cells. Among them, Pax3 and Pax7 (paired/homeo domain) transcription factors are thought to be master regulators of myogenesis (Buckingham and Relaix 2007). Pax3 is already expressed early during embryonic development and is essential for muscle formation (Schienda et al. 2006) at the exception of the head muscle (Sambasivan et al. 2009). Pax7 function essentially takes place during perinatal growth (Seale et al. 2000). Moreover, both factors have clearly defined functions during adult myogenesis as well (Kassar-Duchossoy et al. 2005, Kuang et al. 2006, Relaix et al. 2006). A double mutant mouse for Pax3 and Pax7 has been generated (Relaix et al. 2005) resulting in the loss of muscle tissue at (E)12.5 due to the absence of founder stem/progenitor population (Gros et al. 2005). In 2000, Rudnicki lab (Seale et al. 2000) generated a

Pax7 germ line mutant and demonstrated that Pax7 is required for SC specification. Later on, Pax7 has been shown to be necessary and sufficient to induce myogenic differentiation in a non myogenic cell population (Seale et al. 2004). Moreover, Pax7 is expressed in adult SCs (Zammit et al. 2006). The use of a conditional knock-out strategy, where the expression of the Cre recombinase activity is under the control of tamoxifen to temporally control Pax7 expression loss, has shown that neither Pax7 (nor Pax3) are required for adult SC function during regeneration (Lepper et al. 2009). Furthermore, this study demonstrates that Pax7 plays only a critical role during the transition progenitor to adult SC state during perinatal development. A more recent study has used a mouse model in which Pax7 expressing cells are ablated after injection of diphtheria toxin (diphtheria toxin receptor is under the control of Pax7 promotor) (Sambasivan et al. 2011). Upon cardiotoxin injury, the regeneration capacity was shown to be lost in this mouse model due to the ablation of the Pax7 expressing cells. On the other hand, transplantation of freshly isolated SCs could rescue the observed phenotypes. In a similar conditional knock-out model (use of diphtheria toxin fragment A to generate cell-autonomous death), it was shown that ablation of Pax7 expressing cells leads to loss of regeneration potential up to 2 months following acute injury (Lepper et al. 2011). The high myogenic potential of the Pax genes has also been shown by their transduction into embryonic stem cells that were then able to contribute to fiber formation in vivo upon transplantation (Darabi et al. 2011).
Furthermore, the importance of Pax3 and Pax7 level of expression has also been investigated in vitro by using overexpressed or dominant negative isoforms (Collins et al. 2009). It appears that high levels of Pax3/7 enhances proliferation and prevent myogenic differentiation. In agreement with this, expression of a dominant negative isoform results in inhibition of proliferation.
Overall Pax7 has only a crucial function during perinatal growth but it remains an excellent marker of the cells with regenerative capacity in adulthood.

In addition to the Pax genes, there is in vertebrates a family of conserved bHLH transcription factors known as myogenic regulatory factors (MRF). They are the gatekeepers of muscle cell identity. The four major MRFs are Myf5, Mrf4 (also known as Myf6), MyoD and Myogenin (Mgn) (Rudnicki et al. 1993, Kablar et al. 2003, Kassar-Duchossoy et al. 2004, Gensch et al 2008, Kuang et al 2007). The three first markers Myf5, MyoD and Mrf4 represent marker of commitment whereas Mgn is a marker of differentiation. In early 90', the enhanced myogenic capacity of MRFs has been demonstrated by their ability to convert non-muscle cells into muscle cells (Choi et al 1990, Davis et al 1987). Firstly, it has been established that the combined inactivation of MyoD and Myf5 result in a complete loss of the skeletal muscle formation (Rudnicki et al. 1993). On the other hand, inactivation of Myf5 disturbs the appearance of the first wave of muscle cells during development whereas the inactivation of MyoD only shows a moderate phenotype (Braun et al 1992). In one of these studies a triple mutant mice has been generated (Myf5, Mrf4 and MyoD) leading to the loss of myoblast and muscle fibers whereas a stem/progenitor cell population persists without

showing any sign of myogenic commitment (Kassar-Duchossoy et al. 2004). In the same study, it was shown that these cells fail to differentiate into myoblast even in an in vitro culture system. Nevertheless, the phenotype was partially rescued by Mrf4 expression. The different phenotypes observed in the single and combined knock-out mouse models allow to conclude that these genes have partially overlapping functions or that several parallel cell lineages exist with different requirements (Kuang et al. 2007). Mgn is required later in the differentiation process. Mgn knock-out mice die at birth, exhibiting severe skeletal muscle deficiencies (Hasty et al. 1993, Venuti et al. 1995). However, conditional knock-out study has demonstrated that Mgn is only required before embryonic development (Knapp et al. 2006) and thus not necessary during adulthood. MRF expression is described as a landmark of myogenic differentiation (Fuchtbauer and Westphal 1992, Yablonka-Reuveni and Rivera 1994, Zammit et al. 2004).

Moreover, recent studies have established that microRNAs control the expression of MRFs. For example, microRNA 206 contributes to Pax7 inhibition during differentiation (Chen et al. 2010) while microRNA 31 is responsible of Myf5 sequestration in SCs (Crist et al.2012). MicroRNA 206 is also able to affect the expression level of Pax3 (Boutet et al. 2012). Furthermore, a mechanism of alternative polyadenylation was described in SCs to escape this regulation with high level of Pax3. MicroRNA 489 has been established to regulate SC quiescence by controlling the expression of the oncogene Dek (Cheung et al. 2012).

Adult myogenesis in healthy and diseased contexts

Minor damages to muscle fibers, are patched by the family of type II transmembrane protein called dysferlin proteins (Han et al. 2007), while more extensive injury results in the activation of muscle-resident stem cells that marks the initiation of the regenerative response. Recent studies have shown that adult muscle regeneration mainly depends on one population of stem cells, the SCs (Figure 3) (Montarras er al. 2005, Sacco et al. 2008, Sambasivan et al. 2011, Murphy et al. 2011, Lepper et al. 2011). Upon sustaining damage, activated SCs start to proliferate and generate committed myoblasts, which differentiate into myocytes. To repair the damage, these myocytes fuse with each other to make new myofibers, or fuse with the residual myofibers (Figure 3).

The repair process in healthy muscle is completed within one to three weeks, depending on the extent of the damage. However, the regenerated muscle fibers of dystrophic muscle (see below for the description of the disease) remain unstable due to underlying genetic defects, and therefore continue to accumulate damage. As a result, dystrophic muscle engages in continuous rounds of degeneration and regeneration. These ongoing cycles of muscle degeneration and regeneration characterize dystrophic muscle, and are thought to result in exhaustion of the SC pool (discussed below). It progresses to loss of function of the affected muscle and eventually to muscle atrophy. It is unclear whether ongoing muscle regeneration also occurs during the

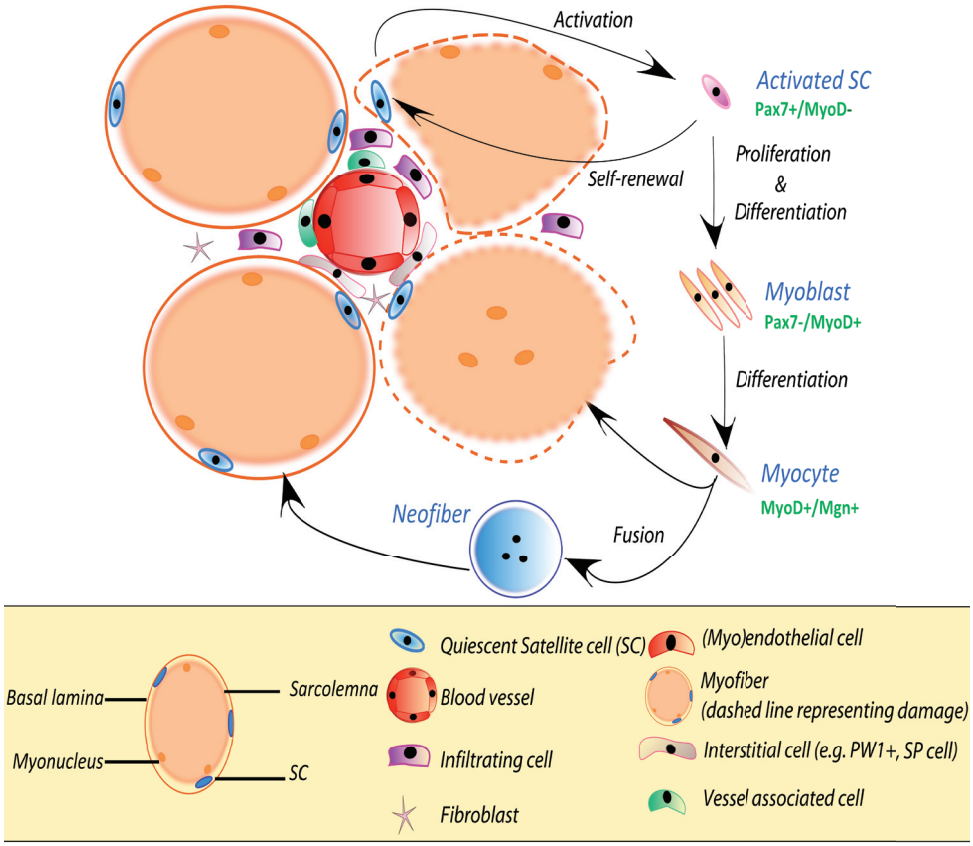

Figure 3: Skeletal muscle regeneration and muscle resident stem cells
The figure depicts a cross-section of skeletal muscle showing myofibers surrounding a blood vessel and the localization of several types of muscle-resident stem cells. The satellite cells (SCs) are located at the periphery of the myofibers, beneath the basal lamina, and become activated upon damage. The activated SCs start expressing myogenic factors, including MyoD and progress to become myoblasts. Myoblasts upregulate the expression of the differentiation factor myogenin (Mgn) and differentiate into myocytes that finally fuse together (forming neofibers) or to damaged myofibers.

The figure is adapted from Kuang et al. Trends Mol Med 14: 82-91.

disease progression of all muscle disorders. In conditions where mainly atrophy has been observed (and loss of SCs is not implied), the affected muscles will have reduced regenerative potential, and muscle wasting will be progressive.
It has been proposed that muscle is capable of complete regeneration when SC numbers are at least 10-20% of those in young adults. This may indicate that the number of regenerative-competent SCs decreases below the critical threshold during disease progression, implying that even a modest increase in stem-cell number may have a beneficial effect in diseased muscle. This observation may provide a basis for cell

therapy of muscle disorders by using muscle-regenerative cells.

Muscle disorders and mechanism of satellite cells exhaustion

Muscle disorders

Muscle disorders are a group of inherited or acquired diseases with a great variety of disease manifestations. Their common denominator is a progressive loss of muscle structure and function often associated with fibrogenesis and adipogenesis, for which no sufficient therapy is currently available (Emery et al 2002). As we discuss below, SCs include the predominant muscle stem cell population responsible for postnatal muscle regeneration. Acute or chronic muscle damage results from the disruption of muscle structural organization, inducing muscle-fiber necrosis, infiltration of inflammatory cells, and deposition of non-myogenic material (e.g. connective tissue, fat, and glycogen deposits). Many hereditary and acquired neuromuscular disorders (e.g. muscular dystrophies, toxic, inflammatory and metabolic myopathies, and neuropathies leading to muscle denervation) are associated with muscle damage. Muscle damage is also seen in systemic conditions such as ageing, cancer and endocrinological disorders. Neuromuscular disorders are a heterogeneous group of rare disorders that may occur at any age and may significantly reduce life expectancy, especially when the cardiac and respiratory muscles are involved (such as in Duchenne Muscular Dystrophy (DMD) and Pompe's Disease).

Muscle disorders are associated with a lengthening list of defects in genes that encode cytoskeletal, lysosomal, sarcomeric and membrane-associated proteins. The clinical and pathophysiological hallmarks of these myopathies can vary widely (Van der Ploeg and Reuser 2008, Manzur and Mutoni 2009, Bushby 2009). However, irrespective of the mechanisms involved, the common denominator is a muscle-wasting phenotype. With regard to several of the inherited muscular dystrophies, it is thought that disease progression is largely determined by exhaustion of the stem-cell pool and the resulting progressive loss of muscle-regeneration potential. On the basis of this assumption, it is possible that attenuation of muscle damage or restoration of muscle-regenerative potential is key to the effective treatment of neuromuscular disorders.

Satellite cells exhaustion mechanism

Muscle regeneration is mediated by muscle SCs, which characteristically reside beneath the basal lamina and were first described over fifty years ago by Alexander Mauro (Mauro 1961). SCs are characterized by the expression of the paired box transcription factor Pax7 across species, including man, mouse and chicken (Yablonka-reuveni 2011). Several recent studies have shown that SCs are bona-fide stem cells and generate differentiating progeny (myoblasts and myocytes) and new SCs via self-renewal (Collins et al. 2005). As stated above, muscle regeneration does not proceed in the absence of SCs and loss of SC number or activity is thought to be responsible of muscle wasting observed in diverse conditions affecting skeletal muscle. There may

be several mechanisms, both cell-intrinsic and -extrinsic, underlying SC exhaustion in dystrophic muscle and here we will discuss several of the mechanisms that have been proposed.

In some hereditary myopathies, the association between the gene defect and SC exhaustion is very clear. In these cases, the 'disease' gene is normally expressed in SC in healthy individuals. The absence or loss of function of this gene directly affects SC function. For instance, lamin A/C deficiency in Emery-Dreifuss myopathies induces premature SC differentiation and cell cycle exit (Frock et al. 2006). As a result, the SC pool is depleted and the muscle regenerative potential is progressively lost.

Other disease-causing genes, such as dystrophin, are not expressed in the SC compartment, but only in the terminally differentiated myofibers. While loss of function of these genes is directly related to myofiber stress and damage, the lack of expression of these genes does not directly affect SC behavior. In these cases, the progressive muscle wasting alters the muscle architecture in a process that involve inflammation, fibrosis or deposition of non-myogenic material (adipogenesis), as described for DMD (Heslop et al. 2000). As SCs reside in a special niche formed by the basal lamina, SC function and survival is dependent on the availability of this niche. Detrimental changes in the muscle architecture inhibit the potential of SCs to regenerate the muscle, or even induce SC death.

The importance of the SC environment in determining the muscle regenerative response has also became clear from heterochronic transplantation studies. These studies showed that the age of the host determines the efficiency of muscle-regenerative response (Carlson and Faulkner 1989). Aged muscle progenitors were capable of efficient muscle regeneration when transplanted into a young host. More recent findings using a heterochronic parabiosis approach further substantiated these early findings and showed that circulating factors play key roles in determining the regenerative potential of aged SCs (Conboy et al. 2003, Conboy et al. 2005 reviewed in Conboy and Rando 2012). The progressive apoptosis of SCs observed in stress urinary incontinence (SUI), which is an age-related myopathy, has been proposed to result from a changing (ageing) environment (Strasser et al. 1999) and would support the findings in the (parabiotic) mouse studies. In conclusion, changes in the availability of 'regenerative factors' in the aged or diseased environment (either niche or circulating factors) limit an adequate regenerative response of SCs and contribute to functional and numerical loss of SCs.

As a third mechanism of SC exhaustion excessive activation/proliferation of the SC pool has been proposed, such as for instance in DMD (Decay et al. 2000) and chronic obstructive pulmonary disease (COPD) (Theriault et al. 2012). As stated above, some dystrophies and muscle-wasting conditions are characterized by continuous cycles of degeneration and regeneration, and lead to excessive use of muscle SCs. This is thought to induce replicative stress and is attributed to telomere erosion (Sacco et al. 2010) or oxidative stress (Rodriguez and Tarnolsky 2003). As a result, the SC pool becomes progressively depleted. The association of replicative stress by telomere erosion with the dystrophic phenotype is underscored by findings in the mouse model

of Duchenne Muscular Dystrophy, the MDX model. Relative to human patients, MDX animals have a very mild phenotype and a near-normal lifespan. However, using MDX/mTRnull (mTR= telomerase RNA component Terc) compound mouse model, the authors showed that loss of telomere maintenance exacerbated the phenotype of the MDX mouse, more closely mimicking the disease progression observed in patients. The study shows that the SC function is compromised and the SC pool becomes depleted with a disease progression. Myoblasts isolated from the MDX/mTRnull mice were also found to have significantly shorter telomeres. Taken together, these findings suggest that loss of telomere length and replicative stress contribute to the muscle pathology in DMD (Sacco et al. 2010). A recent study on a modest number of patients found reduced minimal telomere length in limb muscle in COPD patients, resulting in an exhausted muscle regenerative capacity and a muscle-wasting phenotype (Theriault et al. 2012). This indicates that exhaustion of the SC pool through excessive proliferation is not only restricted to DMD, but may also contribute to loss of muscle function and mass in other muscle disorders as well.

Cell-based therapies

Therapy of muscle disorders

There are currently few treatment options for muscle disorders (Castets et al. 2011). One of the few myopathies for which a relatively effective treatment modality is available is Pompe's disease. Patients with this disease develop skeletal muscle pathology due to storage of glycogen in the lysosomes caused by acid α-glucosidase (GAA) deficiency. The clinical symptoms of Pompe's disease can manifest at any age (Gungor and Reuser 2012). Patients of all ages receive enzyme replacement therapy (ERT). The rapid demise of infants with symptoms presenting at birth is prevented by correction of their cardiac hypertrophy and by the maintenance of their pulmonary function. Most treated infants acquire sitting and walking abilities while they would have had a life expectancy of less than 1 year if untreated (Kishnani et al. 2007). Patients with later onset and less progressive forms of Pompe's disease benefit from ERT and show improved walking capacity and stabilization of pulmonary function (Van der Ploeg, et al. 2010). Recent results suggest that ERT in these patients also prolongs survival (Gungor/ van der Ploeg, personal communication). Despite the success of this treatment, a number of limitations are associated with ERT (e.g. poor responder patients, development of resistance to ERT, high treatment costs) thus explaining the need for novel treatments.

For most of the other muscle disorders, no treatments are currently available and most approaches offer palliative care. However, some of the treatments that are in use, such as for instance glucocorticoid treatment for DMD, actually attenuate disease progression. Glucocorticoid treatment slows down the loss of muscle strength, prolongs ambulation, and supports respiration (Manzur et al. 2008) and even though suboptimal it is currently the standard treatment for DMD (Ruegg and Glass 2011).

It remain, nevertheless, more a confort therapy than a real treatment per se.
Some experimental therapies (such as exon skipping for DMD (Aartsma-Rus 2012) (e.g. clinical trial identifier NCT00159250)) have reached the clinical trial phase, and the hope of a positive outcome is high. Inherited muscular diseases are promising targets for gene-therapy strategies. In most cases, the etiology of the disease involves a single gene (so-called single-gene disorders).
Cell therapy for muscle disorders is one possible treatment being considered as an alternative to ERT, gene therapy or other experimental approaches. Its promise is discussed below.

Rationale for the use of muscle stem cells in the treatment of muscle disorders
Cell-based therapies are particularly promising for the treatment of muscle disorders, as they would enable the robust regenerative properties of muscle-regenerative cells to be exploited. Muscle-regenerative cells are attractive for therapy for three reasons: their ability to generate new myofibers, to repair damaged myofibers, and to correct the genetic defect through cellular fusion. The ability to fuse and share genetic material with the regenerated myofibers is an inherent programmed activity of muscle-regenerative cells and is restricted to muscle regeneration. When cells from healthy donors are used, or when gene-corrected autologous cells are used, this property can be employed to restore expression of the disease-causing genes.
In addition to these properties, most of the cell types that are considered for muscle-regenerative purposes (see below) replenish the stem-cell pool. As discussed previously, exhaustion of the endogenous stem cell pool is thought to contribute to the muscle-wasting phenotype that is common to a subset of muscle disorders. The self-renewing transplanted cells continue to be recruited during ongoing cycles of regeneration and expand the regenerated area. Over time, the condition of the transplanted muscles improves, potentially restoring the function of the affected muscles. Based on these properties, we among others hypothesize that the use of cells with myogenic potential might arrest or attenuate the muscle-wasting process that is common to all myopathies.

Several donor cell types as a source of muscle stem-cell therapy
Skeletal muscle is known to harbor several populations of stem cells, including SCs (Mauro 1961, Yablonka-Zeuveni 2011, Collins et al. 2005), interstitial cells (Mitchell et al. 2010) and vessel-associated cells (Dellavalle et al. 2007), and novel candidates continue to be identified. The predominant muscle-resident stem cells are the SCs, which are mainly responsible for postnatal muscle growth and regeneration as recently demonstrated (Sambasivan et al. 2011, Lepper et al. 2011, Murphy et al. 2011). It is still unclear whether the non-SC populations are involved in physiological and pathophysiological muscle repair in adult muscle and, if so, which role they play. As vessel-associated cells have been shown to contribute to the SC pool early in postnatal life (Dellavalle et al. 2011), it has been suggested that some of the non-SC population are SC progenitors (Relaix et al. 2012)]. But as SC populations (Sacco et al. 2008,

Cerletti et al. 2008) and non-SC populations (Mitchell et al. 2010, Dellavalle et al. 2007) both display potential to regenerate muscle and replenish the endogenous SC pool upon transplantation, both qualify as significant candidate donor-cell populations for cell therapy.
The various muscle-regenerative cells have different properties, and ideally the candidate donor cells should comply with the following features. The cells should:
- have a robust muscle-regenerative potential
- have the potential to expand ex-vivo while maintaining their regenerative properties
- contribute to the stem-cell population and replenish the SC pool
- induce minimal immunogenicity
- have the potential to be delivered systemically, although cells delivered locally may be clinically relevant.

These guidelines will be discussed for two candidate cell populations with distinct properties: cells with the highest myogenic potential after local delivery (myoblasts/SCs), and cells that can regenerate muscle after systemic delivery (vessel-associated cells). The properties of other muscle-regenerative cells are summarized in Table 1.

Muscle stem cells for local delivery: myoblasts/satellite cells

The myogenic lineage constitutes to different cell populations with distinct phenotypical and functional properties. SCs (Pax7-expressing cells in the mouse; Fig. 3) are the predominant muscle resident stem cell population and are capable of proliferation and self-renewal. Upon activation, SCs enter the cell cycle and progress to become myoblasts (pax7-/myod+; Fig. 3), which represent committed progenitors (Wen et al. 2012, Beauchamp et al. 1999). Equipped with limited self-renewal, but extensive differentiation potential, myoblasts undergo a limited number of divisions before differentiating into myocytes (Fig. 3). Myocytes are differentiated muscle cells that have upregulated myogenin and are programmed to fuse either with each other (thereby forming neofibers) or with damaged myofibers.
Based on their extensive proliferation and differentiation potential in vitro, myoblasts have long been considered for muscle cell therapy. Initially, very promising results were obtained by using myoblasts as donor cells for transplantation purposes (Myoblast Transfer Therapy) (Partridge et al. 1989). However, subsequent studies revealed a number of obstacles that complicated their introduction into the clinic. These included poor survival, immune rejection, and limited migration of the donor cells (Qu et al. 1998, Beauchamp et al. 1999).
SCs have greater regenerative potential than committed progenitors (Montarras et al. 2005). Recent studies have indeed demonstrated the remarkable muscle-regenerative potential of freshly isolated SCs (Sacco et al. 2008, Cerletti et al. 2008, Rocheteau et al. 2012), which succeeded in repopulating muscle even after transplantation of single SCs (Sacco et al. 2008). After transplantation into muscle of MDX hosts, SCs were able to restore dystrophin expression (Cerletti et al. 2008). This also rebuilted interest in the therapeutic potential of myogenic cells. Beside contributing to muscle

regeneration, transplanted SCs gave rise to new SCs indicating that SCs retain the potential to self-renew upon transplantation (Cerletti et al. 2008, Sacco et al. 200, Rocheteau et al. 2012). This self-renewal capacity was recently confirmed in a serial transplantation assay (Rocheteau et al. 2012), the most stringent assay to demonstrate self-renewal potential.

Unlike myoblasts, SCs have low immunogenicity. Cerletti and colleagues have shown that healthy SCs transplanted into the muscle of immune-competent MDX mice provided robust donor cell engraftment and contributed to the formation of host-donor chimeric myofibers that lasted up to 4 months after transplantation (Cerletti et al. 2008). The authors even reported reduced inflammation of the host muscle, indicating that the transplanted cells did not generate a strong immune response.

Despite their promising regenerative potential, SCs share a major limitation being their low migratory potential. SCs and myoblasts therefore have limited or no ability to engraft after systemic delivery, while a contribution to muscle regeneration following intramuscular injection is often observed only in the proximity of the injection site. Recently, this was also verified for systemically delivered human muscle progenitors (i.e. SC-derived myoblasts) that failed to engraft in dystrophic muscle (Dellavalle et al. 2007). The same study showed that human muscle progenitors contributed robustly to muscle regeneration after intramuscular injection.

Taken together the properties of SCs and, to a less extent, of myoblasts are most suitable for the treatment of disorders affecting localized muscles, such as stress urinary incontinence (rhabdosphincter mainly affected) or oculopharyngeal dystrophy (affecting primarily the extraocular muscles).

SCs may also be used to regenerate selected muscle in systemic muscle disorders. For instance, it has been suggested, from a DMD patients' perspective, that it would be invaluable to preserve or improve the function of hand and finger muscles (Patel and Morgan 2012). In addition, the diaphragm muscles in DMD or Pompe's disease would be attractive targets for SC-based therapy.

Mesenchymal stem cells for systemic delivery: vessel-associated cells/Mesangioblasts/Pericytes

Currently, the most promising candidates for muscle cell therapy are the cells isolated from the wall of blood vessels in the embryo (De Angeli set al. 1990) (mesangioblasts) or in the adult (pericytes) (Dellavalle et al. 2007). In adults, mesangioblasts are thought to be a subset of pericytes (Dellavalle et al. 2007, Wen et al. 1985). For reasons of clarity, both cell types are discussed here as vessel-associated cells, which can be isolated from vessels throughout the body, are multipotent, and can differentiate into different types of mesodermal cells. When isolated from the vessels present in muscle, these cells were shown to be robustly myogenic in vitro and in vivo (Dellavalle et al. 2007, De Angelis et al. 1990). Interestingly, after transplantation, vessel-associated cells contribute to the SC pool (Dellavalle et al. 2011). This was explained by the fact that vessel-associated cells and SCs share a common origin in the embryo. Even in response to muscle-toxins or dystrophy, these 'vessel-associated cell-derived'

SCs expressed Pax7 and contributed to muscle homeostasis and regeneration (Dellavalle et al. 2011). This may explain their ability to contribute to muscle regeneration under certain conditions, for example after transplantation to distressed muscle.
The muscle-regenerative (and therapeutic) potential of the vessel associated cells is clearly indicated by their ability to restore or ameliorate the dystrophic phenotype after transplantation to dystrophic mice (α-sarcoglycan-null mice (Sampaolesi et al. 2003) and dysferlin-deficient mice (Diaz-Manera et al. 2010)) and golden retriever muscular dystrophy (GRMD) dogs (Sampaleosi et al. 2006). The ability of vessel-associated cells to morphologically and functionally restore the dystrophic phenotype in α-sarcoglycan-null mice (the animal model for limb-girdle muscular dystrophy 2D) indicated that a robust immune response to these cells was lacking or did not limit engraftment. Also, vessel-associated cells from sources other than muscle are shown to have low immunogenicity (Katare et al. 2011, Maier et al. 2011). Given the robust immune response (and hence limited engraftment) observed after myoblast transplantation, this property may be an important attribute for the therapeutic potential of vessel associated cells.
One drawback may be that in absence of well-defined markers, it has been difficult to prepare pure populations of vessel-associated cells with robust reproducible regenerative potential (Meng et al. 2011). Additional cell types may contaminate the isolates and fail to contribute to regeneration, thereby affecting the experimental outcome.
Vessel-associated cells are attractive candidate for therapy due not only to their muscle-regenerative potential but also to their ability to proliferate in vitro. It was reported that they could expand up to 20 population-doublings before undergoing senescence. This was claimed to be sufficient to treat a young patient (Dellavalle et al. 2007).
In conclusion, the properties of vessel-associated cells, particularly their compatibility with systemic delivery, recommend these cells as significant candidates for treating systemic muscle disorders such as DMD and limb-girdle muscular dystrophy.

The ex-vivo expansion of regenerative cells

SCs comprise about ~4% of myonuclei in human adult muscle and only limited numbers of regenerative cells can be obtained from patient muscle samples. This indicates that extensive ex vivo expansion is required to increase cell numbers and thereby the feasibility of cell-therapy. However, culturing freshly isolated (mouse) SCs and human muscle progenitors lead to the generation of committed progenitors whose regenerative potential is reduced (Qu-Petersen et al. 2002, Montarras et al. 2005, Parker et al. 2012). This loss of regenerative potential upon ex vivo expansion is not unique to the culturing of SCs. It is also acknowledged for other stem cell types that are used for therapy, including hematopoietic stem cells (HSCs) (Dahlberg et al. 2011). Even vessel-associated cells, which can be expanded rather extensively ex vivo, eventually undergo senescence while further expansion may be required to treat adult or severely affected patients.
The need for refined culturing techniques is most apparent for SCs, and great pro-

gress has been made in understanding the mechanisms that regulate their stem cell properties. Here we discuss various culturing techniques and how they may be used in future studies to expand cells with the highest regenerative potential.

Understanding the regulation of stem cell fate

The endogenous stem cell pool is maintained in vivo through the tight regulation of self-renewal and differentiation. The regulation of this process is highly complex and is determined largely by environmental factors. The importance of the stem cell microenvironment or niche has been convincingly shown for SCs. SCs are polarized cells with a basal membrane rich in α7/β1-integrin (Song et al., 1992; Burkin and Kaufman, 1999) that is in direct contact with the laminin-rich basal lamina surrounding the myofibers. The apical membrane of the SC expresses M-cadherin and receives signals from the myofiber. Displacement of one satellite daughter cell from the niche after dividing perpendicular to the length axis of the myofiber results in lineage commitment of the apical daughter. The basal daughter remains in the niche (defined by the basal lamina) and retains the stem cell fate. In contrast, SCs dividing in a planar orientation generate daughter cells with identical stem cell fate, as the dividing cells maintain contact with the basal lamina (Kuang et al. 2007).

Other indications for the dominant effect of the environment on stem cell fate were obtained from heterochronic transplantation studies. SC age-related loss of regenerative potential could be restored by heterochronic transplantation of aged SCs into a young environment, while the reverse transplantations were ineffective (Carlson et al. 1989, Conboy et al 2003, Conboy et al. 2005). The importance of the proper environment to dictate the regenerative potential of its associated stem cells is further demonstrated by the success of intact single myofiber transplantation in contributing to new myofibers and the generation of donor-derived SCs (Collins et al. 2005). During the transplantation procedure the SCs remained in their natural niche in these intact myofibers, which is thought to be vital for ensuring their robust regenerative potential. The results of these studies strongly suggest that the signals for governing cell fate and regenerative potential can be identified by dissecting the SC microenvironment. The niche is composed of both soluble and solid biochemical signals (e.g. oxygen, growth factors, nutrients, cytokines, extracellular matrix proteins) and confers biophysical signals (e.g. matrix stiffness, fluidity, oxygen tension).

In addition to signals from the environment, cell-specific factors are critical, and the cell within the niche should be properly programmed to interpret the stem cell signals. This has been shown for bone marrow-derived cells (BMDC), which occasionally occupy the SC niche (Sherwood et al. 2004). These BMDCs did not acquire a myogenic fate during their residency in the SC niche. There are also numerous examples where conditional targeting (e.g. inactivation) of a SC-specific gene that had no affect on the niche, resulted in activation, proliferation and often premature differentiation of SCs. For instance, a recent study targeted Myf5 mRNA expression by inactivating Mir31, which targets Myf5 in quiescent stem cells and prevents accumulation of Myf5 protein (Crist et al. 2012). Myf5 belongs to the family of muscle

regulatory factors (MRFs), which also includes MyoD, MRF4 and myogenin, and is expressed in quiescent SCs and early muscle progenitors. After inactivating Mir31 by the intramuscular injection of specific antagomirs (chemically designed oligonucleotides used to silence Mirs), quiescent SCs re-entered the cell-cycle, and muscle regeneration increased. This was deduced by the presence of an increased number of small embryonic myosin heavy chain (eMHC, detected only in regenerating myofibers) positive myofibers. In addition, two recent studies showed that conditional SC-specific inactivation of RBP-J, a nuclear factor essential in Notch signaling, resulted in SC depletion and loss of muscle regenerative potential (Bjornson et al. 2012, Mourikis et al. 2012), while the niche remained intact in these animals. These studies indicate that targeting certain cell intrinsic factors dictates cell fate, an effect that may be exploited during ex vivo culturing.

Expanding or selecting subpopulations with higher regenerative potential

SC populations are phenotypically and functionally heterogeneous (Biressi and Rando 2010), their regenerative potential varying between SC subpopulations. The heterogeneity in regenerative potential of SC subpopulations is maintained ex vivo (Rocheteau et al. 2012, Beauchamp et al. 1999, Ono et al. 2012), which may allow the selection and expansion of the most highly regenerating subpopulations. It will be then necessary to identify and trigger the proper stimuli favoring the expansion of the population of interest.

A recent study took a label-retention approach to select the slow dividing cell (population able to retain the label upon time) population from SC-derived muscle cultures (Ono et al. 2012). In several types of tissues and cultures there are indications that slowly dividing cells represent the subpopulation with increased stem cell potential. For instance, quiescent HSCs has increased survival after transplantation while short-term culture induced cell cycle reentry and failure of the cells to reconstitute NOD-SCID animals (Jetmore et al. 2002). Also, a slowly dividing population identified in murine SC-derived muscle cultures was shown to harbor increased myogenic potential in vivo and to generate a functional SC population. Because the dyes used for the label-retaining experiments are DNA-binding chemicals this strategy for clinical purposes will be a relevant issue.

FACS sorting is widely used to enrich for cell populations (Kiel et al. 2005, Purton and Scadden 2007) and several cell surface markers, including CXCR4 and CD133, have been reported to allow the isolation of highly regenerative cells directly from donor muscle (Cerletti et al. 2008, Sacco et al. 2008). These sorted subpopulations have a high regenerative potential and it is of clinical interest to expand them ex vivo. However, a minimal ex vivo expansion may be required since the transplantation studies have shown that only a limited number of these cells would be needed to obtain robust engraftment potential. Unfortunately, the FACS-sorted populations either lose their regenerative potential upon ex vivo expansion (Sacco et al. 2008) or they have limited potential to proliferate in vitro (Negroni et al. 2009). So, to maintain the high level of regeneration potential, FACS-sorted populations should be cul-

tured under optimized conditions, as will be described below (e.g. by stimulating self-renewing expansion).
Alternatively, as muscle populations remain heterogeneous in culture and harbor subpopulations with increased regenerative potential (Beauchamp et al. 1999), a FACS-sorting strategy may allow purification of engraftment competent cells from extensively expanded muscle cultures. However, no cell surface marker(s) have been identified so far to use such a strategy.

Inducing self-renewing expansion
Much work has been done to understand the molecular pathway that contribute to the self-renewal of SCs and prevent their premature differentiation. These studies have revealed important roles for soluble signaling molecules, including Notch and Wnt ligands, and also for several membrane proteins such as caveolin-1 and syndecan 3/4 (reviewed by Kuang et al 2008). Most of the knowledge is derived from studies investigating in vivo mechanism, but the importance of these pathways for self-renewal have also been verified in vitro (Parker et al. 2012).
The importance of the Notch pathway in regulating SC behavior and size of the SC pool was shown in earlier studies where pharmacological inhibition of Notch signaling inhibited the proliferation and self-renewal potential of SCs, while the enhancement of Notch activity restored the regeneration potential of aged muscle (Kuang et al. 2007, Conboy et al. 2003). As stated above, SC-specific inhibition of Notch signaling in vivo by conditional inactivation of RBP/J induced premature differentiation. These Notch-inhibited SCs differentiated without first undergoing cell division and fused with adjacent fibers. As a result, the SC pool was gradually depleted (Bjornson et al. 2012, Mourikis et al. 2012). A similar effect was shown on embryonic muscle progenitors after deleting RBP/J (Vasyutina 2007). In Hes1/3 double knockout mice (target genes of Notch signaling), a defect in generating undifferentiated SCs was observed and SC numbers decreased gradually (Fukada et al. 2011). On the other hand, constitutive Notch activation in vivo increased Pax7 expression and promoted SC self-renewal (Wen et al. 2012).
Notch activity was also shown to determine self-renewal and to increase the number of undifferentiated SCs (Pax7+/MyoD-) in vitro (Kuang et al. 2007). A recent study evaluated the role of Notch signaling on SC self-renewal by culturing canine SC-derived myoblasts on polystyrene culture plates coated with IgG-bound Notch ligand Delta1ext (Parker et al. 2012). Upon transplantation, the myoblasts that had been expanded on Notch ligand contributed to muscle regeneration as efficiently as freshly isolated myoblasts. Furthermore, the Delta1ext-expanded cells generated stem cells in vivo. In other words, they were capable of self-renewal as shown by the engraftment of the Delta1ext-expanded cells in secondary recipients (Dellavalle et al. 2007, De Angelis et al. 1999). These experimental outcomes show that Notch signaling is important to SC self-renewal and that manipulation of Notch should be considered for ex vivo expansion protocols.
In addition to Notch signaling, the Wnt pathway is known to contribute to SC self-

renewal and cell fate choice in vivo (Brack et al. 2007, Legrand et al. 2009). Wnt7a, but not Wnt3a, was shown to activate planar cell division (see above), thereby promoting symmetric SC expansion in vivo (Legrand et al. 2009). Therefore the activation of Wnt pathway helps to induce the self-renewing expansion of cultured SCs. Indeed, Wnt7a was shown to promote self-renewing division of Pax7+/MyoD- SCs, but only in isolated myofiber cultures and not in primary myoblasts grown on a regular culture dish. Le Grand and colleagues determined that stimulation of self-renewing division by Wnt7a proceeded through the Wnt planar polarity pathway (PCP). This indicated that maintenance of cell polarity is essential to mediate the effect of Wnt7a. In myofiber cultures, SCs are in their natural environment and cell polarity is maintained (Kuang et al. 2007), while in regular 2D cultures polarity is lost. Although the study of Le Grand and colleagues showed that Wnt activity regulated symmetric self-renewing expansion of SCs, pharmacological stimulation of Wnt activity may not be sufficient. Instead it may be necessary to reconstruct the niche in vitro. For instance, to maximize benefit from soluble factors (such as Wnt7a) that promote self-renewing divisions of cultured SCs, it may be necessary to optimize the culture substrate (discussed below).

Inducing SC activation and proliferation: a two-step approach

After activation, in vivo quiescent SCs enter the cell cycle and proliferate (Shea et al. 2010). Most of the population progresses to committed myoblasts, which continue to divide for a limited number of cycles before differentiating into myocytes. Several studies indicate that these signals also promote SC proliferation in vitro and may be used to rapidly expand the isolated muscle cells. As discussed above, expanding SC-derived cultures under proliferation conditions dramatically reduces their regenerative potential (Sacco et al. 2008) and does not contribute to the generation of highly regenerative cell populations. However, this strategy may currently be the only option for expanding human muscle progenitors.

Unlike murine cultures, human muscle progenitor cells do not proliferate extensively in vitro and undergo a limited number of divisions before entering senescence (Mouly et al. 2005).

Unfortunately, not much is known on the specific factors that promote the proliferative capacity of human cells. Some pathways, including IGF-signaling (Kandalla et al. 2011) and TGF-beta pathway (with myostatin, a member of the TGF-beta superfamily that negatively affects muscle progenitor proliferation) (McFarlane et al. 2011) control the proliferative activity of human muscle progenitors. The maintenance of the proliferative potential of human muscle progenitors is important not only for their eventual clinical applications but even more to facilitate the study of the behavior of these cells in culture.

Once the conditions for efficiently expanding human muscle progenitors have been established, strategies should be followed to restore or increase the regenerative potential just prior to transplantation. Several approaches have been described that can be used to achieve this, including preconditioning, exposing the cells to hypoxia, or

limiting oxidative stress in the transplanted population.
The preconditioning approach is defined as the exposure to a sublethal insult prior to transplantation. This induces a protective response before transplantation that will allow the cells to better survive the hostile environment of the host tissue. Preconditioning has been studied mainly in the context of whole organ transplantations but recent studies suggest that cell therapy strategies may also benefit from this procedure. In a tissue engineering chamber model, preconditioning of myoblasts with the nitric oxide (NO) donor DETA-NONOate increased cell survival (and proliferation) after implantation (Tilkorn et al. 2012).
With regard to hypoxia, we have stated above that preconditioning cells under hypoxic conditions to mimic the oxygen pressure in the host tissue benefits the transplantation of SC-derived myoblasts (Liu et al. 2012). The beneficial effect of hypoxia was reported to increase engraftment almost 2-fold but it needs to be refined.
The increasing resistance to oxidative stress approach may boost the engraftment potential of the cells expanded ex vivo. The damaged or dystrophic host muscle may prove to be a rather hostile environment for transplanted cells, being characterized by necrotic and apoptotic tissue, infiltration of inflammatory cells, and deposits of non-myogenic material. The identification of signals that adversely affect engraftment is as relevant as signals promoting engraftment. The transplanted cells may initially undergo increased levels of oxidative stress, which is thought to reduce the success of engraftment (Bartoszuk-Bruzzone et al. 2012). It has been suggested that engraftment may be positively affected by adapting the conditions to limit the levels of oxidative stress in culture. Cells can be exposed to anti-oxidants, such as N-acetylcysteine or sodium ascorbate, during ex vivo expansion or just before transplantation. Relative to engraftment potential of untreated cells, the transplantation of oxidant-treated cells increased the formation of chimeric donor -host fibers about 1.7 fold.

Effect of stiffness of the culture substrate

The importance of defining the appropriate biophysical properties on the myogenic and regenerative potential of muscle cells has been shown in studies using various types of culture substrate to modify elastic stiffness. C2C12 myoblasts cultured on collagen-coated polyacrylamide gels, which approached the elasticity of skeletal muscle (~12 KPa), differentiated more efficiently than cells maintained on 'hard' plastic (Engler et al. 2004) In addition, a direct correlation has been observed between the stiffness and proliferation rates (higher stiffness leads to increased proliferation).
More recently, freshly isolated SCs were cultured on polyethylene glycol (PEG)-based hydrogels with the same rigidity of muscle in vivo (~12 KPa). The cells self-renewed in vitro and contributed more efficiently to muscle regeneration in vivo than SCs cultured on regular plastic substrates. In both studies, the use of both softer and harder substrates resulted in decreased performance in the in vitro and in vivo assays used, indicating that there is an optimal culture substrate formulation. As suggested, it will be interesting to determine whether the number of regenerative cells may be further increased by combining elastic substrates with chemically-defined media.

Generation of muscle progenitor cells by reprogramming somatic cells

In recent years it has become possible to use transient expression of 3-4 transcription factors to reprogram somatic cells to induced pluripotent stem (iPS) cells (Takahashi et al. 2006, Takahashi et al. 2007). Phenotypically and functionally, iPS cells resemble embryonic stem (ES) cells and can be expanded in vitro for many passages while maintaining both pluripotency and the ability to differentiate into cells of all three germ layers. This also eliminates the ethical considerations associated with ES cells. On the basis of these properties, iPS cells can be proposed as an attractive alternative to somatic cells.

The clinical application of iPS technology faces two major challenges: 1) how these cells can be derived without altering the genome, and 2) how they can be differentiated to homogeneity of the desired cell type. Common methods of generating iPS cells use retroviral or lentiviral gene delivery with the risk of insertional mutagenesis. Proper differentiation is important not only to obtain the cell type of choice but also to eliminate remaining pluripotent cells, which can form teratomas when placed in the wrong (non-embryonic) environment. Recently, important progress has been made. Various methods for non-viral gene expression have been reported, including those using Cre recombinase-mediated transgene excision (Soldner et al. 2009) and gene expression via the non-integrating Sendai virus (Nishimura et al. 2011) as well as mircroRNA (Anokye-Danso et al. 2011).

A number of reports document the successful generation of myogenic progenitors from mouse and human iPS cells and engraftment of these cells in mouse models for human muscular dystrophies (Barbari et al. 2007, Mizuno et al. 2010, Quattrocelli et ak. 2011). Major differences between various studies include the protocol used for generating myogenic progenitors, the efficiencies of these efforts, and the capacities of generated cells for long-term engraftment and functional improvement.

An efficient method that results in successful long-term engraftment and functional improvement (i.e. 8 months in the mouse) was reported recently by Darabi et al. who, on the basis of previous observations using mouse ES or iPS cells (Darabi et al. 2008, Darabi et al. 2011), used inducible expression of pax7 during embryoid body formation of human iPS cells. A straightforward FACS sorting approach based on co-expressed GFP proved sufficient to purify myogenic progenitors to homogeneity. No teratomas were observed after transplantation. The endogenous markers used for purification in the mouse were PDGFa+/Flk-, though it is unclear whether these markers may be used in human as well. Importantly, intramuscular injection into the Tibialis Anterior muscle of a mouse model for Duchenne Muscular Dystrophy resulted in successful engraftment and the partial restoration of dystrophin expression. Donor dystrophin expression was still present 46 weeks after transplantation. Similarly, muscle function improved and a fraction of engrafted cells contributed to the endogenous SC population, suggesting that the iPS-derived progenitors self-renewed in vivo.

This work thus presents an important proof of principle for using iPS cells in the

long-term treatment of muscular dystrophy. Challenges for the future include: efficient 1) generation of human iPS cells, 2) gene correction, 3) cell differentiation without functionally changing the human genome, and 4) the efficient delivery of cells to various muscles using intravenous or intra-arterial administration.

Summary on cell based therapies for muscle

Despite its promise and potential, cell-based therapy for muscular dystrophies is still in its beginning. Although the clinical efficacy of myoblasts has turned out to be rather disappointing, the identification of additional cell types or populations especially satellite cells and vessel-associated cells that can regenerate muscle provide new hope for cell-oriented therapy. Their specific properties would indicate use in the treatment of distinct muscular diseases, which require either systemic (vessel associated cells) or local delivery (SCs). The progress in the field of cell-based therapy for skeletal muscle is underscored by the stage I clinical trials with vessel-associated cells for the treatment of DMD that started in 2011. The results are awaited impatiently. SCs have not advanced to this stage as of yet and several issues require attention.

Most of the work on SCs has been performed with murine cells, and it must still be determined whether the findings described above can be applied to human SCs. Although it has been established that mouse and human SCs share many properties (reviewed by (Boldrin et al. 2010)) – including the ability to regenerate muscle upon transplantation – there are some striking differences. The isolation of human SCs is complicated by the lack of highly specific markers, and despite some strong initial indications that these cells too are bona fide stem cells, it remains uncertain whether they self-renew in vivo (Ehrhardt et al. 2007).

The progress with human muscle progenitors is dependent on methods to overcome their limited proliferative potential in culture. In the short-term, the 'two-step' approach discussed above may be the most feasible strategy for human muscle progenitors. However, strategies such as preconditioning and oxidative stress increase regenerative potential only modestly. The expansion of self-renewing cells seen in murine cultures would greatly increase the regenerative potential of the cultures that will be used for transplantation. In this respect, the identification of RCs in human muscle progenitor cultures is very promising (Abou-Khalil et al. 2009). Like their counterparts in mice (Yoshida et al. 1998), human muscle progenitor cultures have been shown to harbor a population of RCs. RCs are mononuclear cells that, under differentiation conditions, escape from differentiation and are thought to have properties of muscle stem cells. It would be of major future interest and of clinical importance to identify the mechanisms or factors that contribute to their specific maintenance or expansion ex vivo.

The next major milestone that can be envisioned for human muscle progenitors would be to evaluate their therapeutic potential in a relevant (pre-)clinical setting that involves the isolation of human SCs, their expansion and finally their transplantation into a suitable animal model. The putative therapeutic potential of expanded human SC-like muscle progenitors will only be evaluable under these conditions. The

importance of the immune system and its avoidance to engraftment success dictates that an animal model should be used that develops a relevant (i.e. human) immune response against the transplanted cells. The animal model should also make it possible to quantify the change in muscle function after cell transplantation. Given these requirements, it will be valuable to develop a humanized mouse model with a muscle phenotype (Brehm et al. 2010). Such model will also be valuable to the various laboratories that aim to use human muscle regenerative cells for therapy. The developement of alternative model physiologically relevant like it has been done with the use of a dystrophic dog model (Sampaleosi et al. 2006) should also be encouraged.
A general issue of importance associated with cell therapy is safety. The transplantation of C2C12 myoblasts, a myoblast cell line established in the late 1970s by Yaffe and Saxel was associated with muscle regeneration, but also with a propensity to generate tumors under certain conditions in vivo. It is thought that the cells may acquire a certain level of genomic instability and subsequent tumorigenic activity during the extended in vitro expansion culture. Indeed, it has recently been shown that MDSCs acquired a transformational phenotype when expanded over 200 population doublings ex vivo (Deasy et al 2005). This further underscores the importance of defining optimized conditions for expanding cells with the highest regenerative potential that may already achieve a functional effect at reduced numbers and require minimal expansion ex vivo.
A major advantage of including an ex vivo expansion phase is that quality control parameters can be implemented and Good Manufacturing Practice (GMP) guidelines be applied (see http://www.emea.europa.eu/), enabling the generation of highly reproducible cell products. More than any other technology, iPS offers the potential to generate large batches of well-defined regenerative cells that can be stored until use.
In conclusion, muscle regenerative cells remain attractive novel tools for the treatment of muscle disorders and much progress in understanding the behavior of these cells in vitro and in vivo has been made. However, it is also clear that several challenges, both with respect to practical issues and regulations, remain before introduction of a cell-based therapy for the treatment of muscle disorders becomes reality. In two chapters of this thesis two different approaches have been developed to isolate/enrich cells with engraftment potential from long-term cultures, which constitutes a good first step in the development of cell-based therapy.

Cancer stem cell (CSC) concept, clonal evolution model and Tumor Initiating cells (TIC)

Muscle structure, mass and function can be affected by a wide range of progressive degenerative diseases, described previously. Besides genetic diseases, gene alteration can lead to malignant growth.

Normal differentiated cells are constrained by the Hayflick limit and cannot divide indefinitely. Malignant cells, on the other hand, acquire certain mutations that will allow them to express cancer hallmarks described in figure 4 and evolve progressively to a neoplastic state (Hanahan and Weinberg 2000 updated 2011).

The first and most fundamental feature of cancer cells involves their ability to continuously proliferate enabling tumorigenic growth. This feature is intrinsically required for these cells to escape the proliferation control, which occurs in normal tissues. Malignant cells can acquire this feature via different ways. For example, oncogenic cells can manipulate their environment so that the cells within this environment start to produce growth factors necessary for their proliferation (Cheng et al 2008). Another way to overcome proliferation limits could be achieved by becoming hyper responsive to growth factors via increasing the levels of signaling receptors at the cell surface. Having established the basic feature of tumorigenicity, the neoplastic cells might acquire more somatic mutations allowing them to become independent of their environment, to gain their migratory and invasive potential and to eventually become metastatic.
Nevertheless, not all the cells within the tumors share the same features and the percentage of cells carrying all the cancer hallmarks remains unclear. The existence of intratumor heterogeneity is widely accepted (Nowell et al 1976, Marusyk et al. 2012) and moreover this heterogeneity leads to variations in treatment efficacy. It is not clear how intratumor heterogeneity is obtained. Two concepts attempt to explain

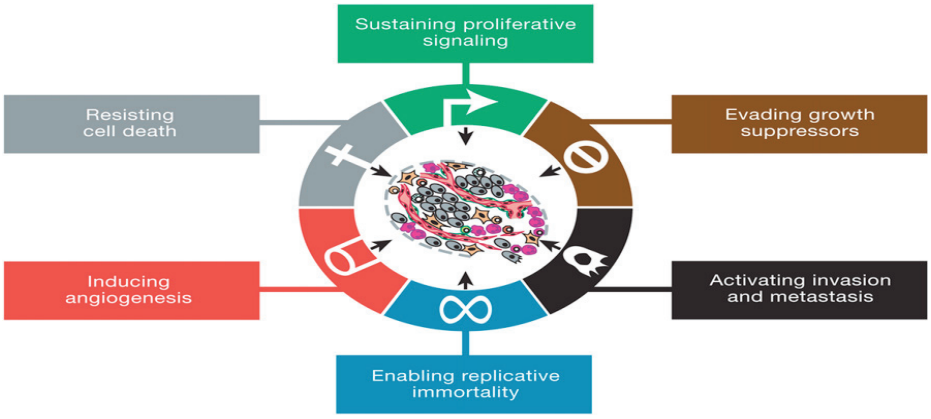

Figure 4: Hallmarks of cancer. Adapted from Hanahan and Weinberg 2011
Suggested functional capabilities required for cancer development.

this: the clonal evolution model and the cancer stem cell (CSC) hypothesis (Figure 5).

The clonal evolution model is based on Darwin's evolution theory (Greaves and Maley 2012). Upon time, several random somatic mutations occur, in series or in parallel, and subsequently a subclonal selection takes place. Thus, this model suggests a progressive accumulation of mutations with concomitant successive clone selection

steps, which increase the aggressiveness of the tumor. The pancreatic cancer (adenoma, carcinoma, metastasis) constitutes a perfect example of disease progression according to this model (Berrozpe et al. 2006).

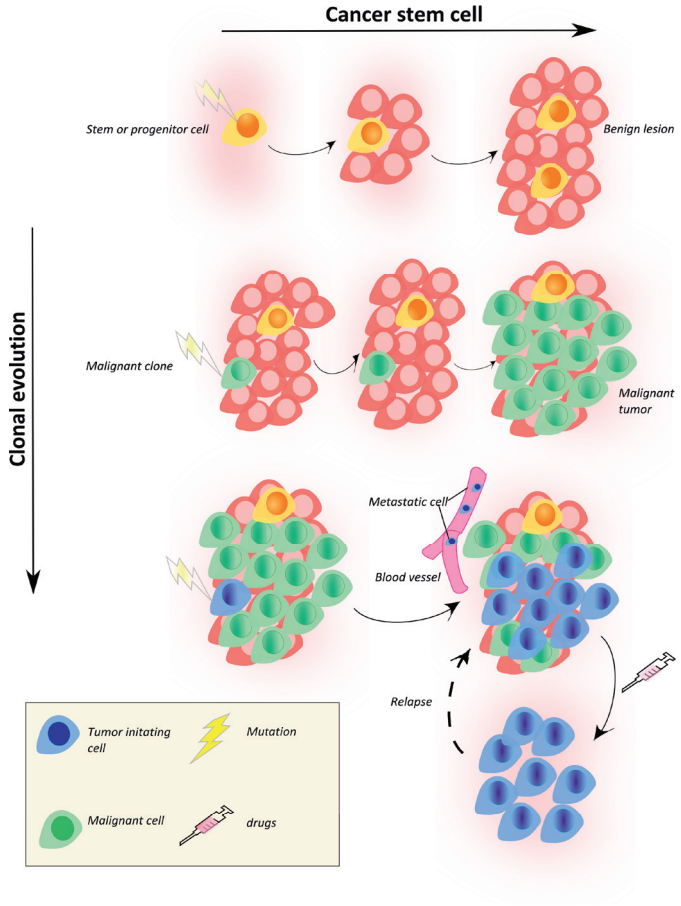

Figure 5: Clonal evolution and cancer stem cell concept. Adapted from Clevers 2011.
top to bottom: clonal evolution drives tumor progression. Left to right cancer stem cell concept. Serie of mutations leading to the generation of a malignant and invasive population. The different population represented in the final tumor reflect the intratumor heterogeneity.The cancer stem cell properties are represented: self-renewal, drug resistance and capacity to give rise to various populations.

On the contrary to the well-established clonal evolution model, the CSCs are a subject of debate (reviewed by CT Jordan 2009). CSC origin, nature, existence and representative percentage inside a tumor are actively discussed. Furthermore, the possibility that most cells within the tumor are endowed with stem cell properties is not favorable from a therapeutic point of view. The CSCs are defined as malignant cells able to self-renew and to give rise to heterogeneous progeny (classical features of any stem cell categories) (Clarke et al. 2006). Other or secondary features frequently as-

sociated with CSC are increased drug resistance and increased metastatic potential.

Bonnet and Dick published the first conclusive evidence for CSCs in 1997 (Bonnet and Dick, 1997). In this study they isolated a subpopulation of leukemic cells expressing CD34 and lacking CD38 expression, and established that the CD34+/CD38- subpopulation was capable of tumor initiation after transplantation in NOD-SCID mice. The existence of leukemic stem cells prompted further research into other types of cancer. CSCs have recently been identified in several solid tumors, including breast, colon, ovarian, pancreas and prostate cancers as well as melanoma. All those studies have demonstrated the ability of a specific subpopulation, based on the expression of a particular subset of markers, to generate tumors in vivo after transplantation in immunodeficient host.
Nevertheless several studies from Morrison lab have testing the ability of specific markers (more than 50) to define tumor-initiating cells in immunodeficient host (NOD-SCID Il2 rag -/-) (Quintana et al. 2008, Quintana et al. 2010). No major differences were observed in the ability of the cells to form melanomas, even sometimes no major differences in metastasis ability (comparison between the CD271 expressing and non expressing cells). Those studies suggest that the heterogeneity observed in some cases might not come from the intrinsic ability of the cells but more from the environment the cells are challenged with. These observations are a strong argument in favor of a clonal expansion theory more than CSCs existence.
An increased resistance to drug, which after treatment leads to tumor relapse, also characterizes CSCs. This property has been shown in RMS (Kin Wah Lee, 2011).

Upon time, a new concept emerged due to the controversy in the field: the TIC (Zhou et al. 2009). The appearance of the TIC population inside a tumor can be justified by the clonal evolution concept as well as the CSC concept. Those cells have been identified in various tumors types like colon cancer (Ricci-Vitiani et al. 2007) or melanomas (Schatton et al. 2008) in breast cancer (Al-Hajj et al. 2003) or brain tumors (Singh et al. 2004). The study referenced above and a study of 2006 (Balic et al. 2006) demonstrated together that in breast cancer patients a population of CD-24low/CD44+ cells can be found in metastasis (pleural effusions) and that the early disseminated tumorigenic cells find in the bone marrow harbor a hypothetical breast cancer potential.
However, irrespective of the tumor model to cells which allowed their appearance, the identification and elimination of this highly tumorigenic population remain clinically relevant and will constitutes a step forward in the treatment of cancer.

Rhabdomyosarcoma

Rhabdomyosarcomas (RMS) are predominantly pediatric tumors that express markers of myogenic differentiation (Huh et al. 2010). RMS is the most frequent soft tissue sarcoma of childhood (Huh et al. 2010) and is characterized by myogenic gene expression and myogenic differentiation. More than 150 years ago, Weber reported for the first time a localized enlargement of the tongue of a 21-year-old man consisting of striated muscle cells at various stages of myogenic differentiation. Almost 100 years later, Stout et al. have described more extensively the unique morphology of RMS based on the analysis of series of tumors (Stout et al. 1946).
RMS has an annual incidence of seven per million in children aged 14 years or younger (Merlino et al. 1999). The occurrence of RMS in adults, on the other hand, is rare. Two peaks of incidence can be observed: one between 2 and 4 years of age and a second between 12 and 16 years of age (Rodeberg et al 2006). The current therapy used is based on a multimodality treatment consisting in an operative resection of the primary tumor associated to chemotherapy and radiotherapy adapted in function of the RMS type.
These tumors can develop anywhere in the body, including sites where striated muscle are not normally present. The most frequent tumor locations are the head and neck (40%), including the orbit and para-meningeal regions, the genitourinary tract (20%) including the bladder and/or prostate, the uterus and vagina, para-testicular regions, and the limbs (20%) and trunk (10%). Histopathological analysis, and light and electron microscopy are used to identify the characteristic skeletal muscle features such as cross striations, sarcomeres z-bands. Immunohistochemistry for specific markers such as MyoD, Myogenin and Desmin are used to confirm the diagnosis (Wexler 2006, Dagher et al. 1999, Kumar et al. 2000, Molenaar et al. 1985).
Based on the International Classification of Rhabdomyosarcoma, RMS can be divided in 4 different subtypes. The alveolar rhabdomyosarcoma (ARMS) and undifferentiated sarcoma are both characterized by a poor prognosis due to their metastatic potential. The third subtype is the embryonal rhabdomyosarcoma (ERMS) which has an intermediate prognosis and is the most commonly encountered. Finally the botryoid and spindle cell rhabdomyosarcoma can be found. These two are usually considered as subsets of ERMS. ERMS tumors are generally associated with a better prognosis.

Besides distinct histological features the two main subtypes ERMS and ARMS can be separated by distinct morphological and clinical features as mentioned above as well as by genetic features. ARMS is characterized by the unique translocation of PAX3-forkhead (FKHR) (the t(2;13) translocation) or PAX7-FKHR (=t(1;13) chromosomal translocation). This translocation encodes a fusion protein PAX3 or PAX7 and FOXO1A resulting in a hybrid transcription factor with enhanced stability, abundance, transcriptional activity and additional target genes (Calhabeu 2012, Tiffin et al 2003). Conditional mice were developed in which the expression of the oncogene PAX3-

FKHR was dependent on the expression of specific myogenic transcription factors (Keller et al. 2004, Keller et al 2004, reviewed in Keller and Capecchi 2005). Pax3-FKHR expression during early embryogenesis (Pax3-dependent) results in severe birth defect due to the absence of the endogenous Pax3. Expression of the oncogene during prenatal state (Pax7-dependent) also fails to induce tumor formation. In a parallel study where the fusion protein Myf6-FKHR is formed (Myf6-dependent), the mice developed tumors but in a low frequency and after a long time. These results seem to argue that mature skeletal and not embryonic or post-natal muscle stem cells could be the cells of origin of ARMS.

An alternative explanation to the results obtained was that Pax3-FKHR expression alone was not sufficient for tumor formation. Using conditional knock-out alleles of either p53 or CDKN2A (encoding p14/16 ink4A) in combination of the Pax3-FKHR knock-in allele, tumor formation was accelerated and tumors formed displayed histological and immunohistochemical features of ARMS.

ERMS tumors are genetically more diverse and harbor features of genomic instability that is more frequently associated with adult carcinogenesis. The genes, which can be found frequently mutated, include RAS (Stratton et al. 1989, Schaaf et al. 2010), p53 (Felix et al 1992), Rb1 (Kohashi et al. 2008) and PTCH1 (Hahn et al 1998). A recent study showed that KRAS expression in p16/19 knock-out background SCs give rise to pleiomorph RMS (Hettmer et al. 2011). In the same study, they also show that when KRAS is expressed in non-SC population, non-myogenic sarcomas developed. It has been shown in Zebrafish that the ERMS propagating population is confined to a Myf5 expressing cell population (Ignatius et al 2012). Another study with a p53 loss and a Sonic Hedgehog pathway gain-of-function (through the loss of Ptch1) in myoblasts and SC lineages shows that the mice develop tumors with human ERMS features (Rubin et al 2011). All these studies not only show that ERMS can arise from different combinations of genetic aberrations but also that the origin of the cell population might vary and be myogenic or non-myogenic.

Moreover, some of these studies highlight the fact that RMS is composed of different cell populations with various tumorigenic, metastatic and resistance to therapy potential (Keller et al 2004, Keller et al. 2004, Rubin et al 2011, Ignatius et al. 2012). TICs are characterized by an increased resistance to drug, which after treatment leads to tumor relapse. This property has been shown in RMS (Kin Wah Lee, 2011). A chapter of this thesis is dedicated to the identification and characterization of TIC in RMS.

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Chapter 2

Cultured muscle stem cells characterized by persistent lack of CD24 expression contribute to muscle regeneration and self-renew in vivo

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Cultured muscle stem cells characterized by persistent lack of CD24 expression contribute to muscle regeneration and self-renew in vivo

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Submittted

Abstract

Muscle stem cells (satellite cells (SCs)) have the ability to engraft upon transplantation and contribute to the regeneration of injured muscle. The loss of regenerative potential upon expanding SCs ex vivo limits the use of SCs in the treatment of degenerative muscle diseases. However, increasing evidence suggests that subsets of SCs have high regenerative potential compared to the population as a whole. To test for the presence for cells with high regenerative potential in muscle cell cultures, we screened for cell surface markers that were expressed by subsets of SC-derived myoblasts. We show that CD24 is heterogeneously expressed in SC-derived and C2C12 myoblast cultures. Clonal analysis identified a rare population of cells that, in contrast to the main population, did not express CD24 upon passaging. These persistently CD24-negative (CD24-ve) cells expressed the satellite cell marker Pax7 at high levels and differentiated efficiently in vitro. Upon transplantation into injured muscle of immunodeficient mice, CD24-ve cells contributed robustly to the formation of new muscle fibers. In addition, upon serial transplantation, CD24-ve cells continued to participate to muscle regeneration, suggesting that CD24-ve cells underwent self-renewal in vivo. Muscle cells that maintained high level expression of CD24 lost the ability to differentiate in vitro and in vivo. Our results demonstrate that cells with stem cell characteristics can be purified from extensively expanded cultures using the single marker, CD24. The ability to enrich and expand cells with high regenerative potential ex vivo is an important step towards development of cell-based therapies for muscle degenerative diseases.

Introduction

Cell-based therapies are promising alternatives for the treatment of muscle degenerative diseases, including muscular dystrophies. The unique properties of muscle progenitors, which are programmed to fuse and repair damaged myofibers offer the possibility to not only deliver therapeutic genes, but also restore the regenerative capacity of muscle and reverse the clinical course.

Myoblasts, which are committed myogenic progenitors, were initially chosen as sources for muscle therapy and the first results were promising (Partridge et al., 1989). However, subsequent studies dampened the enthusiasm for myoblast transfer therapy as engraftment was limited as result of immunogenicity, high mortality and poor migration of transplanted myoblasts (Skuk and Tremblay, 2010). The field was reignited by recent studies showing the regenerative potential of freshly isolated satellite cells (Cerletti et al., 2008; Sacco et al., 2008). The transplanted satellite cells (SCs) contributed to new myofiber formation, even at single-cell dosages, and were shown to replenish the SC pool. A recent study showed that freshly isolated SCs

could be serially transplanted, demonstrating self-renewal potential of transplanted cells (Rocheteau et al., 2012). Satellite cells constitute only 2-5% of the myonuclei in healthy skeletal muscle, indicating that expansion is required to obtain sufficient number of cells for therapeutic application. However, ex vivo expansion of SCs resulted in a dramatic loss of regenerative potential (Cerletti et al., 2008; Montarras et al., 2005; Rando and Blau, 1997; Sacco et al., 2008). As a result large doses of cultured muscle cells, including primary cultures (Gussoni et al., 1992; Rando and established cell lines (Morgan et al., 1992; Wernig et al., 1991), were required to obtain engraftment and muscle repopulation. Despite the disappointing results with ex vivo maintained muscle cells, these studies also indicated that a minor population (about 1% of the transplanted population) actually engrafts and contributes to muscle regeneration (Beauchamp et al., 1999). A recent study showed that primary muscle cultures maintained a slow-dividing label-retaining population that harbored the potential to engraft and contribute to muscle regeneration upon transplantation (Ono et al., 2012). So far these engraftment-competent cells have not been characterized in detail precluding their robust and reproducible purification.

In the present study we aimed at identifying and characterizing cells with regenerative potential present in long-term muscle cultures. We were particularly interested in identifying a cell surface marker that would allow prospective isolation of the engraftment-competent cells. We found that CD24, a heavily glycosylated cell surface protein, can be used to enrich cells with increased regenerative potential. Clonal analysis showed that the majority of cell restored heterogeneous CD24 expression of the parental cultures, but that a minority of clones never re-expressed CD24 (annotated further as CD24-ve cells). CD24-ve cells expressed Pax7 homogeneously and showed a remarkable efficiency of myogenic differentiation in vitro and in vivo. Importantly, transplanted CD24-ve cells generated a pool of cells that remained mononuclear and became quiescent after regeneration was completed. These donor cell-derived 'reserve' cells contributed to muscle regeneration upon transplantation to a secondary host, suggesting that CD24-ve cells underwent self-renewal in vivo and that this population maintains the stem cell properties of self-renewal and differentiation potential even following repeated passaging.
Our results show that cells with regenerative potential can be enriched from heterogeneous muscle cultures using a single marker, CD24. The identification of CD24-ve cells in primary muscle cultures verifies the relevance of this population of cultured muscle stem cells and offers the possibility to isolate these cells from diverse muscle backgrounds. The maintenance of the regenerative potential after extensive ex vivo expansion signifies an important step forward in developing efficient cell-based therapies to treat muscular dystrophies.

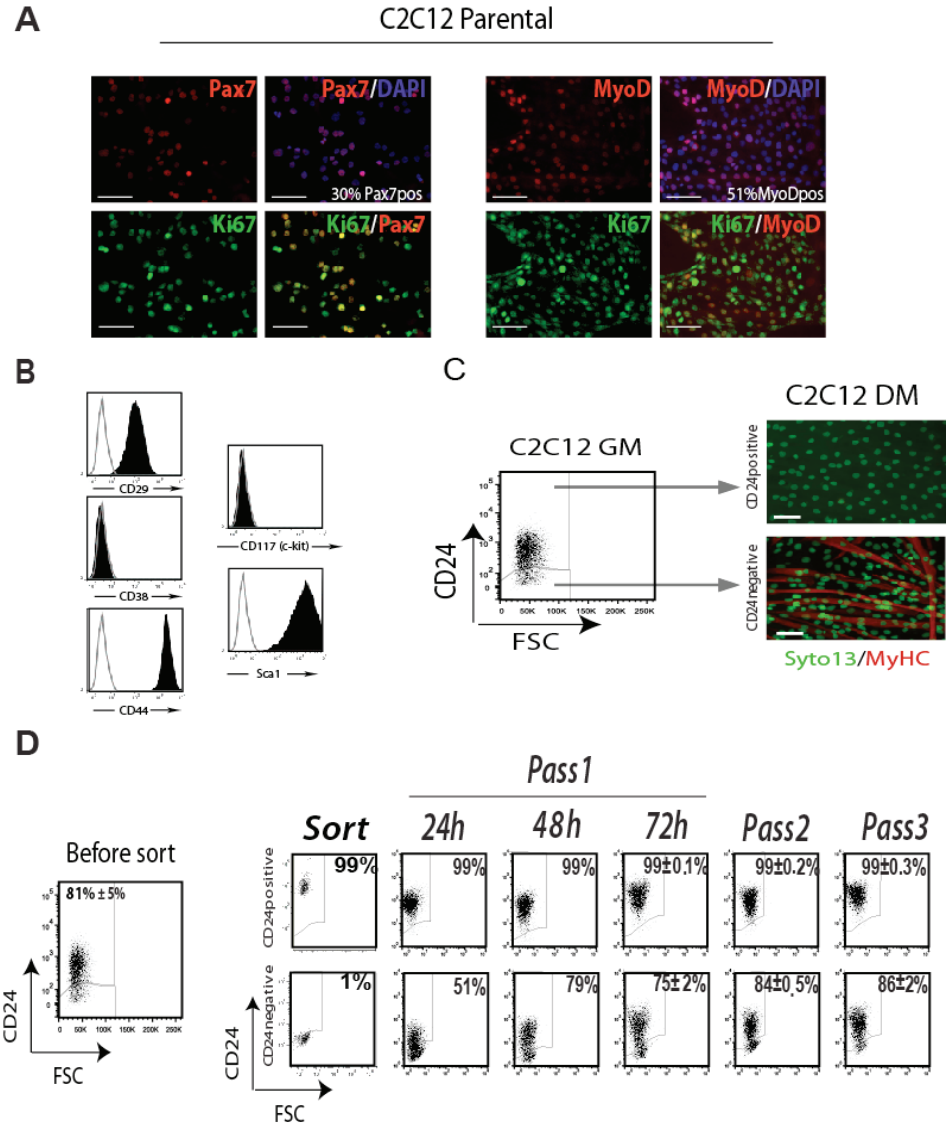

Figure 1: CD24 expression discriminates myogenic and non-myogenic cells
(A) Pax7 (red) and MyoD (red) expression was stained in proliferating C2C12. Ki67 (green) was stained as proliferation marker, DAPI was stained to visualize nuclei. (B) C2C12 cultures were stained for several surface markers as indicated in the picture. Staining intensity was analyzed by flow cytometry.
(C) CD24 expression in C2C12 cells as determined by flow cytometry. Myoblasts were sorted based on CD24 expression (gates indicated in the figure) and the CD24-sorted cells were plated in differentiation medium (DM) for 4 days. Cells were fixed and stained for MyHC and syto13 (nuclei). GM= proliferation medium.(D) C2C12 cells were sorted on CD24. Sorted populations were passaged and CD24 expression was determined by flow cytometry. Average percentage of CD24-positive cells (±SD) of three independent samples is indicated in the plots.

Results

The cell surface marker CD24 discriminates between differentiation-competent and incompetent cells in C2C12 cultures

We hypothesized that the regenerative population of a subpopulation of cultured muscle cells shown in previous studies (Beauchamp et al., 1999; Morgan et al., 1992) is represented by an immature undifferentiated population of cultured muscle cells. As described previously (16), muscle cultures are a mix of undifferentiated cells, expressing the satellite cell marker Pax7, and committed differentiated cells expressing MyoD or even myogenin (MyoG). The expression of these myogenic markers, including Pax7, MyoD and MyoG, is temporally regulated during muscle differentiation and serves as readout for the state of differentiation. To verify the heterogeneous nature of muscle cultures, we stained Pax7 and MyoD in C2C12 muscle cultures. We found that a large population of the cells expressed MyoD defining a differentiating population of myoblasts (~51 %; Figure 1A). MyoG was not expressed in proliferating cells, but was expressed after cells were induced to differentiate (data not shown). About one third of the cells expressed the satellite cell marker Pax7 (Figure 1A), expression of which is associated with regenerative potential in vivo (Rocheteau et al., 2012).

To be able to prospectively isolate the putative regenerative (Pax7-expressing) population, we proceeded to identify a selective cell surface marker expressed by a subset of cells. We screened a number of markers associated with stem cells, including as c-KIT (CD117), CD29, CD38, CD44 and Sca1, but these markers were either homogeneously expressed or not expressed at all (Fig. 1B). In contrast, the cell surface marker CD24 was heterogeneously expressed in C2C12 cultures (Fig. 1C). To test whether the CD24-defined subpopulations are functionally different, CD24-sorted populations were plated directly in differentiation medium. The CD24-negative myoblasts differentiated efficiently as indicated by the formation of myotubes. The multinucleated structures stained strongly for the terminal differentiation marker MyHC (Figure 1C). In contrast, CD24-positive sorted cells failed to differentiate and did not express MyHC (Fig. 1C. These results demonstrate that the single marker CD24 discriminates between differentiation-competent and -incompetent cells in C2C12 myoblast cultures.

CD24-negative population self-renew in vitro

To further characterize the CD24-sorted populations, we determined the CD24 expression in the subsequent passages after sorting. CD24-negative sorted populations readily re-expressed CD24 up to the level of parental cultures within 3 passages (Fig. 1D). In contrast, the CD24-positive sorted cultures remained highly CD24-positive (~95% CD24-positive cells), although at advanced passages (>10) the percentage of CD24-positive cells dropped to 90%. Our results demonstrate that CD24 is expressed dynamically in the CD24-sorted populations, but that a CD24-negative population is maintained during passaging and self-renews (Figure 1D).

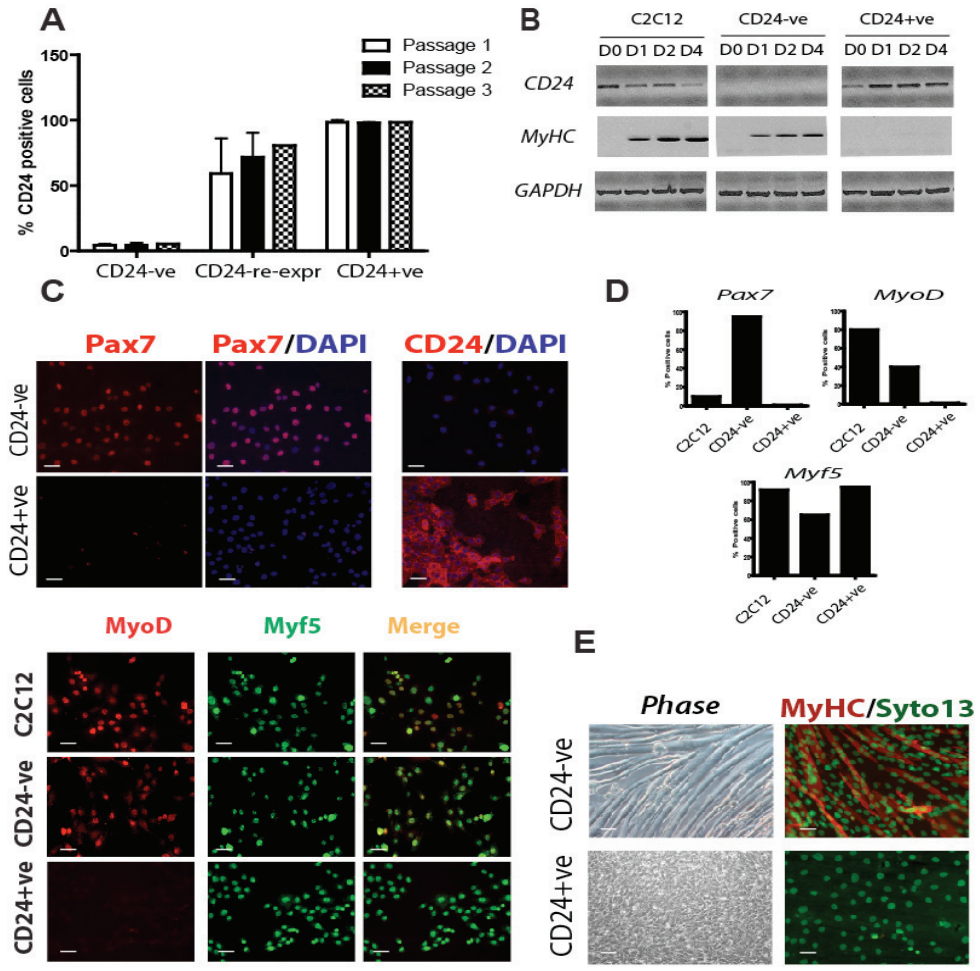

Figure 2: Muscle cultures contain three CD24-defined cellular subtypes
(A) C2C12 cells were single-cell sorted on CD24-ve and CD24+ve clones were allowed to form cultures. CD24 expression was determined in three subsequent passages. The figure depicts the average of three independent cultures for each of the three types of CD24-defined clones.
(B) RT-PCR analysis of CD24-ve and CD24+ve defined clones showing differential CD24 and MyHC expression during differentiation. Cells were switched to DM at day 0 (DO). GAPDH is shown as loading control.(C), (D) Characterization of C2C12 CD24-ve and CD24+ve clones. Proliferating clones were fixed and stained for CD24 (red), Myf5 (green), MyoD (Red) or Pax7 (red). Nuclei are stained with DAPI (blue). The histograms depict the number of Pax7-, Myod- or Myf5-positive cells as percentage of total nuclei (average of at least two independent experiments per marker). (E) Differentiation potential of C2C12 CD24-ve and CD24+ve clones. Cells were plated at high density and 48h later switched to DM. After 4 days cultures were fixed and stained for MyHC (red) and syto13 (green; nuclei).

Figure 1C shows that not all the cells in the CD24-negative sorted population differentiated, suggesting functional heterogeneity within this population. To examine this heterogeneity we performed a clonal analysis. C2C12 cultures were single-cell sorted on CD24 and the expression of CD24 of the clones in the first passage after sorting was determined (see also Fig. S1). CD24-negative sorted cells generate two types of clones: the majority of clones re-expressed CD24 up to the level found in the original culture and a minority of clones with low level of CD24 expression (Fig. S1A). CD24-positive sorted cells predominantly generated cultures that maintained high levels of CD24 expression.

To determine the stability of CD24 expression in the clones, we tracked changes in CD24 expression of more than 100 clones during subsequent passages. This analysis identified three distinct types of clones in C2C12 cultures (Fig. 2A): rare clones that never re-expressed CD24 (annotated as CD24-ve; 2-5% of the population), clones with heterogeneous CD24 expression (60% of clones) and clones generating homogeneously CD24-positive cultures (CD24+ve clones; 35% of clones). The clones that generated cultures with heterogeneous expression appeared similar to the parent cultures with respect to differentiation potential and are further regarded similar to parent C2C12 cultures. Absence of CD24 expression by CD24-ve cultures was demonstrated by RT-PCR (Fig. 2B), immunostaining (Fig. 2C) and Western blot analysis (see Fig. S1B). These analyses also confirmed CD24 expression in parent and CD24+ve cultures (Fig. 2B-C, S1B. Taken together, our data shows that C2C12 cultures harbor a rare population of cells that are characterized by lack expression of CD24.

Next we wondered if CD24-ve and CD24+ve cells were at different stages of myogenic differentiation. To characterize these clones, the expression of myogenic markers was determined, similarly as described above for the parental cultures (see Fig. 1A). Three individual C2C12 CD24-ve clones were expanded and analyzed shortly after sorting. CD24-ve cells homogeneously expressed Pax7, while MyoD and Myf5 (Fig. 2C) were expressed at variable levels, indicating heterogeneity within this population. Myogenin was not expressed in proliferating CD24-ve cells (Fig. S1C).

Proliferating CD24+ve cells were negative for Pax7 and MyoD, but myogenin and Myf5 were expressed (Fig. S1C-D and 2C). In differentiation conditions, CD24+ve cells expressed low levels of myogenin (data not shown) and MyHC RNA (Fig. S1E). Parent cultures expressed the myogenic transcription factors heterogeneously as shown in Figure 1A. Taken together CD24-ve cultures are enriched with immature, uncommitted cells. CD24+ve cells are committed myogenic cells, but are arrested in completing myogenic differentiation.

To verify that clonal analysis did not compromise the differentiation capacity, C2C12 CD24-ve and CD24+ve clones were induced to differentiate. CD24-ve cells formed multinucleated myotubes highly efficiently, while CD24+ve cultures failed to differentiate (Fig. 2E). As expected the differentiation potential of CD24-ve and parental cells was comparable as judged by the similar fusion index, defined by the percentage of nuclei in myotubes (data not shown).

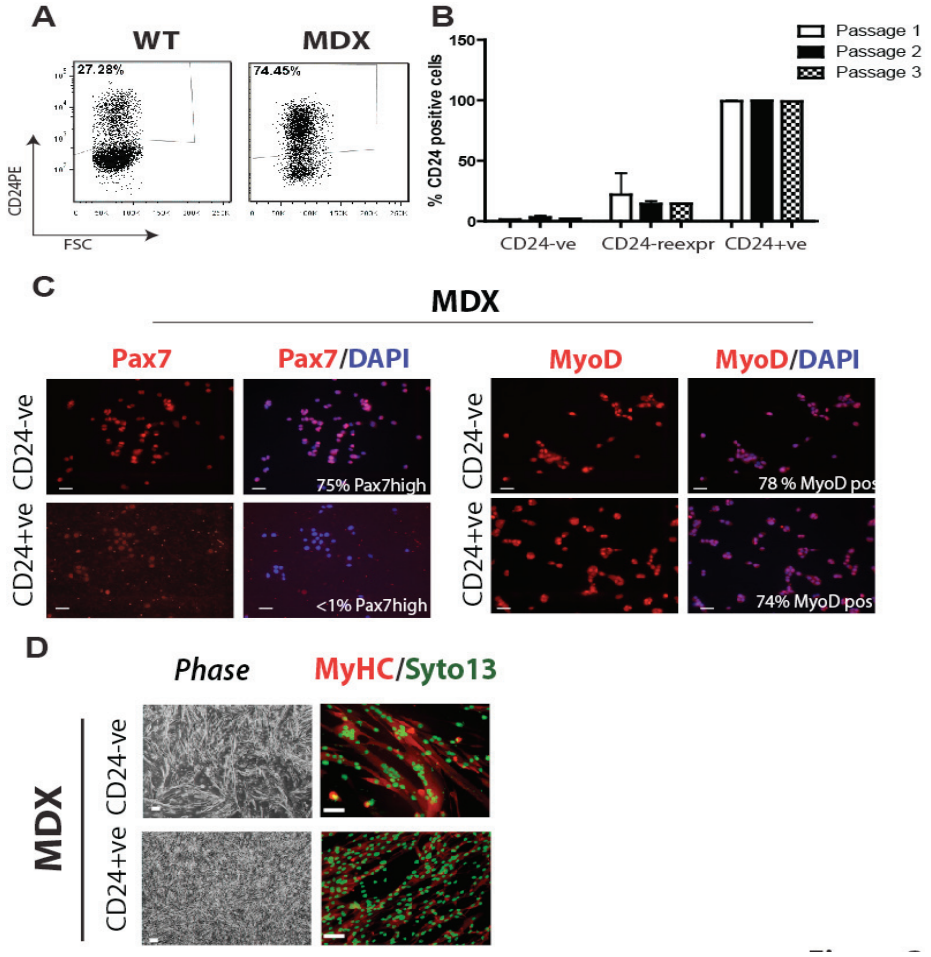

Figure 3 CD24-ve cells are identified in primary muscle cultures

(A) Representative FACS plot of WT and MDX primary cultures showing heterogeneous CD24 expression.

(B) Primary WT and MDX cells were single-cell sorted on CD24 and clones were allowed to form cultures. CD24 expression was determined in three subsequent passages. The figure depicts the average of at least three independent cultures for each of the three types of CD24-defined MDX clones.

(C) Characterization of MDX CD24-ve and CD24+ve clones. MDX CD24-ve and CD24+ve (at least 2 clones of each subtype) cells were fixed and stained for Pax7 and MyoD. Quantification of the staining is indicated in the picture (% positive cells of total DAPI-positive cells).

(D) Differentiation potential of CD24-defined primary muscle clones. MDX CD24-ve and CD24+ve clones were plated at high density and switched to DM 48 h later. After 3 days differentiated cultures were stained for MyHC (red) and syto13 (green, nuclei).

CD24-ve cells are present in primary muscle cultures

C2C12 myoblasts are immortalized cell lines (Yaffe and Saxel, 1977). We wondered if cells with the properties of CD24-ve cells could be identified in satellite cell-derived primary muscle cultures,. To this end, we established primary limb muscle cultures from WT (C57/Bl6) and MDX animals (dystrophin-/-; animal model for Duchenne Muscular Dystrophy). WT and MDX cultures expressed CD24 heterogeneously (~25 and 75% CD24-positive cells, respectively (Figure 3A). To identify cells with CD24-ve properties, primary cultures were sorted clonally on CD24. Figure S1F shows the level of CD24 expression in the CD24 single-cell sorted clones in the first passage. This analysis showed that the majority of CD24-negative clones (76% and 71 % of clones, respectively) re-expressed CD24. One third of clones expressed low levels of CD24 (<5% CD24-positive cells). We expanded eight clones (3*WT and 5*MDX) with low CD24 expression and found that 7 of these clones remained CD24-negative/low during subsequent passaging (Fig. 3B). These clones were designated WT or MDX CD24-ve clones. In addition, clones that expressed CD24 heterogeneously and CD24+ve clones were identified in primary cultures (Fig. 3B).

From a clinical perspective we were particularly interested in the MDX cultures, as we wondered if CD24-ve cells could be isolated from cultures established from dystrophic muscle. To determine the state of differentiation of CD24-sorted MDX clones, we analyzed the expression of Pax7 and MyoD in MDX CD24-ve and CD24-ve cultures. Similarly as was shown for C2C12 CD24-ve cultures MDX CD24-ve cultures expressed Pax7 strongly and MDX CD24+ve were Pax7low/negative (Fig. 3C). To compare the differentiation potential of CD24-ve and CD24+ve cells, different clones were induced to differentiate. In line with our findings for C2C12 clones, MDX CD24-ve cultures differentiated very efficiently, while MDX CD24+ve cells differentiated poorly as judged by limited formation of MyHC-positive myotubes and poor fusion (Fig. 3D). These results suggest that CD24-ve and CD24+ve cells are present not only in muscle cell lines, but also in satellite cell-derived primary cultures from dystrophic muscles.

CD24-ve engraft, survive and expand in vivo

After having established the myogenic potential of CD24-ve cells in vitro, we wondered if CD24-ve would functionally engraft in vivo. Since previous transplantation studies indicated that transplantation of cultured muscle cells resulted in massive cell death in the first 24-48h (Beauchamp et al., 1999; Montarras et al., 2005), we first assessed whether CD24-ve cells survive and engraft following transplantation. To this end, we transplanted C2C12-derived CD24-ve and CD24+ve cells in BaCl2-injured Tibialis Anterior (TA) muscle. Donor cells were identified by the CD31neg/CD45neg (Lin-neg)/TER119neg/Sca1-pos/GFP-pos signature. The number of donor cells was quantified 24h, 7 days and 21 days after transplantation (Fig. 4A). Shortly (1 h) after transplantation about 23% of the delivered cells could be recovered, indicating that the majority of cells was lost during or immediately after transplantation. One day after transplantation still about 20% of the delivered cells could be detected, dem-

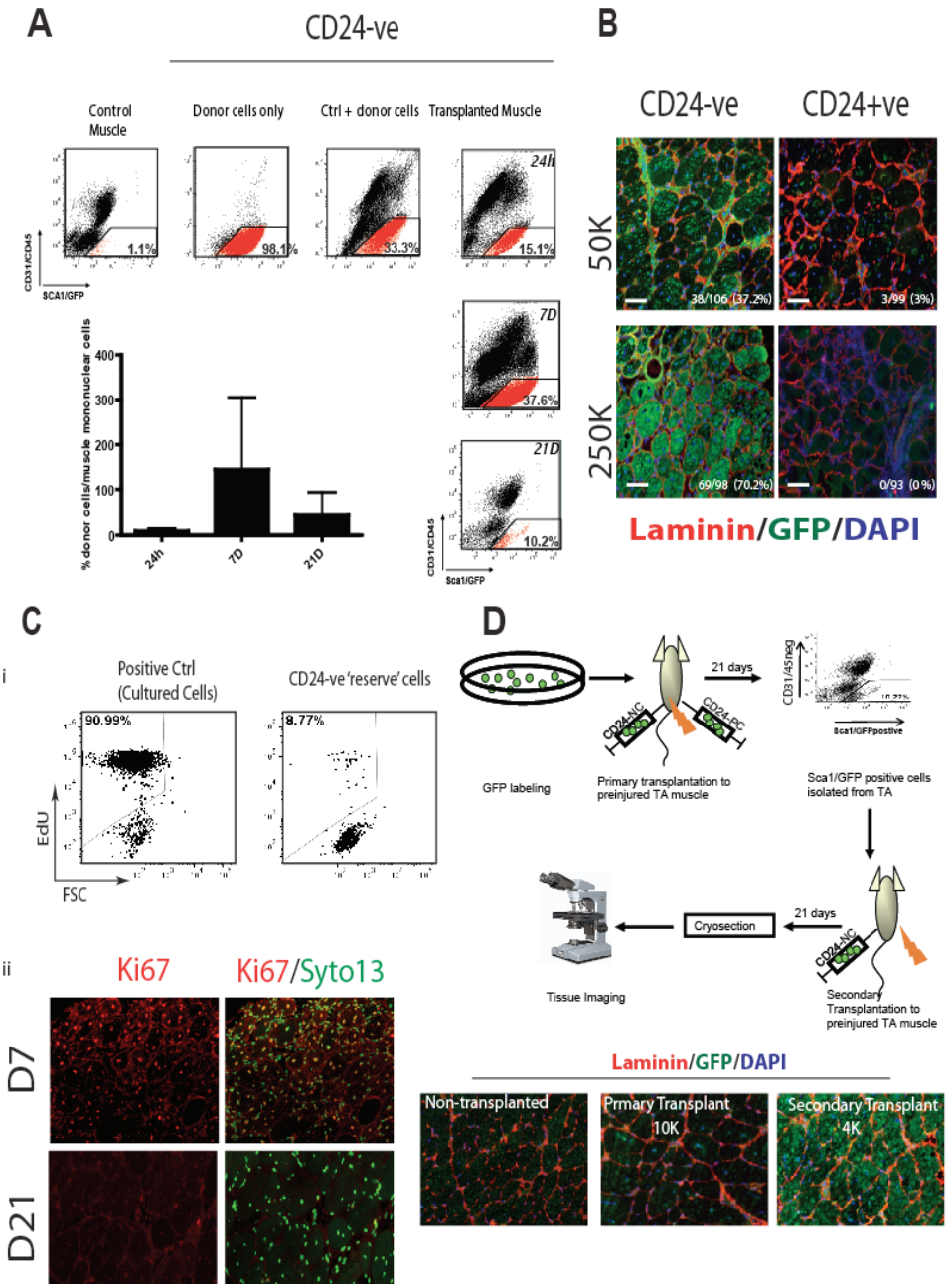
A
CD24-ve
Control Muscle
Donor cells only
Ctrl + donor cells
Transplanted Muscle
1.1%
98.1%
33.3%
24h
15.1%
7D
37.6%
21D
10.2%
CD31/CD45
SCA1/GFP
% donor cells/muscle mononuclear cells
400
300
200
100
0
24h
7D
21D
B
CD24-ve
CD24+ve
50K
250K
38/106 (37.2%)
3/99 (3%)
69/98 (70.2%)
0/93 (0%)
Laminin/GFP/DAPI
C
i
Positive Ctrl (Cultured Cells)
CD24-ve 'reserve' cells
90.99%
8.77%
EdU
FSC
ii
Ki67
Ki67/Syto13
D7
D21
D
21 days
CD24-NC
CD24-PC
GFP labeling
Primary transplantation to preinjured TA muscle
CD31/45neg
Sca1/GFPpostive
Sca1/GFP positive cells isolated from TA
Cryosection
21 days
Tissue Imaging
Secondary Transplantation to preinjured TA muscle
Laminin/GFP/DAPI
Non-transplanted
Prmary Transplant 10K
Secondary Transplant 4K

Figure 4 CD24-ve contribute to muscle regeneration and generate a quiescent reserve population

(A) Fate and survival of transplanted cells. The figure depicts the gating strategy to identify CD24-ve cells (CD31neg/45neg/TER119/Sca1pos/eGFPpos), but was similar for CD24+ve cells. eGFP-labeled CD24-ve cells were transplanted into preinjured TA muscle of NOD-SCID animals. The contralateral leg was injured but not transplanted (control muscle). Cells were harvested at indicated timepoints after transplantation. Cultured (trypsinized) CD24-ve cells were added to control muscle suspensions at transplantation doses prior to staining to the control muscle suspension (ctrl + donor cells) and used as internal reference. Cultured donor cells were stained for these markers as positive control (donor cells only). The histogram shows the quantification of donor cells/internal reference. (B) CD24-ve cells contribute to muscle regeneration. eGFP-labeled CD24-ve or CD24+ve cells were transplanted at different doses to preinjured TA muscle of NOD-SCID hosts. Muscle was harvested 21 days after transplantation and contribution to new muscle formation was determined by the presence of eGFP-positive myofibers. Sections were stained with laminin (red) to visualize myofibers. Nuclei were stained with DAPI. The picture shows muscle after transplantation with 50000 (50K) and 250000 (250K) cells. For different transplantation doses see the text. Similar results were obtained with cells labeled with a different reporter (pCAGGS-GFP-IRES-GFP). The number of animals per dose is provided in Table S3.(C) CD24-ve donor reserve cells are quiescent. i: Proliferation activity of the 'reserve pool cells' generated by transplanted CD24-ve cells. Muscle was digested 21 days after transplantation and Sca1pos/GFPpos cells were isolated by FACS sorting. Cells were plated for 24h in medium containing EdU, after which cells were collected and fixed. As positive control, logarithmically growing donor cells were trypsinized and plated in EdU-containing medium and stained. The percentage in the plot indicates the cells incorporating EdU. ii: Sections from CD24-ve transplanted muscle at 7 and 21 days after transplantation and stained for Ki67 (red). Nuclei were counterstained with DAPI.(D) Self renewal potential of CD24-ve cells in vivo. CD24-ve cells were recovered 21 days after transplantation (for gating strategy see Fig. 4A) and directly transplanted to preinjured TA muscle of secondary NOD-SCID hosts. Secondary hosts were sacrificed 21 days after transplantation and muscle sections were stained for eGFP (green) and laminin (red). Nuclei were stained for DAPI (blue). The formation of GFP-positive myofibers/section was determined as indication for selfrenewal in vivo. GFP-stained non-transplanted muscle and muscle transplanted with 10K CD24-ve cells directly from culture (primary transplantation) are shown as reference.

onstrating that the engrafted cells survived efficiently. There was a strong increase in donor cells in the next 6 days, suggesting robust proliferation of the surviving cells (Figure 4A). There was no difference between the initial engraftment between CD24-ve and CD24+ve cells, at least up to day 7 after transplantation (Fig. S2A). We conclude that CD24-ve and CD24+ve cells engraft and expand efficiently in vivo.

CD24-ve cells contribute to muscle regeneration

Then we wondered if transplanted CD24-ve cells would contribute efficiently to muscle regeneration. To determine this, CD24-ve and CD24+ve cells were labeled with a fluorescent eGFP reporter. The labeled populations were transplanted to preinjured muscle of immunodeficient hosts. Three weeks after transplantation when muscle regeneration is completed, muscle sections were analyzed for donor-host hybrid myofibers. At the lowest dose (5000 cells) CD24-ve cells no hybrid myofibers were detected. Transplantation of 10000 cells resulted in contribution to the formation of GFP-hybrid myofibers (7% of fibers). While after transplantation of the lower doses CD24-ve cells (<50000 cells) GFP-positive fibers were found predominantly close to the needle track (data not shown), at higher doses hybrid myofibers were found across the section (Fig. 4B). Transplantation of 250000 CD24-ve cells resulted in contribution to 70 % of the fibers and homogeneous GFP expression in hybrid fibers (Fig. 4B). Transplantation of CD24+ve cells did not result in contribution to muscle formation in vivo, not even at the highest dose. Unsorted cells, as expected, also contributed to myofiber formation (Fig. S2B), but the myofibers were only weakly GFP-positive as compared to a similar dose of CD24-ve cells. These data show that transplanted CD24-ve cells contributed robustly to muscle regeneration.

Transplanted CD24-ve cells generate a quiescent reserve population

It has been suggested previously that transplanted muscle cells generate a 'reserve' pool of cells that can persist long-term (Morgan et al., 1993; Yao and Kurachi, 1993). The analysis of engraftment showed that a population of donor cells remained mononuclear (non-fused) after regeneration is completed (21 days; Fig. 4A). To determine the nature of the in vivo reserve cells we first assessed their proliferation status. Donor cells were recovered 21 days after transplantation by FACS sorting (gating strategy depicted in Figure 4A) and pulsed with EdU, a nucleoside analog of thymidine. EdU incorporation was determined 24h later. As positive control cultured CD24-ve cells were trypsinized and EdU-pulsed under the same conditions. Fig. 4Ci shows that CD24-ve reserve cells hardly incorporated EdU, while the positive control cells readily incorporated EdU (>90% of cells were labeled). As a further control donor cells were also EdU-pulsed 7 days after transplantation. This showed that ~20% of the transplanted cells incorporated EdU at that stage (Fig. S2C). To verify the non-proliferative nature of the non-fused donor cells, sections from transplanted muscle were stained for the proliferation marker Ki67. Sections prepared from muscle 7 days after transplantation showed numerous Ki67-positive nuclei. However, Ki67 staining was largely absent in muscle at 21 days after transplantation (Fig. 4Cii). These results

demonstrate that the CD24-ve donor reserve cells entered quiescence after regeneration is completed.

CD24-ve cells self-renew in vivo

To determine if the reserve population is functional, we recovered CD24-ve donor reserve cells by FACS sorting as described above. The recovered re-entered the cell cycle after an initial lag phase and were expanded in vitro. Figure S2D shows that the CD24-ve donor reserve cells differentiated efficiently as judged by the formation of multinuclear MyHC-positive myotubes, demonstrating that their myogenic potential was not compromised by in vivo passaging.

Next we asked if this residual donor cell population had in fact self-renewed in vivo and represent bona fide stem cells. The most stringent test for self-renewal is serial transplantation and is used frequently to demonstrate stem cell activity of hematopoietic and cancer stem cells. CD24-ve donor cells were FACS-sorted (Lin-neg/Sca-1pos/GFPpos) 21 days after the primary transplantation and directly transplanted into pre-injured muscle of secondary hosts. While only about 10% of the cells transplanted to the primary host was obtained after 21 days, transplantation of this limited number of 'reserve' cells resulted in robust repopulation in muscle of the secondary host, as judged by contribution to virtually all of the fibers across the section (Fig. 4D and Fig. S2E). This indicated that the CD24-ve donor reserve cells not only repopulated damaged muscle efficiently but migrated far from the needle track. In fact, the 4000 cells from the secondary transplantation contributed as efficiently to the formation of eGFP-positive fibers as after transplantation of 250000 CD24-ve cells directly from culture. Importantly, the CD24-ve cells transplanted to the secondary host also generated a reserve pool (Fig. S2F), suggesting that the CD24-ve donor reserve cells retained self-renewing potential following transplantation to the secondary host. Taken together, these results demonstrate that CD24-ve cells upon transplantation both contributed to muscle regeneration and generated a quiescent self-renewing population of stem cells.

Discussion

The regenerative potential of freshly isolated muscle satellite cells is remarkable and suggests great therapeutic potential. However, the limited number of satellite cells available in vivo and the rapid loss of the regenerative potential during ex vivo culturing limit the clinical use of this stem cell population. The present study demonstrates that cells with muscle regenerative potential are actually maintained during ex vivo passaging and can be purified by the use of a single marker, CD24.

An early study showed that in human muscle CD24 is only expressed in regenerating or diseased muscle (Figarella-Branger et al., 1993), suggesting the relevance of CD24 as a marker in the myogenic lineage. However, in study of Figarella-Branger et al. the

use of CD24 for discriminating muscle cells with differential myogenic potential was not explored. The cell surface marker CD24 is used as a marker to enrich for stem cells in normal and malignant tissues, including the mammary gland and colon (Al-Hajj et al., 2003; Shackleton et al., 2006). Depending on the tissue or species self-renewing stem cells resided in the CD24-negative (Al-Hajj et al., 2003; Sato et al., 2011) or -positive (Shackleton et al., 2006) population. The explanation for these paradoxical observations may lie in the transient expression of CD24 during differentiation. For instance, hematopoietic stem cells still lack CD24 expression, while during B-cell differentiation CD24 starts to be expressed by pro-B-cells. CD24 is then again downregulated in mature B-cells and plasma cells (Nielsen et al., 1997). We have indications that CD24 is also transiently upregulated during the early stages of myogenic differentiation in vitro (data not shown). Furthermore, the transient expression of CD24 during differentiation supports the heterogeneity observed within the CD24-negative sorted population of cultured muscle cells. In this study the CD24-ve muscle stem cells comprise <10% of the CD24-negative sorted muscle cells. This indicates that the CD24-negative population may be dominated by differentiated cells and appear as a whole more committed as compared to CD24-positive cells (early progenitors). A similar observation has been reported for the use of CD133, where tumor initiating cells in metastatic colon cancer were found in both the CD133-negative as well as CD133-positive fraction (Shmelkov et al., 2008). Subsequent studies demonstrated that the AC133 epitope, recognized by the most commonly used antibody to detect CD133, is lost upon cancer stem cell differentiation (Kemper et al., 2010). This suggests that the CD133-negative population of colon cells is heterogeneous and contains differentiated cells that are less or even non-tumorigenic that contaminate the comparison of stem cell activity in CD133-sorted populations. These observations may explain the contradictory results observed in the use of cell surface markers and indicate that markers should be used with caution. Most studies combine two or more markers to discriminate stem cells from differentiated progeny. In this study we used a clonal analysis approach and identified cells that persistently lack CD24 expression excluded dependence on the expression of a single marker.

Obviously a second (positive) marker to prospectively identify muscle regenerative cells would be of great value. In particular, as CD24-ve cultures became heterogeneous (Pax7 expression decreased and MyoD upregulated) at later passages (>10), suggesting that CD24-ve need to be transplanted soon after purification from the heterogeneous cultures to ensure increased regenerative potential. To identify additional markers for regenerative cells, we validated cell surface markers used to purify SCs directly from murine muscle, integrin alpha7/beta1 and CXCR4 (Cerletti et al., 2008; Sacco et al., 2008). These markers were expressed homogeneously or not expressed at all by cultured muscle cells (Figure 1 and data not shown) and could therefore not be used for selecting the minor population of regenerative cells. CD34 expression was used previously to isolate cells with regenerative potential from short-term myoblast cultures (Jankowski et al., 2002). In our hands more than 50% of C2C12 cells expressed CD34 (data not shown), indicating that CD34 expression does not

identify the CD24-ve population. In fact, CD24-ve cells expressed CD34 heterogeneously, but we cannot exclude that CD24-ve cells constitute a subpopulation of CD34-expressing cells. CD24+ve cells expressed CD34 homogeneously. Based on our data, CD34 does not identify the same population of cells and can certainly not be used as (single) positive marker to select regenerative cells from ex vivo maintained muscle cultures. As opposed to the use of a well-defined marker, a recent study reported the use of a label-retention strategy to select slow-dividing cells from short-term primary cultures and showed that this population contributed to muscle regeneration upon transplantation (Ono et al., 2012). Based on the reported frequency of 4%, we wonder if label-retaining cells and the CD24-ve population overlap, at least in primary cultures. Although the use of label-retention avoids the need for specific markers, it remains unclear from this study if the label-retaining cells represent a stable population (the label-retaining population was defined as the 4% of cells with the highest PKH26 signal) that is maintained during extensive passaging. The ability to purify regenerative cells from long-term cultures, a major aim from this study, enables robust expansion to generate clinically relevant numbers and, if needed, correction of an underlying genetic defect. In addition, it will be interesting to determine if the label-retention strategy allows isolation of a population with similar properties and purity from subsequent donor cultures with different genetic backgrounds as we have shown with the use of a single cell surface marker. By absence of a positive marker, it will be worth exploring different approaches that stimulate self-renewing expansion of freshly sorted isolated cells (Gilbert et al., 2010) to preserve the homogeneity and regenerative potential of purified CD24-ve cells.

The poor survival, limited migration potential and the incompatibility of satellite cells with systemic delivery has been used to argue against the therapeutical use of muscle satellite cells (Beauchamp et al., 1999; Dellavalle et al., 2007; Huard et al., 1994). Our results indicate that CD24-ve cells engraft upon transplantation, expand in vivo and contribute to hybrid muscle formation. As a large portion of the cells were lost during the transplantation procedure, the engraftment and regenerative efficiency of CD24-ve cells can be enhanced further by optimizing the method of cell delivery. With respect to the migration potential of CD24-ve cells in vivo, indeed at lower doses CD24-ve cells only contributed to regeneration at or around the needle track. However, at somewhat higher doses the CD24-ve cells and in particular the CD24-ve reserve population generated in vivo, repopulation was observed across the diameter of the transplanted muscle. This demonstrates that within one muscle CD24-ve cells have the ability to migrate far from the site of delivery. Taken together, the regenerative potential of CD24-ve cells is not limited by poor survival, limited migration potential, while transplantation of selected (e.g. most heavily affected; e.g. rhabdosphincter in stress urinary incontinence) muscles avoids the need for systemic delivery.

Collins et al. have shown that myofiber-associated satellite cells are bona fide stem cells and demonstrated that the transplanted cells self-renewed and generated differentiating progeny (Collins et al., 2005). Studies with freshly isolated satellite cells

showed that the transplanted cells expanded again in response to a secondary injury indicating that some of the transplanted cells were reactivated during the secondary cycle of regeneration (Sacco et al., 2008). More recently, serial transplantation of freshly isolated satellite cells was reported (Rocheteau et al., 2012), further demonstrating the self-renewal potential of transplanted SCs. To our knowledge, self-renewal of cells extensively passaged in vitro has not been demonstrated by any of these approaches. Our studies shows that CD24-ve efficiently self-renew in vivo, similarly as freshly isolated SCs (Rocheteau et al., 2012). The ability of transplanted cells to generate self-renewing donor cells in vivo, is critical for ensuring long-term maintenance of host-donor hybrid muscle. Based on the study of Sacco et al. (Sacco et al., 2008) self-renewing donor cells present in transplanted muscle may continue to contribute to expanding the repopulated area with time as result of ongoing muscle regeneration that characterizes dystrophic muscle. It remains to be determined if the quiescent CD24-ve 'reserve cells' detected at 21 days after transplantation assume a 'satellite cell position' at the periphery of the fibers. That could constitute the intriguing possibility that primary cultures maintain a subpopulation of cells that have maintained most if not all of the properties of satellite cells in vivo.
The data presented here show that cells with regenerative potential are preserved during ex vivo culturing. These regenerative cells can be enriched from long-term cultures with diverse genetic backgrounds using a single marker CD24. This straightforward approach to obtain regenerative cells from heterogeneous cultures at significant numbers opens avenues for the development of cell-based therapies for muscular dystrophies. In particular, the specific properties of the CD24-ve cells make these cells interesting candidates to reverse the muscle pathology in conditions restricted to one or few muscles such as in stress urinary incontinence resulting from poor sphincter function (Chartier-Kastler et al., 2010).

Material and Methods

Materials and Antibodies

Fibroblast growth factor (FGF) basic was purchased from Preprotech. Ham's F10 medium, Dulbecco's modified Eagle's medium (DMEM) and DPBS were purchased from Lonza (Benelux BV, The Netherlands). Extracellular matrix (ECM), PNGase F and N-acetyl-D-glucosamine (GlcNac) were purchased from Sigma (St. Louis, USA). A hybridoma for anti-CD24 (clone M1/69) was a kind gift from Dr. P. Altevogt (University of Heidelberg, Germany), origin and dilutions of all other antibodies in Table S2. Processing of hybridoma cultures are described in Supplemental data. The EdU staining kit was (Life Technologies, Bleiswijk, The Netherlands) used according to the manufacturers' protocol. pEGF-N1 plasmid was from Promega, while the pCAGGS-GFP-IRES-GFP was constructed by Dr. C. Dupont (Dept. of Reproduction, Erasmus MC, The Netherlands).

Cell culture

Growth medium (GM): C2C12 were cultured in DMEM supplemented with 20% fetal calf serum (FCS) and 1% penicillin/streptomycin (PS). Primary cultures were grown in Ham's F10 supplemented with 20% FCS and 1% PS. Differentiation medium (DM): DMEM medium containing 2% horse serum and 1% PS. The differentiation experiments as well as the maintenance of the primary cultures were performed on ECM -coated dishes.

Antibody production

All subsequent steps were performed at Bioceros (Utrecht, The Netherlands): Hybridomas (anti-chicken Pax7 and anti-mouse CD24) were cultured in the presence of 1% FCS and gentamycine (50 ug/ml). Ammonium sulfate 0,75 M was added to the clarified supernatant and this was loaded on a thiophillic agarose resin and eluted using 50 mM Tris-HCl pH 8.9. Purified antibodies were subsequently dialyzed against PBS and filtered through a 0,2 um filter.

Myogenic Cell Preparation

Limb muscle from 6-9 weeks old mice were collected and myoblast isolated as described previously (Rando and Blau, 1994). In short: dissected hindlimb muscles were digested in 0.2% Type II Collagenase in DMEM for 45 min at 37°C, followed by a second digest in 1% Dispase/0.2% Type II Collagenase for 30 minutes at 37°C (all enzymes from Invitrogen, Carlsbad, CA). Muscle mononuclear cells were mechanically dissociated from myofibers after the final digestion by passing the tissue suspension through a 20G needle. After washing cells were plated on ECM-coated dishes in preconditioned medium (CM; sterile-filtered medium preexposed for 48h to proliferating muscle cells of syngeneic animals) supplemented with bFGF (20 ng/ml). Cells were allowed to adhere for 72 h, when cells were washed and maintained in bFGF-supplemented CM. During the first 5 passages, myogenic cells were enriched by a preplating procedure described previously (10).

Plasmid transfection

Cells were transfected with either pN1-EGFP (Clontech, USA) or by a CAGGS-eGFP-IRES-eGFP constructed by Dr. C. Dupont (Dept. of Reproduction, Erasmus MC). Cells were plated at subconfluent densities and plasmids were mixed with Lipofectamine2000 in Optimem (Lonza, USA) according to the manufacturers protocol (Lifetechnologies, USA). The next day, the cells were split and selected in G418 (pN1-eGFP) or puromycin (CAGGS-eGFP-IRES-eGFP). Clones were plated using the BD FACS sorter (ARIAIII) and homogeneously positive clones that maintained phenotypic and functional characteristics of the population before transfection (CD24 expression, differentiation potential) were selected for further study.

RT-PCR

Cells were harvested in Trizol reagent (Life Technologies). Total RNA was prepared according to the manufacturers' protocol. Primer sequences and reaction conditions are provided in Table S1.

Western Blotting

Western blotting was performed as described previously (Schaaf et al., 2010). Cells were washed twice with cold PBS and lysed in for 20min in ice-cold RIPA lysis buffer (20 mM Tris•HCl, pH 8.0/150 mM NaCl/1% Nonidet P-40/1% SDS/0.5% deoxycholic acid) supplemented with a protease inhibitor mixture and phosphatase inhibitors (Sigma, St. Louis, USA). Protein lysates from muscle were prepared by homogenation of tissue in RIPA using an Ultraturrax. Protein concentration was determined using Bicinchoninic Acid (BCA) protein assay kit (Thermo Scientific, USA). 20 µg cell lysate (80 µg for detecting CD24) was electrophorized on SDS/PAGE and blotted on immobilon-P membranes. Membranes were developed with specific antibodies described in the text.

Immunostaining

Immunostaining was performed on fixed cells (4% PFA, 10 min) after permeabilization subsequently with 0.25M NaOH and 0.1% triton and 1% SDS for 10 min and block with 5% goat serum (GS) in PBT. Cells were incubated in primary antibodies overnight at 4 °C (Table S2). Nuclei were stained with fluorescence with 4', 6-diamidino-2-phenylindole (DAPI) or Syto13 (Life Technologies Europe, Bleiswijk, The Netherlands). Images were taken on a Zeiss Axio Imager Z1 fluorescent microscope.

FACS analysis and sorting

For FACS, cells were trypsinized, washed and blocked 30 min on ice with 5% NGS. Cells were then stained with appropriate antibodies in DMEM/2% FCS in suspension for 1 h on ice. Subsequently, the cells were washed twice with PBS, resuspended in PBS. Hoechst 33258 (Sigma) was added to exclude dead cells. Cells were analyzed or sorted using a FACS Aria III cell sorter (Becton-Dickinson). Clonal analysis was performed by sorting single cells into 96-well plates containing conditioned medium (medium that was exposed for 48h to logarithmically growing cells).

Animals and Animal Procedures

Mice: MDX (C57BL/10ScSn-Dmd /J obtained from Jackson, Maine, USA), C57bl6 (Erasmus MC in-house colony), and CD24KO (gift from Dr. J. Sleeman, Karlsruhe Institute of Technology, Germany).

Regeneration experiments: Animals were anaesthesized (O2/Isoflurane). TA muscle was injured by injection of 50 µl barium chloride (1.2% in MilliQ) distributed over 15 sites. Animals were post-treated with Buprenorphine (0.1 mg/kg sc) and Enrofloxacine (10mg/kg sc). Food and water was provided ad lib. Animals were were sacrificed. Injured muscles were resected and processed for (immune) histochemical analyses,

RT-PCR and Western Blotting as described. For cryosection muscles were embedded in OCT and snap-frozen in N2-chilled isopentane. Muscles were subjected to a secondary and third injury (as described above) 21 days after an initial injury and allowed to recover for a further 21 days.
Transplantation experiments: Cell were trypsinized, resuspended in serum-containing medium and counted. Cells were suspended in 60 ul PBS/10 % mouse serum and kept on ice until transplantation. Tibialis Anterior muscle of host animals were injured one day prior by injection with 50 ul 1.2% BaCl2 (distributed across the length of the muscle) under O2/Isoflurane anesthesia. The cells were loaded in insulin syringes (30 cc) and the needle was injected through the skin from ankle to knee and vice versa. Cells were deposited by withdrawal of the needle without leakage. NOD-SCID mice were used as hosts for the transplantation assay.
Animals were sacrificed at indicated times and muscle was processed for immunohistochemical analysis or myogenic cell preparation. For animal numbers see Table S3.All procedures involving experimental animals were approved by the local ethical committee. Details of animal experimentation, including the number of animals used in the experiments, are provided in the Supplemental Information.

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Supplementary information

Table S1: Antibodies and dilutions

Primary Antibodies

Target	Raised in	Dilution	Clone	Distributor
CD24-PE	rat	200	M1/69	BD
Myogenin	rabbit	200	polyclonal	Santa Cruz
MyoD	mouse	200		BD
MyHC	mouse	200	MF20	DSHB
Myf5	rabbit	250	polyclonal	Santa Cruz
Pax7	mouse	100		DSHB
Ki67	rabbit	400	polyclonal	Millipore
Laminin Alpha	rabbit	250		Sigma
Laminin Alpha	chicken	500		Abcam
GFP	rabbit	500		Abcam

Secondary Antibodies

Target	Fluorochrome	Dilution		Distributor
Goat-anti Mouse	AlexaFluor488/555	500	IgG (H+L)	Invitrogen
Goat-anti Rabbit	AlexaFluor488/594	500	IgG (H+L)	Invitrogen
Donkey-anti-chicken	AlexaFluor594	500	IgG (H+L)	Invitrogen

Distributors (if not mentioned in the main text): Abcam, Cambridge, UK; DSHB, Iowa City, Iowa, USA; Santa Cruz, Heidelberg, Germany

Table S2: Primer sequences used in RT-PCR analysis

gene	sequence
Pax7-F	*GAAAGCCAAACACAGCATCGA*
Pax7-R	*ACCCTGATGCATGGTTGATGG*
Myf5-F	*TGAGGGAACAGGTGGAGAAC*
Myf5-R	*GCAAAAAGAACAGGCAGAGG*
Myh1-f adult	*CTTCAACCACCACATGTTCG*
Myh1-r adult	*AGGTTTGGGCTTTTGGAAGT*
MyoD-F	*GAGCAAAGTGAATGAGGCCTT*
MyoD-R	*CTGGGTTCCCTGTTCTGTGT*
CD24a F	*ACATGGGCAGAGCGATGGT*
CD24a R	*CTAACAGTAGAGATGTAGAAG*
actin beta F ex3	*GGACTCCTATGTGGGTGACG*
actin beta R ex4	*GGGGTGTTGAAGGTCTCAAA*
GAPDH F	*ACCACAGTCCATGCCATCAC*
GAPDH R	*TCCACCACCCTGTTGCTGTA*

Table S3: Animal numbers used in experiment

Primary Transplantation

No cells (x1000)	CD24-ve	CD24+ve	Unsorted
5	2		1
10	2		1
50	3	2	
100	1	1	
250	1	1	1

Engraftment efficiency

Time after trspl	CD24-ve	CD24+ve
24h	2	1
7D	3	2
21D	4	2
1h	1	

EdU incorporation

Time after trspl	CD24-ve	CD24+ve
7D	1	1
21D	2	1

Serial Transplantation

Host	CD24-ve	CD24+ve
2nd	2	1
3th	1	

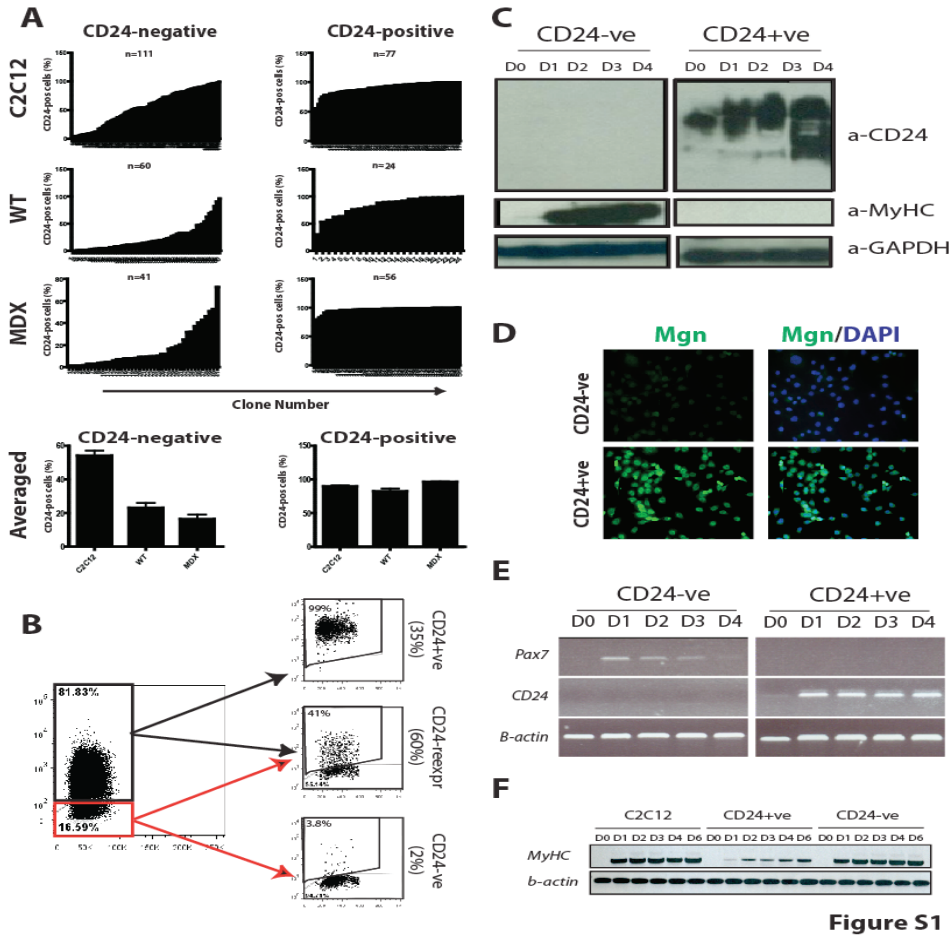

Figure S1

Figure S1:
(A)Clonal analysis of C2C12 and primary WT and MDX muscle cultures. CD24 expression in single-cell sorted derived clonal cultures (number of clones analyzed is indicated) in the first passage. The lower histograms depict the averaged percentage of CD24-positive cells in all CD24-negative sorted and CD24-positive sorted clones, respectively.(B) CD24 expression of single-cell sorted clones in the first passage. Three types of clones were identified and are depicted in the figure. The frequency of the respective clone types is indicated between brackets. (C) Western blot analysis of differentiating C2C12 parental, CD24-ve and CD24+ve cells. The blots were developed using CD24 and MyHC specific antibodies. GAPDH is shown as loading control. (D) C2C12 cells in proliferation (GM) or after 4 days of differentiation (DM) were stained for myogenin (red/green as indicated). Nuclei were counterstained with DAPI (blue). Pictures were taken with a 10x objective.(E)RT-PCR analysis of Pax7 and CD24 expression in differentiating C2C12 CD24-ve and CD24+ve cells. Samples were taken at indicated timepoints after switching the medium to differentiation medium (DM). Beta-actin is shown as loading control.(F) MyHC is expressed at low levels in 'differentiating' CD24+ve cells. C2C12, CD24+ve and CD24-ve cells were induced to differentiate and CD24 RNA levels were determined at different time points in DM. The figure depicts an enhanced image of RT-PCR analysis to visualize low expression. Beta-actin is shown as loading control.

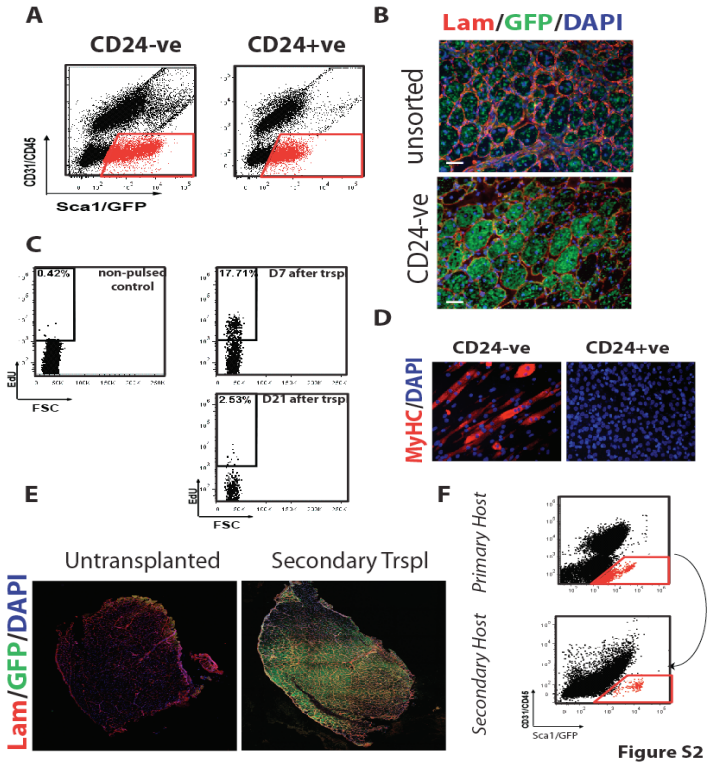

Figure S2:

(A) Both CD24-ve and CD24+ve cells engraft and expand similarly upon transplantation. The FACS plots depict the analysis of muscle 7 days after transplantation. Donor cells were identified based on CD31neg/CD45neg/TER119neg/Sca-1pos/GFPpos signature. Gated populations are indicated in red. (B) Unsorted C2C12 cells contribute to regeneration. The picture shows a section of a muscle transplanted with 250000 unsorted GFP-labeled C2C12 cells. A section from a muscle transplanted with 250000 GFP-labeled CD24-ve is shown as reference.(C)Transplanted cells proliferate in the first 7 days after transplantation. Cells were transplanted as described. At day 7 and 21 after transplantation, cells were recovered by FACS sorting (using the Lin-neg/Sca-1pos/GFPpos signature) and plated in EdU (10 μM) containing medium. The next day the cells were collected and EdU incorporation was determined by flow cytometry according to manufacturer's instructions. (D) Reisolated CD24-ve reserve cells maintain the capacity to differentiate. CD24-ve and CD24+ve cells were transplanted to injured muscle and isolated by FACS sorting 7 days or three weeks (only CD24-ve cells) later. The isolated cells were expanded and induced to differentiate for 4 days. The picture depicts MyHC (red) staining of cells isolated 7 days after transplantation. CD24-ve cells isolated three weeks after isolation differentiate similarly efficiently upon recultering (not shown). Nuclei are shown in blue (DAPI). (E) Transplanted CD24-ve cells robustly contribute to regeneration across the transplanted muscle in the secondary host. The confocal picture shows a cross-section of TA muscle (fibers were stained with laminin (red), nuclei with DAPI (blue)). Non-transplanted muscle stained with GFP-antibody is shown as reference. Pictures were taken on a Leica (Leica Microsystems BV, Rijswijk, The Netherlands) SP5 confocal microscope. (F) CD24-ve efficiently self-renew and can be serially passaged in vivo. CD24-ve were transplanted and 'reserve' cells were isolated 3 weeks after transplantation and directly transplanted to the muscle of a secondary host. The formation of self-renewing 'reserve' cells in the primary and secondary host was determined by flow cytometry and is identified as a distinct population (red gated population) in the Lin-neg/Sca-1pos/GFPpos gate.

Chapter 3

A quiescent population of muscle stem cells identified in vitro in C2C12 cultures

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A quiescent population of muscle stem cells identified in vitro in C2C12 cultures

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Abstract

Muscle satellite cells (SCs) mediate the repair of severely damaged muscle and have the potential to engraft and regenerate injured muscle upon transplantation. Based on these properties SCs are considered as vectors in the treatment of muscle-degenerative diseases. The limited number of SCs in vivo demands for extensive expansion to significant numbers. However, ex vivo culturing of SCs is associated with loss of regenerative potential, preventing the clinical use of these cells.

In the present study we show that cells with similar properties than quiescent satellite cells are preserved in C2C12 in vitro culture. This population named qRCs is a subset of the previously described heterogeneous reserve cell population. qRCs can be enriched from the heterogeneous reserve cell population based on a significantly decreased adherence potential. qRCs express homogeneously the satellite cell marker pax7, and display enhanced in vitro differentiation potential as compared to 'classical' reserve cells and the parental population. qRCs are in the G0 phase of the cell cycle when isolated from differentiation cultures. After replating they re-enter the cell cycle with a delay of ~40 h and generate progeny with increased potential to differentiate. In contrast, 'classical' reserve cells are arrested in G1, but readily re-enter the cell cycle under proliferation conditions.

Thus, our culture conditions revealed the presence of a quiescent satellite like cell population in long-term cultures. Such culture conditions might be applied in the future to develop culture strategy to periodically enrich for muscle stem cells from long-term cultured patient biopsies. It would not only allow gene-correction approaches and the use of autologous cells, but also to produce large amount of cells with regenerative potential.

Introduction

Muscle-degenerative diseases, or myopathies, are characterized by progressive muscular degeneration (Fauci et al. 2008, McNally and Pytel 2007). The clinical and pathophysiological spectrum of these myopathies is broad (Monaco et al. 1988,Reuser et al. 1978). Nevertheless, muscle wasting remains the common denominator characterising these conditions. In muscular dystrophies, this symptom is thought to arise from repetitive activation of the muscle regenerative pathway, ultimately resulting in an exhaustion of resident muscle stem cells(Sacco et al. 2010). As the disease progresses, muscle weakness becomes generalized, fibrosis develops (Mann et al. 2011) and fat accumulation occurs (Warren et al. 2004). Life expectancy of these patients is reduced and to date, no therapy is able to reverse the skeletal muscle pathology (Fauci et al 2008,McNally et al. 2007). Thus the use of a stem cell based therapy has been pursued as an interesting novel approach for myopathies. Preserv-

ing muscle stem cell potential ex vivo or stimulating stem cell self-renewing would be a great benefit for these patients (reviewed in (Schaaf et al. 2012)).

Among muscle regenerative cells, satellite cells (SC) represent the predominant muscle resident stem cell population, which have a role in maintaining tissue homeostasis and are the sole mediators of muscle repair after severe trauma or during disease. (Lepper et al. 2011, Sambasivan et al. 2011). Therefore they represent ideal candidates for cell-based therapy assays. However, the number of SCs available is limited and represents two to five percents of all myonuclei in a healthy skeletal muscle (Schultz et al. 1996). Moreover, the regenerative potential of SCs is rapidly lost in culture in vitro (Beauchamp et al. 1999, Lepper et al. 2011). Nevertheless, successful regeneration has been achieved after transplantation of single SCs in normal and dystrophic mouse models (Cerletti et al. 2008; Sacco et al. 2008). This demonstrates the strong regenerative and therapeutic potential of the SCs.
SCs form the main resident stem cell population in the skeletal muscle. They were first identified as a distinct cell population located at the periphery of the muscle fiber, underneath the basal lamina (Mauro et al. 1961). They all express the paired box transcription factor Pax7 (Seale et al. 2000). However, the SC population is heterogeneous and displays various levels of regenerative capacity in vivo. The level of myogenic regulatory factors (such as MyoD) notably reflects their level of commitment. Pax7+/MyoD- SCs are quiescent. They upregulate MyoD when they break from quiescence (Zammit et al. 2004). Consequently, SCs expressing high levels of Pax7 or the one relatively quiescent have the highest engraftment efficiency (Kuang et al. 2007, Rocheteau et al. 2012).
Previous studies have shown that cells with SC characteristics are preserved in cultures. Yoshida discovered these cells, named 'reserve cells,' that preserve their stem cell properties upon differentiation culture conditions (Yoshida et al 1998). Reserve cells have since been found in other species including human (Abou-Khalil et al.2009). Reserve cells are mononucleated cells that are isolated based on their rapid re-attachment after trypsinization of complete differentiated cultures. Using the same culture conditions, we have identified in murine C2C12 skeletal muscle line another 'reserve cell' population that adheres slowly (24-96h) and displays properties of quiescent SCs. We therefore named the population, qRC. In previous protocols, qRCs were discarded. Our data indicates that the original fast-adhering reserve cells (referred as activated RC or aRC) represents of population of primed progenitors that lose their myogenic potential upon passaging. Our modified culturing approach allows the isolation of qRCs that maintain myogenic potential over extended culture periods.

Results

Isolation of two distinct populations of reserve cells with differential adherence potential

Freshly isolated muscle cultures harbor some cells with delayed adherence properties (Qu-Petersen et al. 2002; Jankowski et al. 2003 and Ono et al. 2012). These slowly-adherent cells, referred as qRCs, have stem cell like properties. To determine whether extensively passaged cultures still contain a similar qRC population. The experiments wer performed in C2C12 cell line. The C2C12 skeletal muscle line is a common, well-known in-vitro model that is used to study differentiation of skeletal muscle cells and assessment of the contractile forces of the differentiated cells (Li et al., 2008). C2C12 cells are derived from the mouse skeletal muscle C2 cell line, share some similar characteristics with isolated human skeletal muscle cells (Gajsek et al. 2008; Lee 2009). RCs can be isolated by mild trypsinisation protocols to remove the differentiated multinucleated myotubes (Yoshida, 1998). We modified the protocol to isolate cells by trypsinizing complete differentiated cultures, filtering to eliminate multinucleated structures and by replating the mononucleated cells (Fig. 1). The replated cultures were highly heterogeneous in their re-attachment potential and contained cells that adhered rapidly (<1 h after replating, aRCs) as well as cells that adhered slowly (>24h after replating, qRCs). The aRC population is described extensively and is known as the RC population (Yoshida, 1998). The qRC population consisted of small and round mononuclear cells and is, as result of their delayed attachment, usually discarded by protocols used in previous studies (Yoshida et al. 1998). The qRCs attached within 48h after replating and represented a viable population based on exclusion of the vital dye Hoechst as assessed by flow cytometry (Fig. 2A). Taken together, our results shows that two distinct populations of non-differentiating reserve cells are formed during myoblast differentiation.

qRCs (slowly-adhering reserve cells) are in G0 while residual cells are in G1

qRCs attached in the first 24h after replating, but it took >40h before the first doublets were observed (data not shown). This suggests a lag-phase for the qRCs before entering the first cell cycle. aRCs started proliferating directly after plating and needed to be passaged after only two days of culture. To determine if the qRCs were quiescent upon isolation,, aRCs and qRCs were fixed directly after isolation for cell cycle analysis after staining with propidium iodide (PI) and Pyronin Y to determine DNA and RNA contents, respectively. Both RC type populations showed an absence of cells in S-phase (yellow) and G2M-phase (blue) indicating that both RC types are not cycling in the differentiation cultures and are either in the G1 or G0 phase of the cell cycle. RNA transcription is downregulated when cells are in G0, which can be visualized by staining with Pyronin Y. qRCs showed reduced Pyronin Y staining compared to aRCs and the proliferating C2C12 myoblasts (Fig. 2A). This indicates that the qRC population is in G0 phase and therefore quiescent.

To further explore the cell cycle status of the qRCs, cells were labeled with the mem-

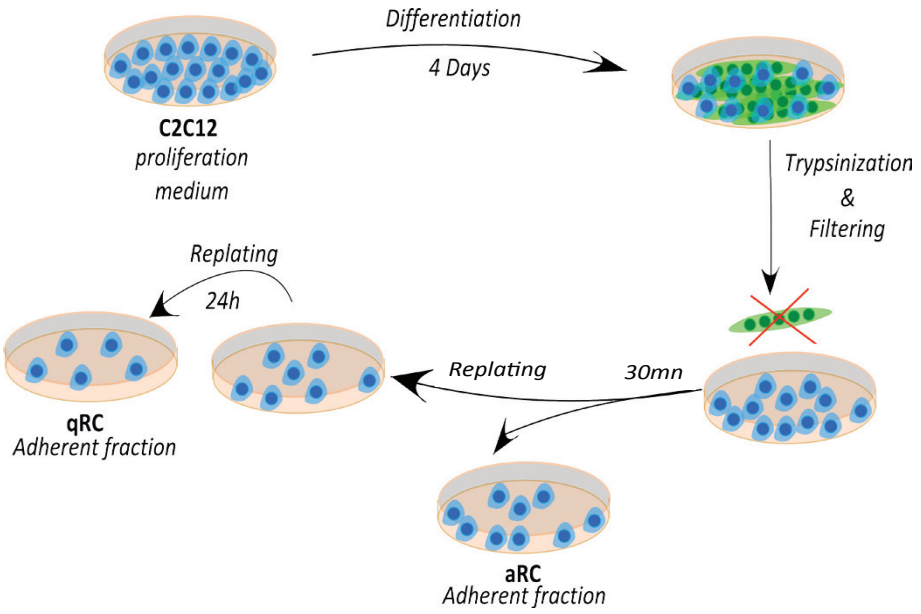

Figure 1: Schematic representation of preplating technique used to isolate the reserve cell populations.

brane dye CFSE. CFSE is proportionally distributed over daughter cells during cell division. CFSE-labeled RCs were plated and intensity of CFSE-staining was determined every 24h by flow cytometry. As shown in Figure 2B qRCs started diluting CFSE 48 h after plating while aRCs had already diluted CFSE twice at this time point of the culture. In the next 24h CFSE was diluted twice by qRCs indicating that after a lag-phase of at least 40h these cells re-entered the cell cycle. Therefore, qRCs enter complete quiescence after four days in differentiation culture, while aRCs seem arrested in G1.

qRCs have an enhanced in vitro differentiation potential

To assess the potential of qRCs and aRCs, the cells were induced to differentiate in culture. Both populations formed myosin heavy chain-positive multinucleated myotubes, but the density and size of the myotubes formed by the progeny of qRC was increased as compared to the one formed by aRCs and parental cells (Fig. 2C). Our preliminary data indicates that at increased passages the qRC-derived C2C12 population retained the ability to differentiate, while the aRC completely failed to differentiate (data not shown), These data suggest that both RC populations retained

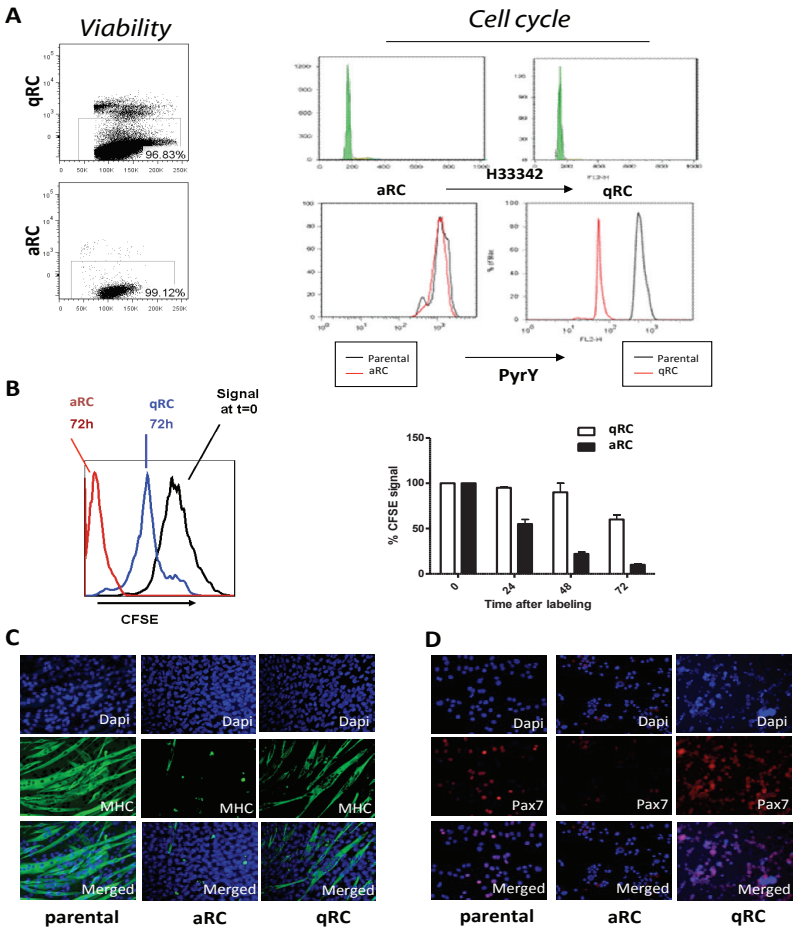

Figure 2: C2C12 Reserve cell population contains two different subpopulations.
(A)C2C12 cells were cultured in differentiation medium for 4 days and quiescent reserve cells (qRC) and activated reserve cells (aRC) were harvested as described. Populations were stained to test cell viability (H33258) or permeabilized and stained with H33342 and Pyronin Y (PyrY) to determine DNA/RNA content (cell cycle)
(B) qRC are quiescent at isolation and re-enter cell cycle with delay.qRC and aRC were labeled with CFSE (when, which time point= important) and every 24h CFSE staining was determined. The histogram plot shows CSFE staining intensity for all populations 72h after labeling (left panel). The bargraph shows the averaged CFSE staining intensity of qRC and aRC population at different time points of at least two independent experiments (right panel).(C) qRC have increased myogenic differentiation potential as compared to aRC. Immunostaining of the myogenic differentiation marker myosin heavy chain (MHC) in differentiated qRC, aRC and parental cells. qRC and aRC were harvested and allowed to re-enter the cell cycle. The different populations were cultured until confluency and exposed to differentiation medium for 4 days prior to analysis. (D) qRC are enriched with Pax7-expressing cells. Immunostaining of Pax7 in qRC, aRC and parental culture. The pictures show staining of (re)proliferating cultures.

myogenic potential, but that self-renewing potential was enhanced in the qRC population.

The qRC population is enriched with cells expressing the satellite cell marker Pax7

The increased myogenic and self-renewal potential of the qRCs suggested that this population was enriched with stem cells. To verify this hypothesis we stained qRC, aRC and parental populations for Pax7 (satellite cell marker) and MyoD (myogenic commitment marker). qRC homogeneously expressed Pax7 (~90% of cells Pax7-positive), while aRC were virtually Pax7-negative. Pax7 was expressed by only 25% of cells in the initial parental cultures (Fig. 2D). MyoD was similarly expressed in qRC, aRC and parental populations. Therefore, the qRC population is enriched for highly myogenic Pax7-expressing cells that in vitro functionally and phenotypically resemble to muscle satellite cells.

Prospective isolation of qRC

Previously, we have found that in proliferating cultures the expression of the cell surface marker CD24 (a known P-Selectin ligand) is associated with reduced myogenic potential in vitro (chapter 2) and in vivo. Quiescent satellite cells in vivo (unpublished observations, Tom Cheung) or in freshly isolated myofibers similarly lack CD24 expression (Fig. S1). To determine if the immunophenotype of qRCs has changed, we stained the cells for CD24. Indeed a decrease in CD24 expression may indicate that qRCs are immune-privileged, which would facilitate their engraftment (Fig. 3A). The analysis shows that qRC cells have reduced CD24 expression (Fig.3A), but upregulate these markers upon proliferation (Suppl. Fig)). Freshly isolated aRC cells expressed CD24 at similar levels as proliferating parental cells (Fig 3A). We also stained the population for the satellite cell surface marker integrin A7 (ITGA7). ITGA7 is used a cell surface marker to purify SC from murine muscle (Sacco et al. 2008, Bentzinger et al. 2012). Our data shows that ITGA7 levels are increased in qRC (Fig. 3B), while aRC have very low ITGA7 levels. Note that the CD24negative population of C2C12 bulk, which is enriched with muscle stem cell activity (Chapter 2) also shows increased ITGA7 levels (Fig. 3A-B). To verify that loss of CD24 expression was specifically associated with the qRC phenotype, we also stained the different cell populations for other cell surface markers commonly associated with myogenic cells, including CD44 and integrin beta1 (Fig. 3C). This analysis indicated that these cell surface markers were expressed at comparable levels in qRC, aRC and parental cells. Taken together, qRC downregulate CD24 and upregulate ITGA7 and acquire a CD24neg/lo/ITGA7hi cell surface signature that may allow direct isolation of qRC from differentiated cultures.

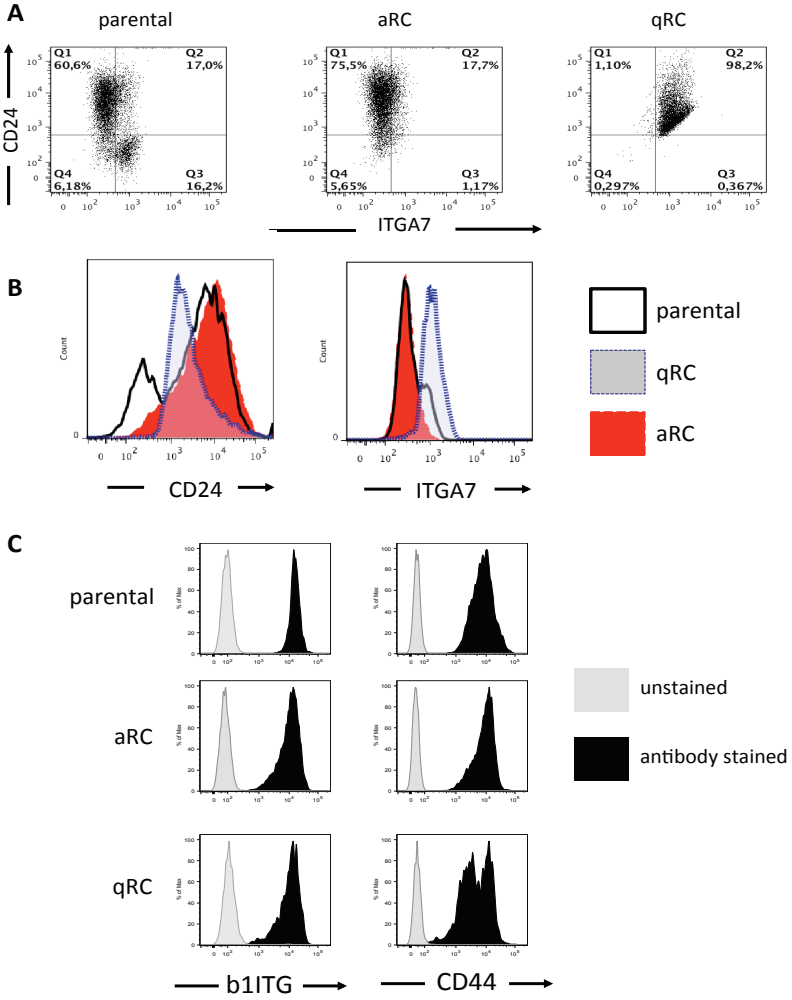

Figure 3: Prospective isolation of qRC

(A) C2C12 cells were induced to differentiate and after 5 days aRC and qRC were harvested as described. C2C12 bulk cells, aRC and qRC were stained for CD24 and integrin A7 and analysed by flow cytometry. The figures shows representative dotplots from 2 independent experiments. (B): Histogram plots of, respectively ,CD24 and ITGA7 staining of the experiment described in A.(C) qRC, aRC and parental culture express level of integrin B1 (ITGB1) and CD44.aRC, qRC and parental C2C12 were harvested and stained with ITGB1 or CD44. FACS analysis shows that both marker were expressed at similar level in aRC, qRC and parental culture.

Discussion

Myoblast transplantation was unsuccessful in the past due to poor survival and poor migration of the grafted cells (Beauchamp et al 1999). In contrast, satellite cells have enormous regenerative potential (Sacco et al. 2008), but their number in vivo is limited. Moreover, satellite cells rapidly lose their regenerative potential upon ex vivo cultures, which limits their use for clinical application and research. The culturing and isolation of self-renewing reserve cells from long-term cultures might offer a solution to these problems by combining the ease of myoblast culture with the myogenic potential of muscle satellite cells.

In previous reports, a homogenous population of non-differentiating cells with reserve cell characteristics has been identified. Our data shows that C2C12 cultures in fact contain heterogeneous RC populations comprising aRCs and qRCs. qRCs were not identified before because they were discarded in previous culture protocols used to isolate RCs. qRCs are quiescent for the first 40h after their recovery from differentiated cultures, while aRCs immediately resume DNA synthesis upon isolation and replating.

In vivo the satellite cell population is heterogeneous (Kuang et al. 2007, Rocheteau et al. 2012). Labeling experiments showed that there are two populations of satellite cells. One population (80% of the satellite cells) divides rapidly and provides new myonuclei. The other population (20% of the satellite cells) divides slowly and most likely replenishes the satellite stem cell pool. Our results indicate that in vitro in cultures, the non-differentiating cell population is equally heterogeneous. The qRCs seem to be the most immature muscle precursor cells while the aRCs represent a more activated population (as shown by the immediate re-activation of DNA synthesis and cell cycle progression when the RCs were placed under proliferating conditions).

Our observations indicate that qRCs generate aRCs during a second cycle of differentiation culture. Furthermore, aRCs also generate qRCs, albeit at a lower frequency (data not shown). This indicates that qRCs and aRCs are hierarchically organized but that aRCs revert at low level to a qRC stage. This is similar to activated (Pax7+/MyoD+) satellite cells that may revert back in vivo to their quiescent (Pax7+/MyoD-) counterparts (Shea et al, 2010, Zammit, 2004). Therefore, qRCs might represent an in vitro equivalent of the satellite stem cell found in vivo. Although the identification of qRCs and aRCs in primary culture remains a first step, cultures of qRCS could provide a very interesting model to study satellite cell population heterogeneity and self-renewal properties in vitro.

Qu et al. isolated muscle derived mononuclear cells directly from murine limb muscle and performed several preplating culture rounds (Qu et al. 1998). They found that cells from the last preplate (cells adhering with the longest delay) were highly myogenic and showed the highest survival after in vivo transplantation. Also, other muscle resident cell populations with poor adherence properties have been described

(Jankowski et al. 2002, Ono et al. 2012). Interestingly, these populations were reported to have stem cell properties. Winitsky et al. studied a population of CD34-CD45- cells directly isolated from muscle (Winitsky et al. 2005). Nomura et al. (Nomura et al. 2008) described a population of cells within human skeletal muscle, which proliferated as non-adherent myospheres and could differentiate into cardiac, smooth muscle and endothelial cells. More recently Ono et al. (Ono et al. 2012) demonstrate that the long self-renewal property of freshly isolated muscle cells resides in the slow-dividing population. When label-retaining cells isolated directly from GFP-positive donor animal are transplanted to an injured host they engrafted very efficiently and contributed robustly to muscle regeneration. A comparison between the populations of Ono isolated from fresh muscle study and the one identified in this study could an interesting future experiment.

All previous reports are based on the culture of freshly isolated muscle cells. Here we demonstrate by using C2C12 cultures that a population of stem cell-like myogenic cells, the qRCs, is retained at long-term and can be harvested from terminally differentiated cultures. The C2C12 is one of the most commonly used cell line for the study of myogenic differentiation in vitro. Being able, with our approach, to isolate qRC from such model represent a proof of principle.

Furthermore, the ability to ex vivo expand cells that maintain high myogenic potential is an important step in the development of a cell-based therapy for muscle degenerative disease. We have preliminary evidence that RCs recovered from long-term cultured primary murine myoblasts robustly contribute to the regeneration of injured muscle (data not shown). The possibility to expand regenerative cells may allow the use of autologous cells to limit the risk of immune-rejection, but also to correct the genetic defects underlying most muscle-degenerative diseases.

The use of this 'cycle' passaging approach offer the possibility to expand cells over many passages with maintenance of stem-cell potential. Our model might allow to study the requirements for maintaining satellite cell identity, modulating stem cell fate choice and selfrenewal under defined conditions that may be ultimately may be used to expand muscle cells for regenerative purposes. The next step will be to evaluate the engraftment capacity of the qRCs and their ability to contribute to regeneration upon transplantation.

Material and Method

Cell culture

Growth medium (GM): C2C12 were cultured in DMEM supplemented with 20% fetal calf serum (FCS) and 1% penicillin/streptomycin (PS). Differentiation medium (DM): DMEM medium containing 2% horse serum and 1% PS. The differentiation experiments were performed in ECM -coated dishes.

Reserve cells isolation

A 4-day-old differentiated muscle cell culture was trypsinized by addition of Trypsin/EDTA (TE) for 4-5 minutes at 37^{0}C. The cell suspension was filtered through a 40µm nylon filter, to filter out the myotubes. The filtered cell suspensions were replated and cells were allowed to adhere. The supernatant containing non-attached cells was replated after 30 min to a new culture plate. The atteched cells were designated as aRCs were cultured further in GM. The non-adhering cells were allowed to attach over night (O/N), after which the supernatant containing slow adherent cells or qRCs was passaged to a new plate to allow attachment and expansion.

Cell cycle analysis

aRCs and qRCs were harvested and washed with Phosphate Buffered Saline (PBS). Cells were spun down and resuspended in 60µL PBS. 140µL ethanol absolute was added drop wise while gently mixing the cells. Fixed samples were stored at -20^{0}C. Prior the staining, samples were spun down at 5500 rotations per minute (rpm) for 10 minutes. Ethanol was removed and samples were washed with Hank's Balanced Salt Solution (HBSS) (GIBCO-BRL, Breda, The Netherlands). Samples were again pelleted and resuspended in 250µL HBSS containing 10µL Hoechst 33342 (1mg/ml) (Invitrogen, Breda, the Netherlands) to stain for DNA content. Samples were incubated for 15 minutes on ice in the dark, after which 250µL HBSS containing 5µL Pyronin-Y (Sigma, St. Louis, USA) was added to stain for RNA content. Samples were incubated for another 15 minutes on ice in the dark after which they were analyzed by Fluorescent Activated Cell Sorting (FACS).

CFSE labeling

A 5mM CFSE stock solution was prepared in DMSO and kept at 4^{0}C. NDCs were isolated from a 3 day-old differentiated culture and kept in suspension. Cells were washed with PBS, pelleted down and resuspended in prewarmed 5% Normal Goat Serum (NGS)/PBS at a concentration of 1 million cells/mL. 2µL of CFSE stock solution was added. Cells were incubated for 10 minutes at 37^{0}C. 5mL ice cold GM was added to quench the staining, followed by 5 minutes incubation on ice. Cells were washed 3 times in GM after which they were preplated to obtain the populations of interest. A reference sample was harvested and fixed directly after staining (t=0) and stored in the dark at 4c. The aRC and qRC samples were harvested after 72h. All the sample were analyzed by FACS the same day.

Immunocytochemistry

Parental cells, aRCs or qRCs (25000 cells each) were plated on glass coverslips which were coated with ECM. Cells were allowed to proliferate for 48 hours before cells were fixed in 2% paraformaldehyde (PFA) and stored at 4^{0}C until analysis.

Prior to staining, cells were washed with PBS. Cells were permeabilized with 0.25M NaOH solution, followed by an incubation with 1% SDS 0,1%triton solution. Samples were blocked with 5% normal goat serum followed by an O/N incubation at

4^0C with primary antibodies (1:100 mouse anti-Pax7/5%NGS (DSHB); 1:20 mouse anti-MHC/5%NGS(DHSB)). After rinsing the samples with PBS/Tween 0.1%, the samples were incubated with the corresponding secondary antibodies (1:500 goat anti-mouse Alexa 594 (Invitrogen, USA)/PBS). Slides were mounted with vectashield containing Dapi (Vector Laboratories) and analysed with the Zeiss Axio Imager Z1 fluorescent microscope.

Single fibers isolation and staining

Single fibers isolation and staining were performed according to the protocol described in Zammit et al. 2004. EDL muscle from 2-3 C57bl6 mice were harvested, digested in a solution of PBS/collagenase II (Gibco). Fibers were dissociated mechanically. From each other.. The single fibers were placed in plating medium (HAMS F10 + 10%HS + 0.5% Chick Embryo Extract (CEE)) during the process. A part of the fibers were then harvested and fixed with 2% PFA (D0 sample). The remaining fibers were then cultured for 48h (D2) and fixed with PFA. Finally immnumostaning was performed.

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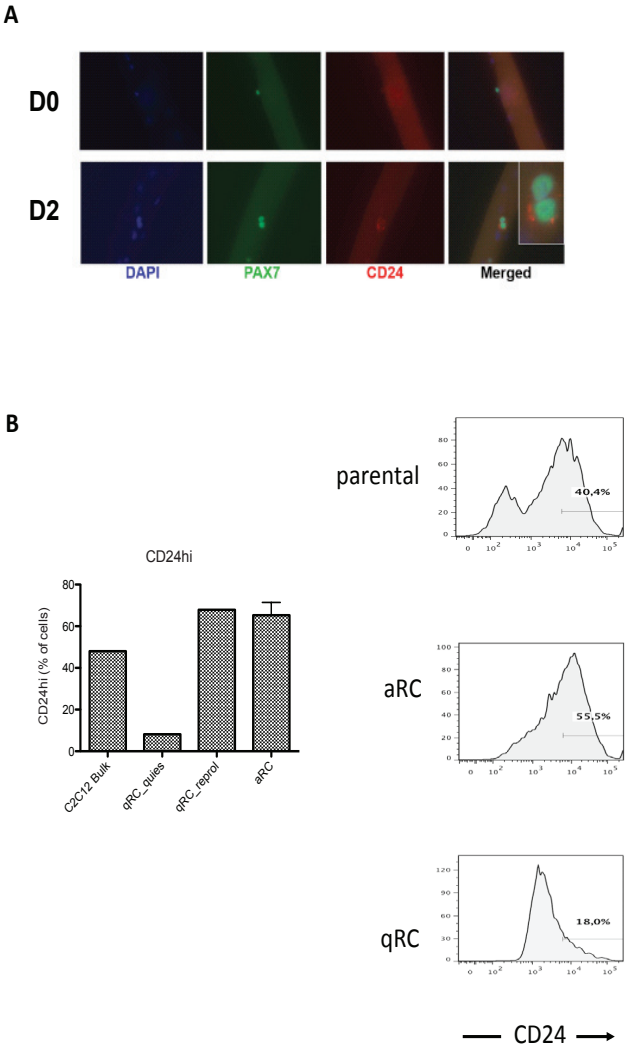

Supplementary Figure

(A) Single myofiber analysis showing that quiescent satellite cells are CD24-negative. Myofibers were prepared as described by Zammit et al (9). The top row are myofibers directly fixed and stained after isolation (D0), the bottom row fibers after 48h in proliferation medium (D2). Doublets indicated proliferated SCs.(B) qRCs display a decreased CD24 expression..Proliferating C2C12 bulk cells, qRC directly after isolation (qRC_quies), qRC at passage 4 after isolation (qRC_reprol) and aRC (passage 4 after isolation) were stained for CD24. The barplots indicates the % of cells expressing high levels of CD24 (CD24hi population defined as upper 50% of parental culture; example of the gating strategy in the right panel).

Chapter 4

Role of CD24 in tumorigenicity of rhabdomyosarcoma

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Expression of Glycosylated CD24 Inhibits Properties of Tumor-initiating Cells and is Downregulated by Oncogenic RAS in Rhabdomyosarcoma

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Abstract

Rhabdomyosarcomas (RMS), pediatric soft tissue tumors characterized by myogenic differentiation, are still fatal to almost one third of patients. Tumor-initiating cells (TIC) play key roles in metastatic growth and tumor relapse and are considered prime targets for innovative cancer therapies. We identified the glycoprotein CD24 as a marker allowing enrichment of TICs from both mouse and human RMS. RMS TICs resided in the CD24-negative compartment and displayed TIC properties of self-renewal, clonogenic potential, efficient tumor initiation and increased drug resistance. Re-expression of CD24 reduced the clonogenic potential and drug resistance of RMS cells, indicating a functional role for CD24 in determining the TIC phenotype. The function of CD24 was dependent on the extent of glycosylation, illustrated by the loss of clonogenic potential following treatment with N-acetylglucosamine (GlcNAc). GlcNAc-treatment increased CD24-glycosylation and extended CD24 cell surface residency and stability, suggesting a role for CD24 in cell adhesion and/or signaling. CD24 expression was specifically downregulated in tumors harboring oncogenic RAS mutations. CD24 overexpression reduced the TIC frequency in oncogenic RAS-positive RMS, indicating that loss of CD24 expression contributed to the tumorigenic activity of mutant RAS. Our findings indicate a novel role for CD24 in regulating the size of the TIC compartment. This study provides a rationale for developing an adjuvant therapy based on increasing the expression and glycosylation of CD24 to reduce the frequency of TIC cells and increase the efficacy of conventional treatments. Such an approach may be particularly relevant for the treatment of RMS, which frequently harbor RAS mutations.

Introduction

Rhabdomyosarcomas (RMS) are malignant pediatric tumors with skeletal muscle differentiation. Despite progress in treatment response, RMS patients are treated with multimodality therapy consisting of intensive (neo-) adjuvant chemotherapy, surgery and radiation treatment, but still these tumors are fatal to almost one-third of patients. Further intensification of chemotherapy regiments will not improve cure rate, but will increase treatment-associated morbidity. It is therefore clear that novel therapeutic approaches are warranted.

Tumors are composed of subsets of cells with varying tumorigenic potential based on their propensity to metastasize or resistance to therapy.[1-5] Cells with increased tumorigenic potential, often referred to as tumor-initiating cells (TIC), are thought to present a major obstacle to therapy as TICs are associated with increased therapy resistance and propensity to relapse the tumor. TICs were first identified in childhood leukemia[4], but recent studies show the presence of TICs also in solid tumors.[6-8] Much debate exists whether tumors harboring TICs follow the hierarchical tumor stem cell

model or evolve by clonal evolution. However, irrespective of the model these tumors adhere to, it is becoming clear that the identification and elimination of this highly tumorigenic population is clinically relevant[6] and constitutes a step forward in the treatment of cancer.

The variation in clinical response and histopathological presentation confirms the heterogeneity of RMS and a recent study shows the functional importance of the different tumor subpopulations in Zebrafish RMS.[9] Ignatius and colleagues showed that non-TICs were important in recruiting TIC cells to new sites of tumor growth.[9] Using the same model, Langenau et al. identified a serially transplantable population of tumor cells in a KRAS-induced Zebrafish RMS model.[10], confirming the presence of cells with TIC properties. Recently, the retention of cells with TIC properties in ERMS cell lines was demonstrated[11, 12], showing that these highly malignant cells even persist and can be studied *ex vivo*.

In this study, we aimed to further characterize the heterogeneity in human RMS in order to define the balance between the TIC and non-TIC compartment. The ability to tip the balance in favor of the non-TIC population will have a clinical impact as elimination of the TIC population reduces the risk on tumor progression and relapse. To this end, we found that the cell surface marker CD24 was strongly and heterogeneously expressed in RMS. CD24 is a heavily glycosylated cell surface molecule that is expressed in developing and regenerating tissues[13, 14] and is used in tumor biology as a marker to identify cells with TIC properties.2, 15, 16 Sorting experiments revealed that CD24-negative (CD24-ve) RMS cells efficiently formed colonies in soft agar and could be serially passaged in immunedeficient animals. In contrast, CD24+ve cells were restricted in their self-renewal potential. Forced expression of (glycosylated) CD24 resulted in loss of self-renewal potential and increased sensitivity for doxorubicin, a chemotherapeutic agent used in the treatment of RMS. Interestingly, the levels of CD24 were reduced in RMS cells expressing oncogenic RAS, an oncogene frequently mutated in RMS. Given the growth-inhibiting role of CD24 in RMS, the inverse correlation between oncogenic RAS and CD24 expression is likely to contribute to the tumorigenic properties of oncogenic RAS.

Taken together, our results indicate that CD24 has tumor suppressive activity in RMS and that expression of CD24 is suppressed by the activity of oncogenic RAS. Increasing CD24 levels or glycosylation, as adjuvant therapy, would decrease the frequency of self-renewing tumor cells and may reduce the risk on tumor relapse mediated by drug-tolerant cells.

Material and Methods

Materials and Antibodies

Dulbecco's modified Eagle's medium (DMEM) and DPBS were purchased from Lonza (Benelux BV, Leusden, The Netherlands). A hybridoma for anti-CD24 (clone M1/69) was a kind gift from Dr. P. Altevogt (University of Heidelberg, Germany) and

was purified at Bioceros as previously described [17], origin and dilutions of all other antibodies in Table S1.

Transcriptome analysis

Transcriptome analysis was performed using both publicly available and home-made datasets from three gene expression platforms: Affymetrix U133A [53], custom cDNA arrays [54] and SAGE libraries. [54] Datasets were normalized and data from normal skeletal muscle was compared with RMS datasets. Genes were considered differentially expressed only if significant in the datasets from all three platforms. The bioinformatical analyses are described in detail in Schaaf et al. (*manuscript in preparation*).

Plasmids, construction and transfection

pEGFP-KRAS^{G12V} was a kind gift from Dr. M. R. Philips (NYU Cancer Institute, USA). pRETRO-SUPER (pRS) and pRS-shKRAS plasmids were a kind gift from Dr. R. Agami (Netherlands Cancer Institute). [55] pGL3-luc is an expression vector containing a full-length luciferase cDNA. Full-length CD24 cDNA (for primer sequences see Table S2) was cloned into the BamH1/Not1 site of pCDNA4/T0 to generate vector for DOX-inducible expression of CD24. Cells were transfected with the appropriate vectors as described previously [17] using Lipofectamine2000 (Lifetechnologies, USA) in Optimem (Lonza, USA). The next day, the cells were split and selected in medium containing the appropriate antibiotic selection agent.

Cell culture

The human embryonal RD, TE381T, Hs729T, T174 (ATCC, Rockville MD), and RUCH2 (kind gift from Dr. Schäfer, University of Zürich, Switzerland), and alveolar RH30 (kind gift from Dr. Lollini, University of Bologna, Italy) rhabdomyosarcoma cells were cultured as described previously. [56] Cells were cultured in a 5% CO2 humidified atmosphere. 100 ng/ml doxycycline (Sigma, St. Louis, USA) was added to the culture medium to induce CD24 expression.

Cell culture assays

The clonogenic analysis was performed by sorting cells (FACS ARIA III, BD biosciences) at limited dilution into 96 well plates using in medium with the appropriate treatment (DOX or GlcNAc). The plated cells were allowed to establish viable colonies, which were visualized and quantified by MTT assay. The frequency of clonogenic cells per sorted population was calculated using http://bioinf.wehi.edu.au/software/elda/. The statistical analysis provided by this tool was used to calculate P-values.

Evaluation of viability (MTT assay), cell proliferation, anchorage-independent growth (soft agar assay) and the generation of inducible cell lines was performed as described previously. [19]

Determination of EdU incorporation was according to manufacturer's instructions (Life Technologies, Bleiswijk, The Netherlands)

RNA, DNA and protein analysis

Cells were harvested in Trizol reagent (Life Technologies). Total RNA was prepared according to the manufacturers' protocol. Primer sequences for RT-PCR analysis are provided in Table S2. NRAS, KRAS and HRAS codons 12, 13 and 61 were sequenced (primers in Table S2). Reactions were run on a DNAengine thermal cycler (Biorad, Veenendaal, The Netherlands) and run at our Center for Biomics.

Western blotting was performed as described previously. [19] Membranes were developed with specific antibodies described in the text. Origin and dilutions of antibodies used, is provided in Table S2.

Immunostaining was performed on fixed cells (4% PFA, 10 min by indirect staining as described previously.[57] Primary antibodies used are listed in Table S2. Images were taken on a Zeiss Axio Imager Z1 fluorescent microscope (Carl Zeiss BV, Sliedrecht, The Netherlands). H&E stainings were performed as described.[19]

FACS analysis and sorting

Cells were trypsinized, washed and blocked 30 min on ice with 5% NGS. Cells were then stained with appropriate antibodies in DMEM/2% FCS in suspension for 1 h on ice. Subsequently, the cells were washed twice with PBS, resuspended in PBS/Hoechst 33258 (Sigma, St. Louis, USA). Cells were analyzed or sorted using a FACS Aria III cell sorter (Becton-Dickinson BV, Breda, The Netherlands).

Grafting and serial passaging in vivo

Grafting experiments were performed as described previously.[19] In short, RMS cells were trypsinized and suspended in PBS supplemented with 10% mouse serum and kept on ice. Within 1 hour cells (in 50 µl) were injected in the Tibialis Anterior (TA) muscles of four to eight weeks old NOD/SCID (Charles River, MA, USA) male mice. Tumor formation was determined by measuring tumors using calipers as described previously.[19] Animals were sacrificed before the tumors reached 1 $cm^{3.}$ All animal experiments were conducted under the institutional guidelines and according to the law and approved by the local animal ethics committee.

Results

CD24 expression is increased in rhabdomyosarcoma

To screen for cell surface markers that are expressed strongly in RMS, we performed a cross-platform transcriptome analysis to look for genes that were consistently differentially expressed between primary human RMS and normal skeletal muscle samples (Schaaf et al. *Manuscript in preparation*). The stringent analysis revealed 38 differentially expressed genes of which 6 genes encoded cell surface molecules (CD24, GAS1, JAM3, NOTCH2, SMBP and TSPAN-9). We decided to focus on CD24 as CD24 is reported as tumor stem cell marker in several tissues and we recently identified CD24 capable of identifying cells with regenerative potential among cultured

skeletal muscle cells.[17]
Increased *CD24 mRNA* and protein expression was verified by gene expression and immunohistochemical analysis of more than 60 primary human RMS samples (Fig. 1A-B). CD24 protein expression was either global or focally concentrated and was restricted to embryonal RMS (ERMS; Fig. 1B). Thus, compared to normal skeletal muscle, both CD24 mRNA and protein is upregulated in RMS.

Reduced CD24 expression in RMS with oncogenic RAS mutation

There was a large variation in CD24 expression within the RMS population. Oncogenic RAS has been reported to decrease CD24 expression[18] and we have shown previously that ERMS-type tumors frequently harbor *RAS* mutations.[19] Based on these observations we re-analyzed CD24 expression in the primary human RMS with and without RAS mutations. This showed that CD24 mRNA and protein expression was reduced in tumor samples harboring activating RAS mutations (Fig. 1B-C). In line with this, *CD24* mRNA levels were reduced in RMS cell lines expressing oncogenic RAS (Fig.1D). In addition, FACS analysis showed that cell lines with mutant *RAS* expressed reduced levels of CD24 on the cell surface (Fig. 1E-F). Immunostainings confirmed the differential expression of CD24 between wild-type and mutant RAS cell lines (Fig. 1E). These data demonstrate that the expression of CD24 and oncogenic RAS in ERMS are inversely correlated.

Oncogenic RAS specifically blocks CD24 expression

We wondered if reduced CD24 expression was directly or indirectly related to the expression of mutant RAS. To explore this, we expressed mutant KRAS ($KRAS^{G12V}$) in myogenic progenitors that as shown in recent studies[20, 21], can be cells of origin for RMS. As myogenic progenitors expressed CD24 strongly but heterogeneously[17] these cells represented suitable tools to investigate the association between mutant RAS and CD24 expression. Expression of $KRAS^{G12V}$ efficiently transformed C2C12 to support anchorage-independent growth and tumor formation in NOD-SCID mice (Fig. 2A). The tumors strongly expressed desmin (Fig. S1) and could be histologically classified as small blue round cell tumors resembling non-alveolar RMS.
Expression of $KRAS^{G12V}$ dramatically reduced CD24 expression at both RNA and protein level (Fig. 2B-C). Flow cytometric analyses confirmed reduced frequency of CD24-expressing cells following transformation by oncogenic KRAS (Fig. 2C). Expression of other cell surface stem cell markers, including c-kit, Sca1, CD29 and CD44 was not affected by oncogenic KRAS (Fig. 2D).
Treatment of the transformed myoblasts with inhibitors of MEK/ERK or PI3K/AKT signaling, two major pathways downstream from oncogenic RAS, did not restore CD24 expression (Fig. S2A). shRNA-mediated silencing of oncogenic KRAS obliterated RAS-downstream RAS-ERK signaling (Fig. 2E, Fig. S2B-C) and eliminated the ability of the cells to form colonies in soft agar (Fig. 2F), but CD24 was not re-expressed (Fig. 2E and S2D). We conclude that oncogenic RAS specifically and irreversibly suppressed CD24 expression.

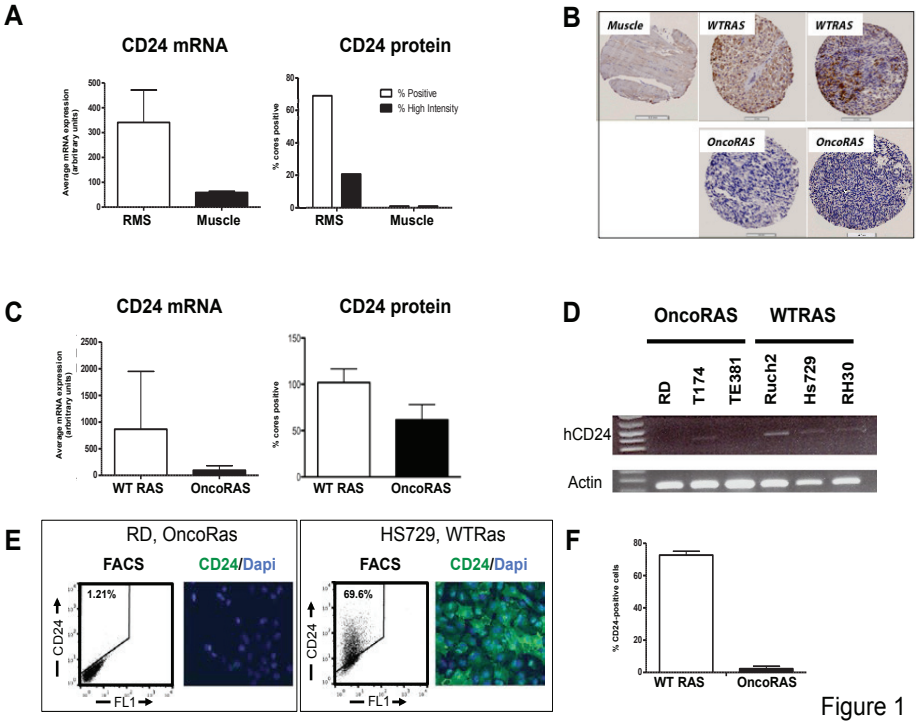

Figure 1

Figure 1 CD24 expression is differentially expressed in RMS and is downregulated in tumors with mutant RAS

(A) Quantification of CD24 RNA (microarray) and protein (tissue microarray) expression in primary RMS and normal human skeletal muscle. Affymetrix microarray analysis was performed as described in methods section. Tissue microarrays containing 63 primary RMS and 6 human skeletal muscle samples were stained with CD24 antibody and scored: 0 no staining, 1-weak staining or staining in a few cells, 2-intermediate staining in most cells or strong staining in few cells, 3-strong staining in a large percentage of cells. The figure depicts the percentage of cores positive (white bars) or with high intensity (black bars), respectively. **(B)** Tissue microarray analysis of CD24 expression in primary RMS and normal human skeletal muscle. Representative cores of primary ERMS tumors with/without RAS mutations stained for CD24 are shown. Hematoxylin was used as counterstain.**(C)** CD24 RNA (microarray) and protein (tissue microarray) expression from ERMS tumors only. Tumors were sequenced to detect NRAS, KRAS or HRAS mutations. Data is presented for tumors with vs. tumors without RAS mutation. Analysis and quantification as in A. **(D)** RT-PCR analysis of CD24 expression in a panel of human RMS cell lines with/without RAS mutation. Actin is shown as loading control. Experiment was repeated twice.

(E) CD24 expression in human ERMS cell lines with (RD) and without (HS729) RAS mutation. Cells were stained and analyzed by flow cytometry to detect cell surface expression of by immunofluorescence microscopy. DAPI was used as counterstain. The figures depicts the results of representative experiment that was repeated at least once.**(F)** Quantification of CD24 expression by flow cytometry of a panel of 6 RMS cell lines that are presented in D is show. The data is depicts the average ± SD and is presented separately for cell lines with and without RAS mutations. The experiment was performed in triplicate and repeated twice.

HDAC inhibitors rescue CD24 expression

Expression of oncogenic RAS is associated with histone deaceylation-dependent chromatin remodeling. [22-24] To determine if CD24 was silenced through HDAC-mediated chromatin remodeling C2-KRAS^{G12V} cells were treated with trichostatin

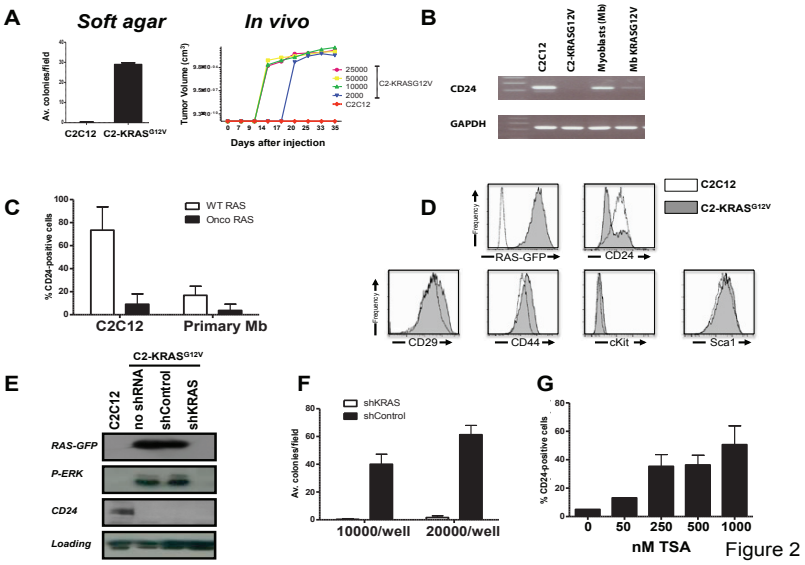

Figure 2

Figure 2 Expression of oncogenic RAS irreversibly silences CD24 expression

(A) Oncogenic KRAS efficiently transforms myoblasts. C2C12 and primary myoblasts were transfected with oncogenic KRAS^{G12V}-GFP and plated in semi solid medium. Colony formation was quantified 3 weeks later (*left panel*). At least 3 fields/well were counted. The histogram depicts the average number of colonies (±s.e.m). For tumor formation, C2C12 or KRAS^{G12V}-transformed cells were transplanted orthotopically to TA muscle of immunedeficient animals at limited dilution. Tumor formation was determined by measurement with calipers (*right panel*). At least 2 animals per cell dose was tested.**(B)** Oncogenic RAS reduces CD24 expression. RT-PCR analysis of CD24 mRNA expression in mock-transfected and KRAS^{G12V}-transformed C2C12 and primary myoblasts. GAPDH is shown as loading control. **(C)** Oncogenic RAS reduces CD24 cell surface expression. FACS analysis of CD24 mRNA expression in mock-transfected and KRAS^{G12V} -transformed C2C12 and primary myoblasts. The figure depicts the average of three independent experiments.**(D)** Oncogenic RAS specifically downregulates CD24. C2C12 and C2-KRAS^{G12V} were analyzed for the expression of various cell surface markers. Experiment was performed twice with similar results. The figure shows histogram plots of a representative experiment. **(E)** CD24 is not reexpressed upon silencing of oncogenic RAS. Western blot analysis of RAS, CD24 and pERK levels in C2C12 and C2-KRAS^{G12V} transduced with shcontrol or shRAS. Total ERK is shown as loading control.**(F)** RAS silencing eliminates tumorigenic potential of C2-KRAS^{G12V} cells. C2-KRAS^{G12V} cells were stably transfected with shcontrol (white bars) or shKRAS (black bars) and plated at 10000 or 20000 cells per well in semisolid medium. Colony formation was quantified 3 weeks later. The figure depicts the averaged results (±s.e.m) of a representative experiment. At least 3 fields/well (4x objective) were counted.**(G)** TSA treatment results in restoration of CD24 expression. C2-KRAS^{G12V} were treated for 72h with increasing concentrations of TSA and CD24 expression was determined by flow cytometry. Experiment was performed in triplicate and repeated once. The figure depicts the results of a representative experiment.

A (TSA) for 72h. Figure 2G shows that TSA treatment resulted in concentration-dependent re-expression of CD24 up to the levels of non-transformed C2C12 (>90% $CD24^{+ve}$ cells as shown in Fig. 2C). These results that CD24 was epigenetically silenced in RAS-transformed myoblasts.

Clonogenic cells enriched in CD24-ve RMS population

To investigate whether the populations with differential CD24 expression were biologically distinct, C2-$KRAS^{G12V}$ cells were sorted on CD24, plated at clonal density and the ability to initiate colony formation was scored. $CD24^{+ve}$ cells displayed a reduced clonogenic potential as compared to $CD24^{-ve}$ cells (Fig. 3A). Interestingly, the few colonies that emerged from *single* $CD24^{+ve}$ cells generally lacked CD24 expression (<25% CD24-expressing cells/colony; Fig. 3A, *lower panel*). In contrast, $CD24^{+ve}$ sorted *populations* that were maintained at subconfluent densities (>10000 cells/cm^2) maintained high levels of CD24-expressing cells for several passages (data not shown).

Next we explored the clonogenic potential of CD24-sorted cells under anchorage-independent conditions. Figure 3B shows that $CD24^{-ve}$ sorted C2-$KRAS^{G12V}$ cells formed colonies more efficiently than $CD24^{+ve}$ sorted population (in number and in size of colonies). These data demonstrated that lack or loss of CD24 cell surface expression promoted clonogenic growth of RMS cells.

Tumorigenic potential of CD24-ve cells

To further test the tumorigenic potential of $CD24^{-ve}$ cells, C2-$KRAS^{G12V}$ cells were sorted on CD24 and CD24-sorted populations were injected orthotopically into the *Tibialis Anterior* (TA) of immunedeficient animals. At lower doses (500-2500 cells) tumors formed much more efficiently after injection of $CD24^{-ve}$ cells (4 out 6 animals) as compared to injection of $CD24^{+ve}$ cells (1 out of 4 transplanted animals formed a tumor). Tumors were formed even after injection of only 500 $CD24^{-ve}$ cells. At higher doses (more than 10,000 cells) both $CD24^{-ve}$ and $CD24^{+ve}$ initiated tumor formation, although the onset of tumor formation was delayed after injection of $CD24^{+ve}$ cells (Table 1). Note that the tumors formed in the primary hosts, irrespective if these were initiated from $CD24^{-ve}$ or $CD24^{+ve}$ cells, lacked expression of CD24 (Fig. 3C and Fig. S3). The $CD24^{-ve}$ cells could efficiently transmit disease upon serial passaging (13 out of 13 animals formed tumors), even at doses as low as 500-1,000 cells (Table 1). The primary tumors did not generate sufficient numbers of $CD24^{+ve}$ cells to evaluate their potential to grow tumors in secondary hosts. These data indicated that $CD24^{-ve}$ cells self-renewed *in vivo*.

Increased tumorigenic potential of $CD24^{-ve}$ RMS cells was also observed in a second model of skeletal tumor muscle formation. Based on the putative origin of RMS in the muscle stem/progenitor cell population[20, 21], we transformed primary muscle progenitors using a random mutagenesis strategy with the mutagen ENU[25]. ENU-transformed myoblasts (ET-Mbs) were highly tumorigenic *in vitro* and formed RMS-like tumors *in vivo* (Fig. S1). ET-Mbs expressed markers of myogenic differentiation, but

were unable to terminally differentiate (Fig. S4). Similarly as wild-type Mbs, ET-Mbs expressed CD24 heterogeneously (Fig. S5A). Upon transplantation CD24^{-ve} ET-Mbs formed primary tumors highly efficiently and could be serially transplanted indicating self-renewal *in vivo* (Fig. S5B and S5C). In contrast, CD24^{+ve} cells only formed small tumors after grafting large doses in primary hosts (Fig. S5B). As was observed for the tumors generated by KRAS^{G12V}-transformed myoblasts, ET-Mb-generated tumors expressed CD24 at very low levels (Fig. S5D) and the rare CD24^{+ve} cells in the primary tumor could not be passaged to secondary hosts (Fig. S5C). Taken together, these results indicated that the CD24^{-ve} population in malignant mouse muscle tumors was enriched with tumor-initiating cells.

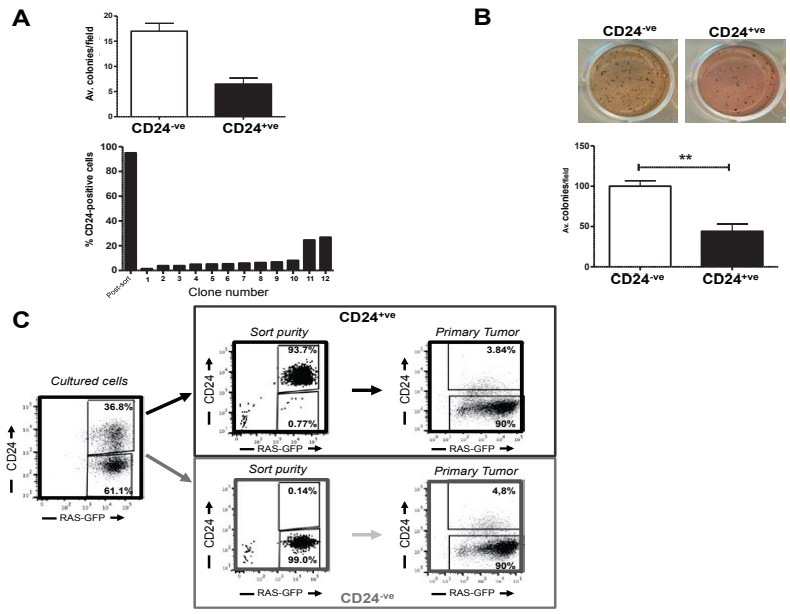

Figure 3

Figure 3 Lack of CD24 expression promotes clonogenic/tumorigenic growth in RMS
(A) Cells with increased clonogenic potential are enriched among CD24^{-ve} RAS-transformed myoblasts. C2-KRAS^{G12V} cells were single cell sorted on CD24 and deposited in 96-well plates. The number of growing colonies resulting from the sorted clones was determined after 3-4 weeks by MTT assay (*upper panel*). The figure depicts the averaged results (±s.e.m) of three experiments. At least 3 fields/well (4x objective) were counted. The lower panel depicts the lack of CD24 expression in growing colonies established by CD24^{+ve} clonally sorted cells. That experiment was repeated with similar results.**(B)** C2-KRAS^{G12V} were sorted on CD24 and plated in soft agar to determine anchorage-independence. Number of colonies formed was counted after 2-3 weeks, at least 3 fields/well (4x objective). The histogram depicts the average number of colonies (±s.e.m) of at least 2 independent experiments. ** $p<0.05$ (t-test). **(C)** RMS tumors *in vivo* lack CD24 expression. CD24-sorted C2-KRAS^{G12V} were injected and tumors were resected one month later. Tumors from CD24-sorted C2-KRAS^{G12V} were resected and digested. CD31^{-ve}/CD45^{-ve}(lin-neg)/RAS-GFP^{+ve} tumor cells were analyzed by flow cytometry for CD24 expression. FACS plots also show CD24 expression of cultured C2-RAS^{G12V} before and after CD24-sort (to show purity of sorted populations). See Figures S3 for the CD24 profile of additional tumors.

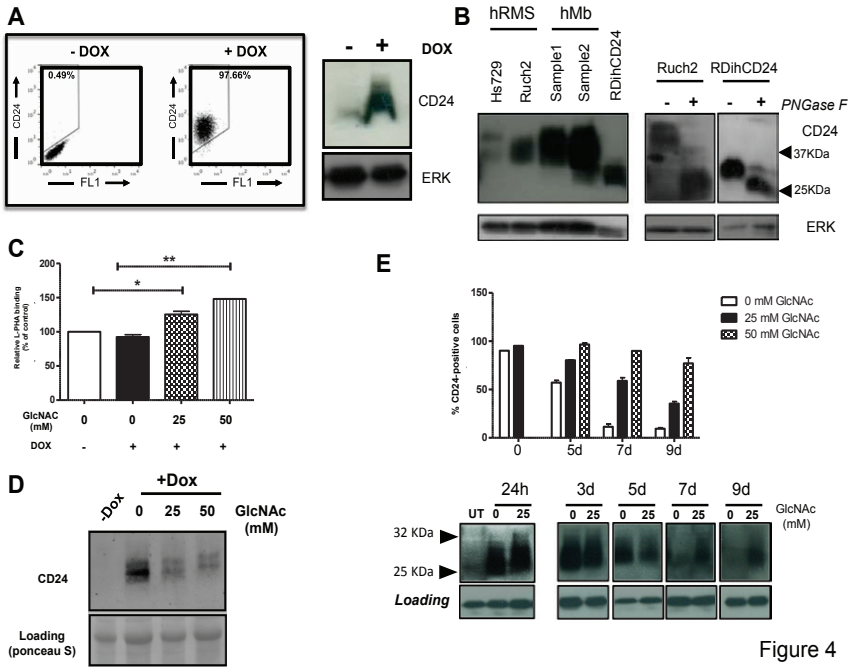

Figure 4

Figure 4 Ectopically expressed CD24 is not fully glycosylated

(A) FACS analysis showing CD24 expression upon treatment of RDihCD24 with doxycyclin (DOX). Cells were treated for 48h with DOX and processed for FACS analysis (*upper panels*). A representative dotplot is shown. The *lower panel* shows a Western blot analysis of untreated and DOX-treated RDihCD24 cell. The membrane was probed with a CD24-specific antibody. The blots are cropped for clarity. Total ERK is shown as loading control.**(B)** Ectopic CD24 is not fully glycosylated in RMS cells expressing oncogenic RAS. Western blot analysis of CD24 expression in lysates from human RMS cell lines (hRMS) with wild-type RAS (Hs729 and Ruch2), human primary myoblasts (hMb) and DOX-treated RDihCD24 (*left panel*). hRMS and RDihCD24 lysates were treated with PNGase F to remove N-glycan moiety to demonstrate CD24 N-glycosylation. The membranes were probed with a CD24-specific antibody (*right panel*). Total ERK was evaluated as loading control.**(C)** GlcNAC treatment increases binding of the lectin LPHA. RDihCD24 cells were treated with increasing concentrations of GlcNAc for 72 h and stained with fluorescent LPHA. LPHA binding was determined by flow cytometry. The figure shows the average + s.e.m. of four experiments. Oneway-anova analysis was performed to determine statistical significance. * $P<0.05$; ** $P<0.001$.**(D)** GlcNAc treatment increases glycosylation of ectopic CD24. Western blot analysis showing a shift in MW of ectopic CD24 upon GlcNAc treatment. Ponceau S staining of the SDS gel is shown as loading control. The blots are cropped for clarity.**(E)** Glycosylated CD24 is retained at the cell surface. RDihCD24 were pretreated with GlcNAc for 48h. Untreated and GlcNAc treated cells were pulsed with DOX to transiently induce CD24 expression. After 24h cells were rinsed extensively, passaged and chased in DOX-free medium (to prevent *de novo* CD24 expression) with or without GlcNAc as indicated. The expression of (ectopic) CD24 was determined at the indicated timepoints. The upper panel shows a FACS analysis of CD24 cell surface expression. The figure shows representative results of two independent experiments. The lower panel shows Western blot analysis of a parallel experiment. The blots are cropped for clarity. Together, the data shows that increased CD24 glycosylation results in extended cell surface residency and delayed protein degradation.

N-glycosylation essential for cellular localization of CD24

To investigate a functional role of CD24 in RMS, we modified C2-KRAS^{G12V} and RD (a human RMS cell line with an endogenous NRAS mutation) cells to allow expression of CD24 upon doxycycline treatment (further referred to as C2- KRAS-^{G12V}-ihCD24 and RDihCD24, respectively). The resulting cell lines robustly expressed CD24 within 24h of treatment with doxycycline (DOX) as determined by FACS or Western Blot (Fig. 4A). To our surprise, CD24 expression did not affect the anchorage-dependent or -independent growth of these RMS cells (data not shown). Western blot analysis revealed that ectopic CD24 was not properly glycosylated. The molecular weight (MW) of ectopic CD24 was markedly reduced compared to the endogenous protein (Fig. 4B). PNGase F (removes N-glycan moieties) treatment of endogenous CD24 reduced the apparent MW of endogenous CD24 from 37 KDa to 25 KDa (Fig. 4B and Fig. S6A), while the MW of ectopic CD24 was hardly affected by PNGase F treatment. These data indicated that ectopic CD24 was minimally glycosylated (Fig. 4B).

Based on our findings with the ability of endogenous CD24 (i.e. glycosylated CD24) expression discriminating between populations with differential tumorigenicity, we wondered if CD24-glycosylation was relevant for its role in tumorigenesis. GlcNAc was added as supplement to the medium of RDihCD24 cells to raise the levels of UDP-N-acetylglycosamine (UDP-GlcNAc), the common donor used to produce N-glycans.[26] GlcNAc treatment resulted in ~1.5 fold increased binding of the lectins E-PHA (data not shown) and L-PHA (Fig 4C) that serves as readout for the expression of cell surface N-glycans.[26] Similar results were obtained when C2-KRAS^{G12V} were treated with GlcNAc (Fig. S6B). GlcNAc-treatment increased the MW of CD24 as detected by Western blot analysis (Fig. 4D and 4E, *left panel*).

The extent of N-glycosylation was reported to determine the cell surface residence and activity of growth factor receptors including TGF-beta receptor and EGFR.[26] To investigate the role of N-glycosylation in determining the cell surface residency of CD24 we pulsed RDihCD24 cells with DOX to induce CD24 expression and chased the cells in DOX-free medium in the presence/absence of GlcNAc. CD24 cell surface expression was maintained in GlcNAc-treated cells, while untreated cells rapidly lost expression of CD24 (Fig. 4E and Fig. S6C). These data demonstrated that the ectopic CD24 is glycosylated and functional following treatment with GlcNAc.

Glycosylated CD24 reduces self-renewal potential

Next we asked if glycosylated CD24 would affect the proliferation potential of RMS cells. Untreated RDihCD24 cells or cells treated with DOX- or GlcNAc-only could be passaged at least five times without loss of proliferative capacity. In contrast, RDihCD24 cells expressing glycosylated CD24 (DOX+GlcNAc-treated) lost the potential to be passaged (Fig. 5A). Similarly, C2 KRAS^{G12V}-iCD24 cells treated

with DOX and GlcNAc simultaneously were depleted upon passaging (Fig. S7A). CD24 was reported to affect several cellular properties, including adhesion[27] or proliferation[28, 29], that may contribute to the loss of CD24-expressing cells during serial passaging (Fig. 5A). To determine if the expression of (glycosylated) CD24 affected plating efficiency, cells pre-treated with GlcNAc and/or DOX were replated at non-confluent conditions and counted after 20h (before the first cell cycle is completed). The expression of CD24 reduced plating efficiency by about 40% (Fig. S7B). In addition, DOX-treated RDihCD24 showed a significant delay in incorporating EdU in the first 24h after passaging (Fig. S7C). The frequency of cells entering and completing S-phase was reduced by almost 40% in cells expressing glycosylated CD24. The expression of CD24 reduced the ability of RMS cells to adhere and to proliferate during serial passaging, which are critical properties of self-renewal potential *in vitro*.

Expression of glycosylated CD24 reduces clonogenic potential

Based on the results described above we hypothesized that the expression of glycosylated CD24 reduced the ability of RMS cells to initiate clonogenic growth. To investigate this RMS cells expressing (glycosylated) CD24 were plated at limited dilution and the colony-forming potential was evaluated. This analysis showed that the frequency of clonogenic cells in CD24-expressing (DOX-treated) cultures was reduced about 4-fold compared to untreated cells. The frequency of clonogenic cells was reduced by almost 30-fold in cultures that were treated with both DOX and GlcNAc to induce expression of glycosylated CD24 (Fig. 5C). In addition, RMS cells that expressed glycosylated CD24 formed less and smaller colonies in soft agar as compared to untreated controls (Fig. 5D). These data show that expression of (glycosylated) CD24 reduced anchorage-dependent and -independent growth.

CD24-mediated cell adhesion was shown to be dependent on integrin expression.[27, 30] We wondered if the reduced clonogenic potential of CD24-expressing cells could be rescued by plating the cells on a laminin-rich substrate (ECM) to facilitate integrin-mediated adhesion. Figure S8D shows that plating of single-sorted cells on ECM-coated plates did not restore the clonogenic potential. However, when the CD24-expressing (DOX and DOX/GlcNAc-treated) cells were plated in conditioned medium from logarithmically growing untreated cells, the reduced clonogenic potential was partially rescued (Fig. S7D). This indicated that soluble factors produced by neighboring cells attenuate the repressing effect induced by membrane-localized CD24.

Expression of CD24 decreases the frequency of drug-tolerant cells

Tumor populations are heterogeneous in their response to chemotherapeutic drugs [31] with the non-TIC population displaying increased drug sensitivity. We hypothesized that $CD24^{+ve}$ cells would be more sensitive to doxorubicin (DRB), a drug that is used in the treatment of childhood RMS. We treated Hs729 and Ruch2 human RMS (both cell lines with wild-type RAS and heterogeneous CD24 expression). At sublethal DRB-concentrations (killing <60% of cells) the surviving populations were enriched with $CD24^{-ve}$ cells (Fig. S8A). When RDihCD24 were exposed to lethal doses of DRB that eliminated more than 99% of cells, drug-tolerant cells emerged in

untreated, DOX-only and GlcNAc-only treated cultures. These drug-tolerant cells re-entered the cell cycle and expanded (Fig. 5D). In contrast, when cells were induced

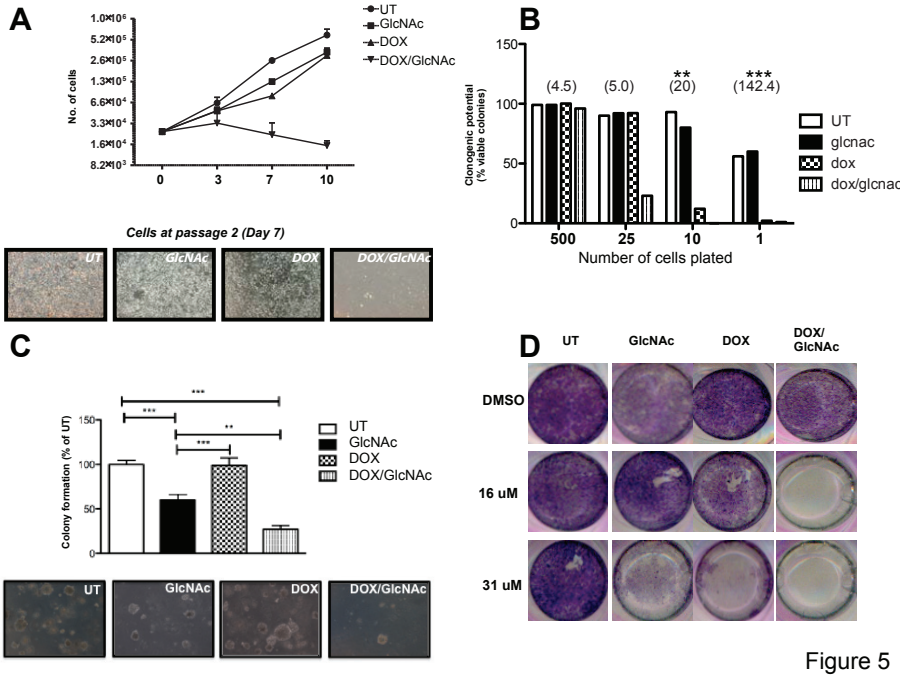

Figure 5

Figure 5 Tumor-initiating cells are enriched in CD24^{-ve} population in RMS

(A) Expression of glycosylated CD24 limits serial passaging. RDihCD24 (human RMS cells with endogenous NRAS mutation) cells were grown with/without DOX (to induce CD24) and with/without GlcNAc (25 mM; to increase N-glycosylation). Each passage the number of cells were counted and plotted. The figure depicts the averaged results (±SD) of a representative experiment that was repeated with similar results. The photomicrographs shown in the lower panel depicts untreated (UT) RDihCD24 cells or cells treated with DOX and/or GlcNAc at passage two of the passaging experiment. A passaging experiment with treated C2-KRAS^{G12V}-iCD24 (RAS-transformed myoblasts) showing similar findings is shown in Figure S8A.**(B)** CD24 expressing cells have reduced clonogenic potential. Cells were pre-treated for 48h with DOX and/or GlcNAc and plated at limited dilution in the appropriate treatment medium. The ability of plated cells to form viable colonies was determined by MTT assay 3-4 weeks after plating. The frequency of clonogenic cells is shown in brackets. For each dilution at least 192 wells were analyzed. UT is untreated. ** P<1e-56; ***P<1e-230. **(C)** Expression of ectopic (minimally glycosylated) CD24 reduces anchorage-independent growth. RDihCD24 cells were plated with DOX and/or GlcNAc in semi-solid medium as indicated in the figure. Cells were fed fresh medium every 3-4 days. After 3-4 weeks colonies were counted (3 fields per condition) and plotted. The histogram shows the average (± s.e.m.) of two independent experiments. The lower panel shows representative photomicrographs of the colonies growing under the respective treatments. UT is untreated. Oneway-anova analysis was performed to determine statistical significance. * P<0.05; ** P<0.001.**(D)** Expression of (glycosylated) CD24 inhibits proliferation and increases chemosensitivity.

Cells were pretreated with either DOX and/or GlcNAc and then exposed to indicated concentrations of DRB for 24h. Surviving cells were stained with Giemsa after two weeks recovery in DRB-free medium. Representative pictures from two independent experiments are shown. UT is untreated.

to express glycosylated CD24 (DOX/GlcNAc-treatment) drug-tolerant cells only appeared after exposure to 3-fold lower doses (Fig. 5D). The drug-tolerance was not stable as cultures established from these drug-tolerant clones regained sensitivity to a second pulse of doxorubicin (Figure S8B). Our data demonstrates that expression of glycosylated CD24 reduced the frequency of drug-tolerant cells, but also that the progeny of the drug-tolerant cells re-acquired drug-sensitivity in line with observation in other studies.[31]

Discussion

Tumor heterogeneity is a complicating factor in cancer treatment and implies the presence of populations with differential tumorigenic potential. An increasing number of cell surface markers are associated with distinct subsets of cells in different types of tumors, but the biological role of these proteins is often unclear. In this study we identify and address the functional relevance of the cell surface marker CD24 that is expressed by non-tumor initiating cells (non-TICs) in RMS. Our data indicates that CD24 expression reduced the number of cells capable of initiating and propagating tumor-growth and also reduced the number of cells with increased tolerance to the chemotherapeutic agent doxorobucin. Our findings show that in RMS CD24 expression antagonizes the properties that are commonly associated with TICs.

The presence of TICs in RMS has been demonstrated in previous studies.[9, 11, 12] In agreement with our findings, RMS cells with TIC properties are retained and can be studied *ex vivo*.[11, 12] The case for TICs in RMS is most convincingly demonstrated by the capability of only 100 $CD24^{-ve}$ cells to initiate tumor growth, while $CD24^{+ve}$ cells were far less capable of tumor initiation or sustaining tumor growth during serial passaging. The $CD24^{-ve}$ cells demonstrated other properties frequently associated with TICs, such as increased clonogenicity, self-renewal potential and resistance to chemotherapeutic drugs. The identification of TICs in both human and mouse RMS, in primary as well as experimental tumors, further underscore the relevance of CD24 as a (negative) marker of myogenic cells with increased tumorigenic potential.

Based on the TIC-repressing activity of CD24 in RMS cells, it is not surprising that CD24 discriminates between RMS TICs and non-TIC cells. CD24 is used as a marker of TIC cells in a range of other malignancies as well. Similarly as in our study, TICs in breast cancer are enriched in the $CD24^{-ve}$ population[2]. However, for other tumors enrichment of TICs has been reported in the $CD24^{+ve}$ population.[15, 32] This paradox may be explained by the transient expression of CD24 during development or postnatal regeneration of several tissues, including hematopoietic [14], neuronal [33] and muscle tissue. [13] As result of this transient expression, the $CD24^{-ve}$ population of these developing tissues may contain both immature and differentiated cells. Developing tumors often display parallels with developing tissues, and CD24 expression indeed decreases in differentiating neuroblastomas {Poncet, 1996 #131}. The presence of non-tumorigenic differentiated cells among $CD24^{-ve}$ cells that vary in frequency be-

tween different tumors or tumor stages, contributes to the observed variation in tumorigenicity of CD24^{-ve} sorted populations. For this reason, CD24 is not very valuable as a single stem cell marker. Similar observations have been reported for other (tumor) stem cell markers, such as CD133. In colon cancer TICs were initially identified in both the CD133-negative as well as CD133-positive fraction.[34] Later it was found that the AC133 epitope, recognized by the most commonly used antibody to detect CD133, is lost upon cancer stem cell differentiation.[35]

Alternatively, the different functions of CD24 in diverse cancer types may contribute to the controversial findings with CD24 as a TIC marker. While some studies report on a growth-stimulating effect [27, 36, 29], others describe CD24-mediated inhibition of tumor cell proliferation.[28] Furthermore, Meirelles reported increased doxorubicin resistance in CD24^{+ve} ovarian cancer cells [37], but the opposite has been described for MDA-MB-231 breast cancer cells.[28] These opposing results suggest that the role of CD24 is cell- or tissue specific. CD24 regulates cellular signaling through binding to src-kinases [31] or through interaction with signaling receptors, such as CD44, CXCR4 and β1-integrin.[38-40] Therefore, the role of CD24 is determined by the tissue-restricted expression of CD24-interacting partner molecules and this will ultimately determine if TIC cells are found in the CD24^{-ve} population or not.

Our results strongly indicate that the acitivity of CD24 is highly dependent on the extent of glycosylation. Lau and colleagues demonstrated that N-glycosylation determines the membrane residence and, thus, the activity of cell surface proteins.[26] Endogenous CD24 expressed in the two independent murine models of RMS included in the present study was extensively glycosylated (data not shown) and highly potent in limiting the tumor-propagating potential *in vivo*. Increasing CD24 glycosylation *in vitro* by treatment with GlcNAc extended the cell surface expression and half-life of CD24 and potentiated its inhibitory effect possibly by enhancing the interaction with signaling partners described above. This indicates that interaction of CD24 with its signaling partners at the cell surface is essential for its antitumorigenic activity. Interestingly, safety and efficacy of oral GlcNAc has been evaluated for the treatment of inflammatory bowel disease in pediatric patients[41] and is also proposed for the treatment of multiple sclerosis.[42] It would therefore be interesting to evaluate the efficacy of GlcNac-treatment as adjuvant therapy in CD24-expressing RMS tumors.

A number of studies report that CD24 expression is associated with increased tumorigenic properties, including metastatic activity [43], invasion [44], and poor prognosis.[44-46] This may indicate that the role of CD24 may be stage-specific. The *in vitro* and *in vivo* limited dilutions experiments demonstrated that lack of CD24 expression promoted clonal growth. Exposure to yet unidentified soluble factors rescued the inability of CD24^{+ve} cells to grow at clonal densities, indicating dependence on surrounding tumor cells. Indeed, at higher cellular densities the proliferation of CD24-expressing cells was unrestricted. These observations are consistent with a model where CD24^{-ve} TICs dominate the initial stages of tumor formation, while during later phases tumors may expand predominantly through extensive proliferation of CD24^{+ve} non-TIC cells. It has indeed been reported that the proliferation rate decreased after CD24

knockdown in lung and pancreatic cancer cells [36] and that breast cancer cells proliferated faster following doxycycline-induced CD24-expression. [47] Our unpublished data indicating decreased proliferation in CD24-deficient myoblasts as compared to their wildtype counterparts are in line with this. [44] The high level of CD24 expression reported in several tumors, including RMS, and the association with poor prognosis[44-46] may indicate that these tumors were rapidly expanding at the time of analysis.
The inverse correlation between oncogenic RAS and CD24 expression in RMS as well as in other cell types [48, 18] is consistent with several components of the RAS/MAPK pathway enriched in the profile of CD24^{-ve} breast cancer cells. [49] RAS mutations and dysregulation of RAS signaling occur frequently in human ERMS [50, 19] and the expression of oncogenic RAS appears to expand the size of the CD24^{-ve} TIC pool. Our results indicated that the RAS-mediated suppression of CD24 is indirect and irreversible. The restoration of CD24 expression in TSA-treated transformed myoblasts suggests an epigenetic mechanism. In line with this, in RAS transformed fibroblasts 55 of 73 RAS-suppressed genes are MEK/ERK independent. Interestingly, 31 of these genes were epigenetically repressed, but the silenced state of these genes was independent of RAS signaling. [51] It has been reported that oncogenic RAS stimulated the nuclear localization and hence the activity of HDAC4 [52], but it remains to be determined if CD24 is silenced through a similar mechanism in RMS cells.
In conclusion, our results demonstrate that expression of glycosylated CD24 expression reduces the frequency of tumor-initiating cells in RMS and increases their drug-sensitivity. Oncogenic RAS increases the number of cells with TIC properties by silencing of CD24 expression. Our data suggest that decreasing the frequency of TICs cells in RMS by pharmacologically increasing CD24 expression and/or glycosylation may be exploited as a novel approach to reduce the risk on tumor relapse and restore the efficacy of established treatments for RMS.

Conflict of interest
The authors declare no conflict of interest
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Supplementary informations

		tumor onset				
Cells injected	n (frequency)	Average (d)	Range (d)	n (frequency)	Average (d)	Range (d)
500-2500	15/18 (83%)	31,4	18-70	1/4 (25%)	21	-
10000	13/13 (100%)	18.3	12-31	3/5 (60%)	25.6	19-30
>10000	6/6 (100%)	23,8	7-36	2/2 (100%)	24	20-28

Table 1: Tumor formation by CD24-sorted RMS cells.
Tumor formation by CD24-sorted C2-KRAS^{G12V}. Cells were sorted on CD24 directly from culture or from the CD31^{-ve}/45^{-ve}/GFP^{+ve} population of primary tumors. The sorted cells were transplanted orthotopically in TA muscle of NOD-SCID animals at limited dilution. Tumor formation was determined using calipers. The average tumor onset, the range of tumor onset and animals analyzed is depicted.

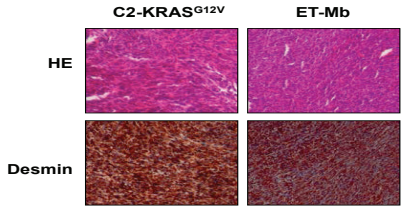

Figure S1

Figure S1:
Immunohistochemical analysis of experimental RMS tumors generated by injection of C2-KRASG12V (A) or ET-MB cells (B). Shown for each tumors are representative pictures of HE and desmin staining.

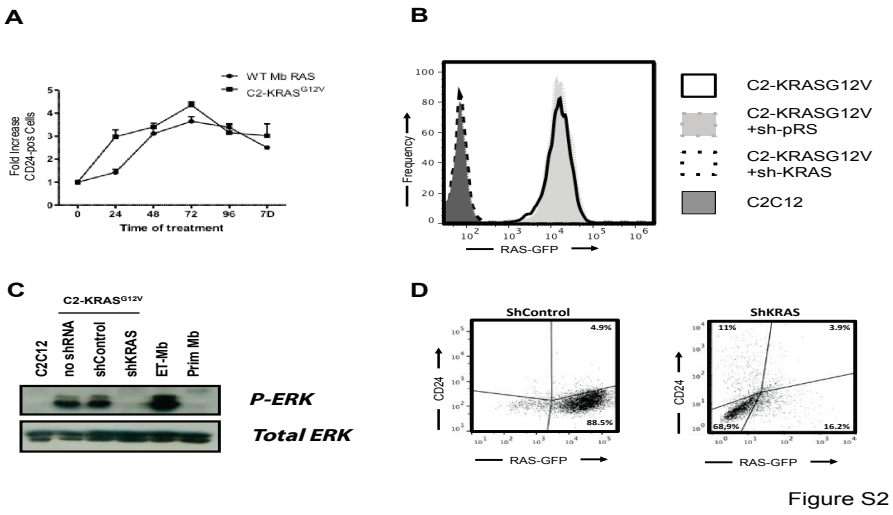

Figure S2

Figure S2:
(A) Inhibition of RAS activity is not sufficient to restore CD24 expression. RAS-transformed C2C12 (C2-KRASG12V) and primary myoblasts (MB-KRASG12V) were treated with PD98059 (MEK inhibitor) and the expression of CD24 in time was determined.(B) Effective shRNA-mediated RAS silencing. Transformed C2-KRASG12V-GFP were stably transfected with shControl (pRS) or shKRAS (pRS-KRAS). Non-transformed C2C12 cells are shown as additional control. The levels RAS-GFP were determined by flow cytometry and histograms are plotted.(C) RAS silencing using shKRAS efficiently silences KRAS expression and activity, but does not restore CD24 expression. Western blot analysis of C2C12 cells, C2-KRASG12V, C2-KRASG12V-pRS and C2-KRASG12V-pRS-KRAS, ENU transformed myoblasts and primary myoblasts. The blot was developed using specific antibodies to determine KRAS levels, KRAS-activity (phosphor-ERK levels) and CD24 levels. This analysis indicated that ET-MBs also display increased RAS/ERK signaling. (D) RAS silencing is not sufficient to restore CD24 expression. C2-KRASG12V were stably transfected with shControl (pRS) or shKRAS (pRS-KRAS) and the level of CD24 expression was determined by flowcytometry. A representative picture out of three independent experiments is shown.

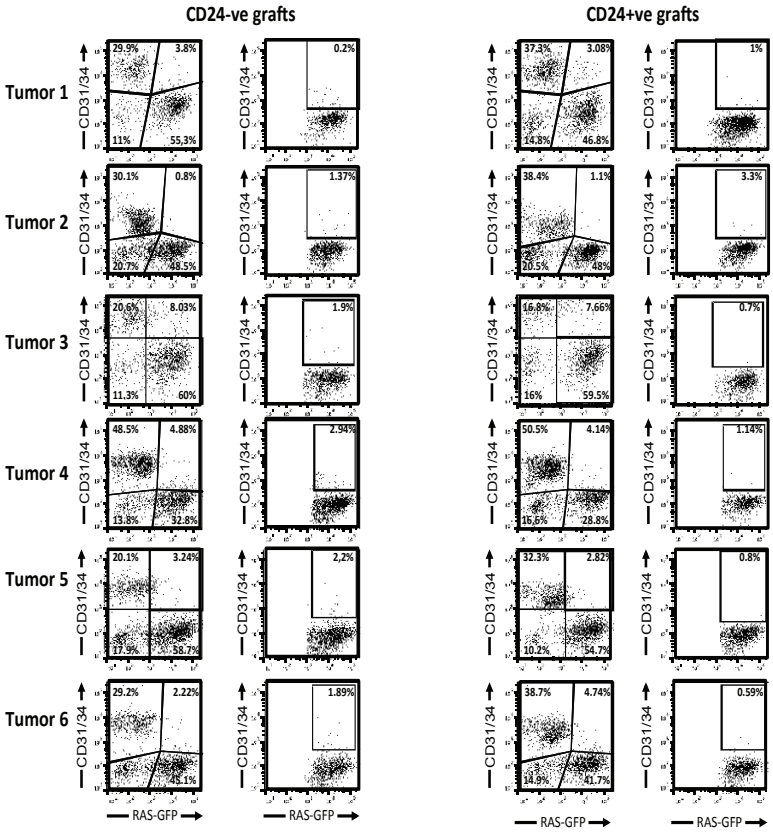

Figure S3

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Figure S3:
Primary tumors formed by grafting either CD24-ve or CD24+ve C2-KRASG12V cells lack CD24 expression. CD24-sorted C2-KRASG12V were injected orthotopically in contralateral hindlimbs of NOD-SCID muscle. Tumors were resected before reaching 2 cm3 and enzymatically digested. CD24 expression in the CD31-ve/CD45-ve/GFP+ve population was determined by flow cytometry. Depicted are dotplots showing CD31/45 vs RAS-GFP staining in the viable cell population (FSC/SSC population) and CD24 expression in the CD31-ve/CD45-ve/GFP+ve population.

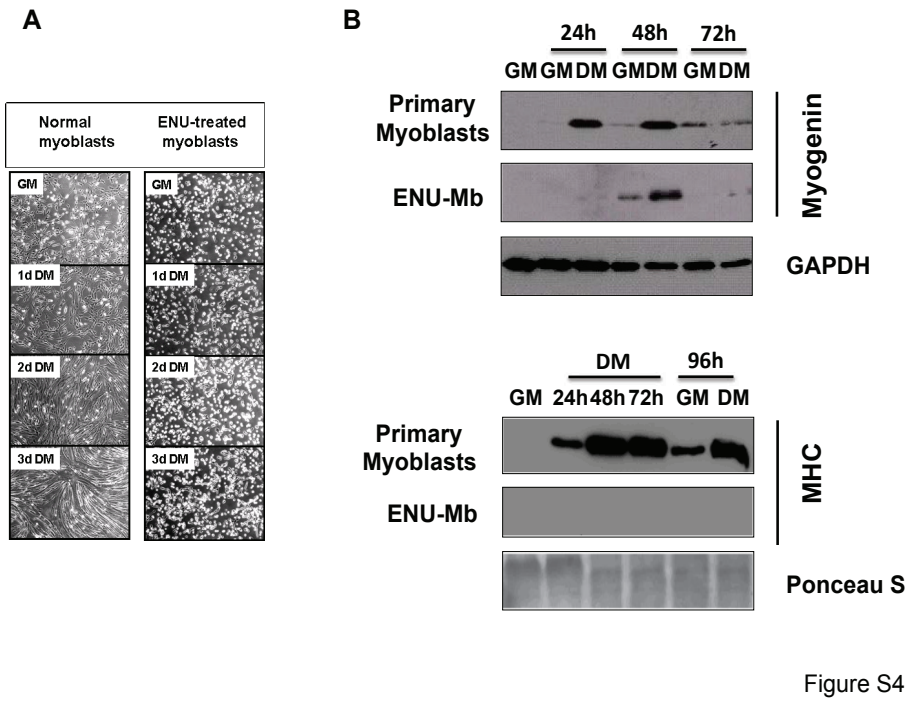

Figure S4

Figure S4:
(A) ENU-treated myoblasts (ET-Mb) do not differentiate. Normal primary myoblasts (C57/Bl6) differentiated and formed multinucleated myotubes after 4 days in differentiation medium (DM). ET-Mb failed to fuse within this period or even after maintenance for one week in DM.(B) Western blot analysis of myogenin (upper panel) and MyHC (lower panel) expression in primary myoblasts and ET-Mb growing in proliferation (GM) or differentiation medium (DM) for indicated time periods. Based on these data we concluded that ENU-treated failed to terminally differentiate indicated by the lack of myosin heavy chain (MyHC) expression. ET-MBs expressed the myogenic commitment marker myogenin (also determined by immunostaining-not shown). ET-MBs efficiently formed colonies in soft agar and muscle-invading tumors upon transplantation (Fig. S1 and data not shown). Histopathologically, the tumors strongly expressed desmin (Fig. S1), resembling non-alveolar RMS. N-, H- and K-RAS were not mutated in ET-MBs, although the ERK pathway was strongly activated (Fig. S2D).

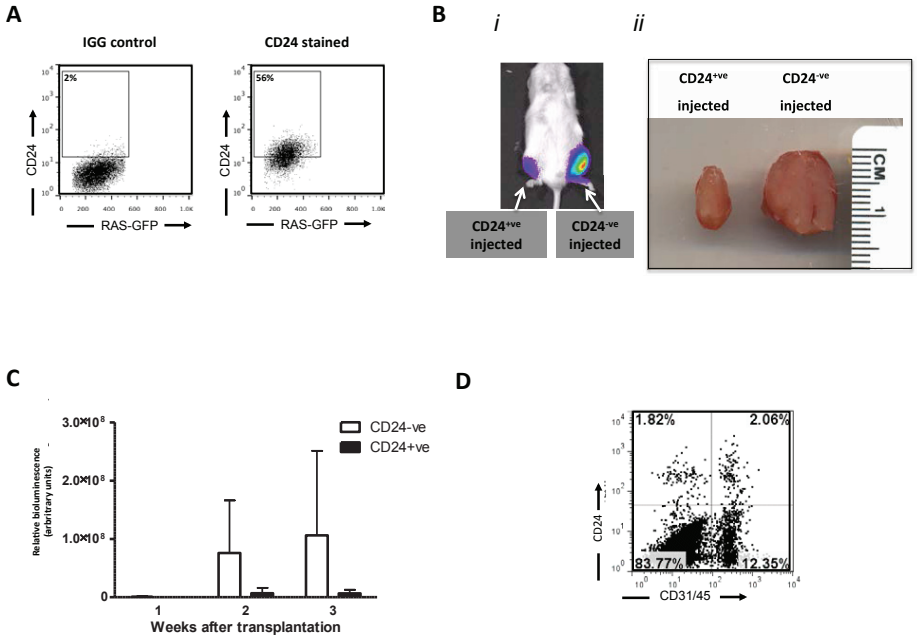

Figure S5

Figure S5:
(A) Heterogeneous CD24 expression in ET-Mb (FACS plot). The left panel shows the IgG staining control.(B) CD24-ve ET-MBs form tumors more efficiently upon grafting. ET-Mbs were sorted on CD24 and injected orthotopically into the TA muscle of NOD-SCID mice. Tumor formation was followed by bioluminescence imaging (BLI; left panel). The tumors were resected before they reached the maximal allowed size of 2 cm3 and photographed (right panel).(C) ET-Mb can be serially passaged to secondary hosts. ET-MBs were injected orthotopically into TA muscle of primary hosts. Tumors were digested and the lin-neg tumor population was sorted on CD24. CD24-sorted tumor cells were engrafted into TA muscle of secondary NOD-SCID hosts. The graphs show the BLI measurements after injecting 10,000 cells of both populations were injected (n=5 animals). (D) Primary ET-MB-dependent tumors express CD24 at low levels. Tumors were resected, digested and cell suspensions stained for lineage markers and CD24. The figure depicts a representative FACS plot of a resected tumor generated after orthotopic grafting of ET-MBs. This indicates that CD24-expression is not compatible with tumor growth in vivo.

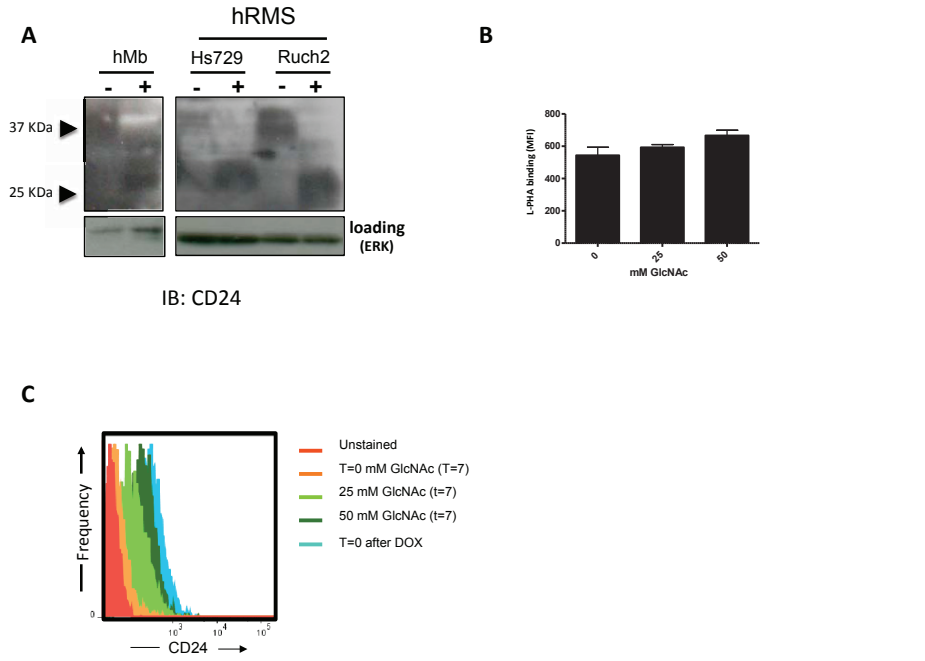

Figure S6

Figure S6:
(A) Endogenous CD24 is heavily glycosylated. The Western blot analysis show untreated lysates or lysates treated with PNGase F from human myoblasts (hMB) and two human RMS cell lines with wild type RAS (Hs729 and Ruch2). The downward shift in Mb after PNGAse treatment demonstrates the extent of N-glycosylation.(B) GlcNAC treatment increases binding of the lecting LPHA. C2-KRASG12V cells were treated with increasing concentrations of GlcNAc for 72 h and stained with fluorescent LPHA. LPHA binding was determined by flow cytometry. The figure shows the average + s.e.m. of four experiments.(C) Glycosylated CD24 is retained at the cell surface. RDihCD24 were pretreated with GlcNAc for 48h. Untreated and GlcNAc treated cells were incubated with DOX for 24h after which the cells were rinsed extensively and passaged to remove DOX. Cells were then maintained in the presence or absence of GlcNAc and the expression of (ectopic) CD24 was determined at the indicated timepoints. The upper panel shows a FACS analysis of CD24 cell surface expression. The Figure shows representative results of two independent experiments. The lower panel shows Western blot analysis of a parallel experiment.

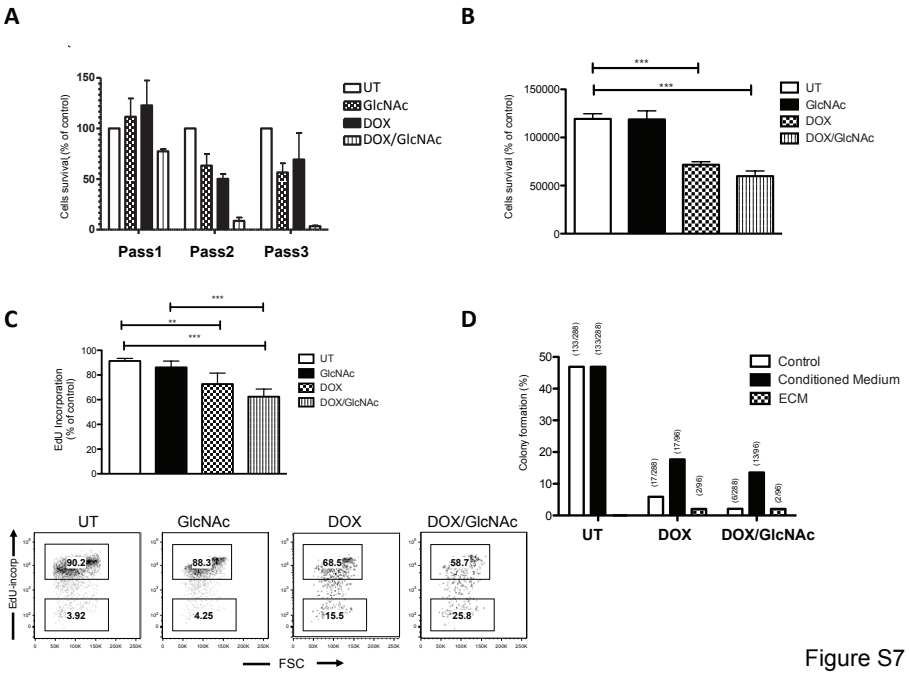

Figure S7

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Figure S7:
(A) Expression of glycosylated CD24 arrests proliferation. C2-KRASG12V cells were grown with/without DOX (to induce CD24) and with/without GlcNAc (25 mM; to increase N-glycosylation). At each passage viable cells were determined by MTT staining. Relative survival of cells for each treatment for each passage is indicated as percentage of untreated (-DOX) cells. An identical experiment with similar results using RDihCD24 cells is described in the main text (Figure 5A). (B) The expression of CD24 reduces plating efficiency. RDihCD24 cells were treated with DOX and/or GlcNAc for 72h and then plated at 10,000 cells/cm2. After 24h cells were trypsinized and counted. The figure depicts an averaged count from two independent experiments.(C) The expression of CD24 reduces proliferation. RDihCD24 cells were treated with DOX and/or GlcNAc for 72h and then incubated with EdU for 24h. Cells were then trypsinized and EdU incorporation was determined by flowcytometry. The upper panel depicts the averaged results of two independent experiments. The lower panel shows representative FACS plots showing the difference in EdU incorporation in CD24-expressing cells. (D) Reduce clonogenic potential is partially rescued by conditionned medium from untreated cells. Soft agar colonies formation assay was realized with pretreated cells using condition medium from logarithmically growing untreated cells.

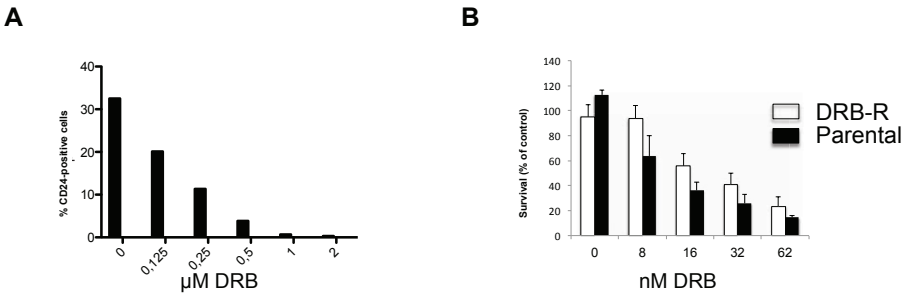

Figure S8:
(A) CD24-ve display increased survival after exposure to chemotherapeutic drugs. Human RMS cell with wild type RAS (Hs729 cells) were exposed to increasing concentrations of doxorubicin (DRB) for 24h and the CD24 expression of surviving cells was determined by flow cytometry. The graph plots % CD24-positive cells in the surviving cultures. The experiment was repeated with similar results. (B) DRB-tolerant cells reacquire DRB sensitivity upon passaging. RDihCD24 cells were treated with DRB (31 nM) for 24h. Cultures were maintained until DRB-tolerant colonies formed. The DRB-tolerant cells were passaged and expanded. At passage 3 after DRB-exposure cells were plated and exposed again to increasing concentrations of DRB. Parental RDihCD24 cells (not DRB-pretreated) were incubated as reference.

Chapter 5

Discussion

Discussion

The work presented in this thesis demonstrated that long-term cultures of normal and malignant muscle maintain a population of cells sharing properties with stem cells.

In chapter 2 and 3 we have identified a muscle stem cell population based on either (lack of) expression of the cell surface protein CD24 or on differential adhesion properties. In the latter approach, we used a preplating technique and identified a novel population of cells resembling quiescent satellite cells in C2C12 cultures. In both approaches, the isolated population was enriched in Pax7-positive cells, displayed self-renewal potential and harbored enhanced in vitro differentiation potential indicating the phenotypic and functional similarity with muscle satellite cells.
C2C12 represents the most commonly used cell line to study myogenesis in vitro. C2C12 represent an immortalized cell line that was generated by serial passaging of primary muscle cultures (Yaffe, 1977). By definition, C2C12 is an archetypical example of a long-term culture and represents a good model to study the maintenance of stem cell-like cells during extensive passaging. Obviously, C2C12 are different in certain aspects from satellite cells in vivo or even from primary cultures established directly from muscle 'biopsies'. However, for clinical purposes the ability of 'cultured' stem cells to both differentiate and contribute to the formation of novel myofibers as well as to contribute to the stem cell pool is of major importance. We have shown that CD24-N cells, identified in chapter 2, retain these essential features even after deriving these cells from the C2C12 cell line donor. For C2C12-derived qRC we have not yet established their regenerative potential in vivo. To determine the putative clinical potential of our approach we have in parallel initiated studies to detect regenerative muscle in advanced passage primary muscle cultures (passage 45 and over). Our current work in the lab seems to show that transplanted GFP-labeled reserve cells (RC) derived from primary mouse cultures contributed extensively to the regeneration of injured muscle of NOD-SCID hosts. In contrast, transplantation of 'bulk' muscle cells from the original parental culture did not result in detectable contribution to regeneration. The poor contribution potential is in accord with the literature (Sacco et al. 2008). The self-renewal potential of reserve cells derived from long-term passaged cultures has not been tested yet, but will be evaluated by serial transplantation as has been shown for CD24-N cells described in Chapter 2. So in contrast to the dogma of the rapid loss of the regenerative potential of satellite cells and their progeny (<48h) ex vivo, our preliminary findings show that the regenerative potential is maintained during extensive passaging. Such regenerative cells can be isolated based on phenotypic (lack of CD24 expression) or functional (poor adherence/reversible quiescence) properties.

The next step will be to determine the regenerative potential of cultured muscle stem cells, e.g. by transplanting the cells into diseased muscle of an animal

suffering from a muscle-degenerative disease, such as the alpha-glucosidase (GAA) knockout mice (animal model for Pompe's disease). This strain of mice is available at our Department and transplantation experiments with wild-type donor cells to reverse the toxic glycogen accumulation in skeletal muscle has been scheduled. The transplanted cells will face an extra barrier as these GAA knockout mice are fully immunocompetent and may mount an immune response against the transplanted cells. Immune rejection is thought to be one of the main reasons for the failure observed in the myoblast transfer therapy trials discussed in the introduction. Our experiments in immunodeficient host demonstrated the intrinsic potential of muscle reserve cells to contribute to muscle regeneration, the potential failure of reserve cells to engraft immunocompetent hosts may warrant the need for immunomodulatory regimens accompanying cell-transplantation,.

The data presented in the chapter 3 suggest that the regenerative potential can be boosted/reinduced by specific, yet unidentified stimuli provided in differentiating cultures, presumably through communication with the multinucleated myofibers (note that cells destined to become reserve cells comprise about 40% of cells in bulk cultures). Identifying these 'inducing' factors will allow the development of a protocol for the efficient and controlled generation of clinical-grade regenerative cells. We will therefore focus our future studies in elucidating the minimal requirements to define stem cell potential in cultured muscle cells.

In parallel, gene expression profiling studies should be done on this population. This analysis might allow, at first, to identify new marker for isolation of satellite cells in vitro and vivo. Secondly, the analysis will also allowed to determine which signaling pathway are important in the maintenance of those population. This will represent a step further in the development of better culture conditions to expand satellite cells in vitro as suggested by the recent study of Parker et al. 2012. In this study, Notch ligand is used to expand freshly isolated satellite cells and demonstration of the maintenance of the engrafment potential after transplantation is done (Parker et al. 2012). In human culture the important role played by the angiopoietin 1/Tie 2 signaling pathway in the control of the proliferation and differentiation of RC has been shown (Abou-Khalil et al. 2009). Those 2 examples nicely illustrated the kind of approach, which might by develop (summarized in Figure 1).

Nevertheless as mentioned during the introduction muscle stem cells are not the only populations that can contribute to muscle regeneration. A number of studies have investigated and demonstrated the muscle-regenerative potential of pericytes, mesangioblasts and PIC (PW1+ve) cells (Dellavalle et al. 2007,De Angelis et al. 1990, Mitchell et al. 2010). A nice study (Delavalle 2007) showed that intraarterial injection of pericytes resulted in some level of engraftment in injured muscle, while intraarterial delivery of freshly isolated SCs was without effect. The same authors showed that SCs after intramuscular injections were far superior. This indicates that the mode of delivery dictates the cell of choice for muscle-regenerative purposes. It should be noted that regenerative pericytes also have limited ex vivo expansion potential, so that

improved culturing methods are needed for further development of pericyte-based cell therapies. Interestingly, in a recent study committed myoblast were transformed into pericytes while maintaining their myogenic memory (Cappellari et al. 2013). This may suggest that the reserve cell culturing approach described in chapter 3 may provide the basis also for cells with pericyte properties.
Furthermore, dystrophic muscle is characterized by intense fibrosis and progressive loss of muscle mass. Several studies have shown that this was the result of a progressive exhaustion of the SC pool due to the chronic recruitment of satellite cells to repair damage (Sacco et al. 2010). Conditional elimination of the SC pool in transgenic animals has demonstrated that the satellite cells are required and responsible of the maintenance of the homeostasis of the muscle tissue (Lepper et al. 2011, Sambasivan et al. 2011). This also suggests that in this environment alternative populations with myogenic potential are not able to compensate the loss of the SC population even though they may be present in the affected tissue. Based on the potential of endogenous SCs to replete both the stem cell pool and restore the myogenicity of damaged muscle, cells with SC-potential represent the candidate of choice when trying to treat patients with advanced stages of muscle degeneration. In this respect, being able to isolate cells for long term culture sharing common features represent a key step forward in the development of cell based therapy.

One of the main drawbacks for the use of SC as a source of cells for cell based therapy is their limited migratory potential and poor survival after transplantation (Beauchamp et al. 1999, Mouly et al 2005). However, our recent data seems to suggest that a substantial contribution can be obtained with a high host/donor chimerism throughout the full muscle after local delivery. Combined with their superior myogenic potential SC or SC-like cells (CD24-N or reserve cells) might represent good candidates for targeted therapy of selected, severely-affected muscles, such as the rhabdosphincter in Stress Urinary Incontinence or the diaphragm muscle in Duchenne Muscular dystrophy. Muscle stem cell-based therapy would in such cases improve the quality of life by relieving major symptoms (in SUI) or extend survival by restoring the myogenicity of a critical muscle. SUI is a common disorder affecting a significant number of women after menopause and presents as a very distressing problem. So far the current therapy based on collagen injection or invasive surgery have shown variable efficacy. A study based on autologous transplantation of myoblast (Strasser et al. 2007) made a first step towards the use of cell based therapy, but the results were modest and it remains unclear if the observed improvement resulted from contribution of the transplanted cells to muscle repair or by providing structural support to the tissue.
The identification of candidate cell populations represents only the first step in the development of a cell based therapy (Figure 1) and several other difficulties will need to be addressed (efficient/safe gene correction, mode of delivery and long-term efficiency). However, the ability to generate sufficient numbers of clinical-grade cells under laboratory-controlled conditions is vital for an eventual introduction of this

new technology into the clinical practice.

In the work presented in chapter 4, using the same single marker, CD24, we discovered that TICS from both mouse and human RMS resided in the CD24-negative compartment, as was the case with stem cells from long-term cultures of muscle cells. Much debate exists whether tumors harboring TICs follow the hierarchical tumor stem cell model or evolve by clonal evolution. However, irrespective of their origin it is becoming clear that the identification and elimination of this highly tumorigenic population is clinically relevant. TICs are associated with increased therapy resistance to conventional therapeutic agents and propensity to relapse the tumor making this highly malignant tumor population a prime target for the development of innovative cancer treatment(Margolin et al. 2011). The potential value and feasiblity of specifically targeting the TIC population has recently been shown in vivo in transgenic mice harboring endogenous glioblastomas. The mice were treated with a combination of a drug that activate the quiescent tumor-initiating cells into proliferation and a drug eliminating rapidly proliferating tumor cells (Chen, Nature 2012). This therapeutic approach completely impeded tumor development and prevented tumor re-growth that was observed after treatment with a single drug targeting the proliferating bulk tumor cells.
In this thesis we demonstrated that the CD24-ve RMS TIC population possessed efficient tumor initiating potential, the ability to self-renew, enhanced clonogenicity, and increased drug resistance. Our data shows that cells with these properties are maintained during ex vivo expansion.
Studies from the Morisson' lab have indicated that in (primary) melanoma the use of a great number of different cell surface markers was unsuccessful in identifying cells with TIC properties (Quintana et al. 2008, 2010). There are many other examples for other tumors showing that the use of cell surface markers did not result in discriminating between TIC and non-TIC populations. In Chapter 4, we demonstrated that CD24-re-expression reduced the TIC properties of clonogenic potential and drug-resistance suggesting that CD24 played a functional role in determining the TIC identity. The functional role in RMS tumorigenesis explains the success of CD24 as marker in discriminating between TIC and non-TIC in our studies. We have successfully applied our CD24-based approach in a range of different human RMS cell lines (which were originally derived from primary human tumors) as well as in two independent experimental mouse RMS-like tumors. CD24 may have a different function in other cells types, which would annihilate its use as marker of TIC cells and explains contradictory findings. For instance in liver cancer, TIC cells have been found in the CD24positive population (Lee et al, 2011).
Alternatively to using phenotypic traits, TIC cells may also be identified based on functional properties.. One of the main properties of normal stem cells population, which has been exploited to identify them in different organs is their slow cycling rate or their ability to return to quiescence in order to replenish the pool. These characteristics have lead the researcher to develop label retaining (LRC) assays to identify

novel stem cell populations in tissue like hair follicle (Cotsarelis et al. 1990, Tumbar et al. 2004) or small intestine (Potten et al. 1992). Similar approaches have allowed to identify stem cell-like populations in cancer. One of the best examples found in the literature comes from a study in which pancreatic adenocarcinoma cells were labeled with Vibrant DiI and a LRC population identified displaying increased clonogenicity potential in vitro and enhanced tumorigenicity in vivo (Dembinski and Krauss 2010, Cicalese et al. 2009). Another example of illustrating the idea of identifying TIC-like cells was shown in a study from the Medema lab, which used a Wnt reporter strategy to identify a TIC-like population in colon cancer (Vermeulen et al. 2010). They showed that cells expressing high Wnt downstream transcription factor TCF/LEF displayed increased clonogenicity and giving rise to heterogeneous progeny of cycling and non-cycling cells.
The immediate value of our findings is that CD24 can be used to isolate TICs, even from (primary) cultured RMS, allows the study for TIC-specific targets by subsequent genome or proteome analyses. Moreover, the use of RMS cultures as sources ensures sufficient number of cells.

In overall, in this thesis, approaches using a single marker (CD24) and a preplating technique were used to enrich for stem-cell like subpopulation from malignant and healthy long-term muscle derived cultures. These approaches are offering the possibility to purify a considerable amount of cells from long-term cultures to study and better understand the control of stem-cell fate in healthy and tumorigenic context.

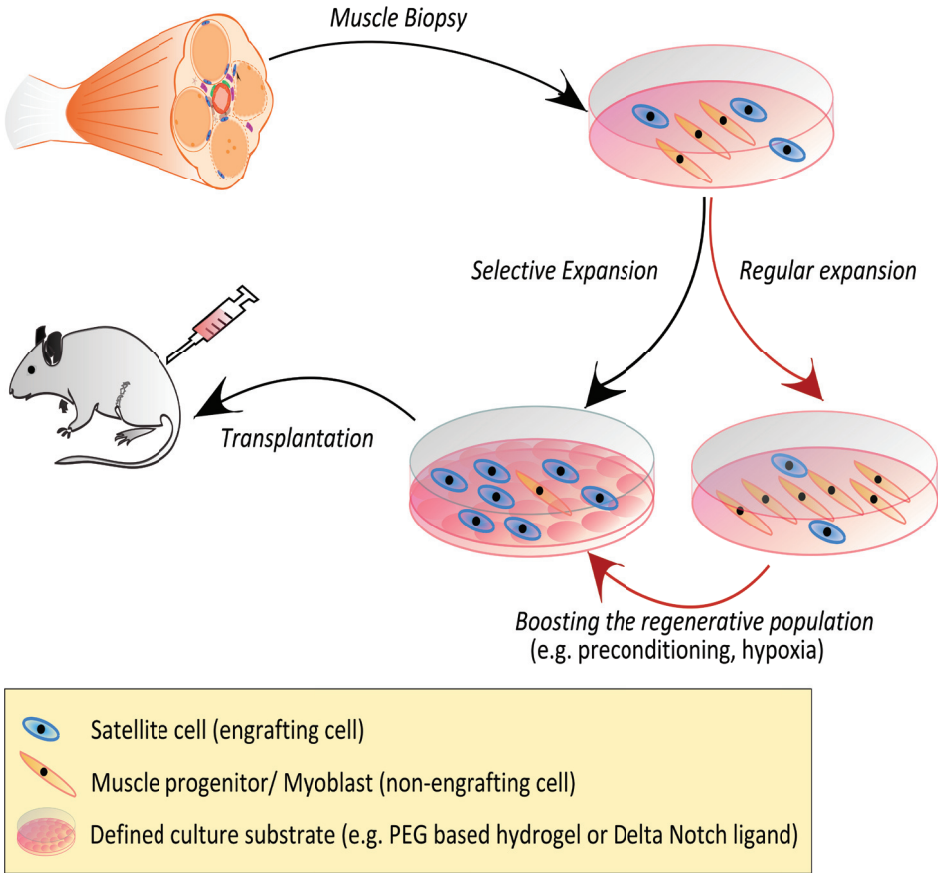

Figure 1: Selective expansion of muscle regenerative cells.
The figure depicts two different strategies to obtain cultures enriched with muscle regenerative cells: a 'two-step' approach and the selective expansion of highly regenerative cells. These procedures start with the establishment of a muscle culture from a small biopsy. The culture is heterogeneous and contains a subpopulation of cells capable of engraftment. Two-step approach: The cultures may be cultured under 'regular' conditions in order to expand the cells rapidly and extensively. Subsequently, strategies, such as preconditioning or exposing the cultures to hypoxic conditions can be applied to boost the regenerative potential of the expanded cells prior to transplantation (referred to as a two-step approach in the text; red arrows). The efficiency of this strategy may be low, because of the extensive loss of regenerative potential in the first step and the modest restoration (~2-fold) of the regenerative potential in the second step of the protocol. Selective expansion: Alternatively, the mechanisms of stem cell self-renewal in vivo may are applied to cultured cells (e.g. use of PEG-based hydrogel-based culture substrates or use of immobilized Notch ligand; see text). We hypothesize that such well-defined culture conditions promote the selective (self-renewing) expansion and, consequently, enrichment of engraftment-competent cells. It is expected that limited numbers of cells from such enriched cultures are required for efficient engraftment and regeneration.

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Appendix

Summary

With more than 600 individual units (i.e. myofibers) and more than 40 percent of total body mass, skeletal muscle represents the largest tissue of the human body. Skeletal muscle contracts in response to nerve impulses in order to play critical roles in physiological phenomena like respiration, movement, glucose homeostasis and thermoregulation.

A considerable number of neuromuscular disorders affect skeletal muscle resulting in the disruption of the structural organization of the tissue, the induction of muscle-fiber necrosis, and the infiltration of inflammatory, adipogenic and fibrotic cells. Muscle-degenerative diseases, or myopathies, are characterized by progressive muscle wasting. Their clinical and pathophysiological spectrum is broad. To date, there is no therapy able to reverse the skeletal muscle pathology observed. The use of a stem cell based therapy has been pursued as an interesting novel approach for myopathies. Muscle regenerative cells are thought to be able to contribute robustly to the regeneration of the damaged fibre and also replenish the stem cell pool. Thus, they are considered as promising in the development of a cell-based therapy.

Among muscle regenerative cells, satellite cells (SC) represent the predominant muscle resident stem cell population, which have a role in maintaining tissue homeostasis and are the sole mediators of muscle repair after severe trauma or during disease. In addition, SCs have the ability to engraft and contribute to the regeneration of injured muscle upon transplantation. Based on these properties, they represent an ideal candidate for a cell-based therapy. However, SCs represent only 2 to 4 percent of the myonuclei in human adult muscle, so that only a limited amount of SCs can be obtained from a biopsy. An in vitro expansion step is therefore required to acquire clinically relevant numbers. Furthermore, SC populations are phenotypically and functionally heterogeneous and consist of both quiescent and actively dividing cells. Upon in vitro expansion of SCs, the majority of the SCs loose their regeneration capacity through differentiation into more committed states (activated SCs or myoblasts). This limits the use of SCs in the treatment of degenerative muscle diseases and necessitates strategies allowing enrichment of a highly regenerative subpopulation.

Here, we show that CD24 is heterogeneously expressed in SC-derived and C2C12 myoblast cultures. Using a clonal analysis strategy we have been able to identify a rare population of cells that, in contrast to the main population remain completely CD24-negative upon passaging. The functional characterization of these persistently CD24-negative (CD24-ve) cells has shown that they expressed the satellite cell marker Pax7 at high levels and differentiated efficiently in vitro. Upon transplantation into injured muscle of immunodeficient mice, CD24-ve cells contributed robustly to the formation of new muscle fibers. Upon secondary transplantation, these CD24-

ve cells participated robustly in muscle regeneration, suggesting that CD24-ve cells underwent self-renewal in vivo. The population of muscle cells that maintained high level expression of CD24 completely lost their myogenic potential in vitro and in vivo, suggesting a functional role of CD24 in myogenic differentiation.

In parallel to the use of CD24 to purify a population with stem cell characteristics, we developed a strategy based on a preplating approach to enrich for SCs. By this way, we showed that cells with properties of quiescent satellite cells are preserved and can be enriched from the C2C12 muscle cell line. We established that thise so called reserve cell population obtained from the non-differentiating fraction of differentiating cultures is heterogeneous. It consists of a previously reported reserve cell population (aRC) attaching rapidly after replating and a slowly adherent reserve cells fraction (qRC). Further analyses have shown that the qRC were enriched in Pax7-positive cells. Cell cycle characterization indicated that the qRC were in G0 phase of the cell cycle, in contrast to the aRC which were arrested in G1. This qRC demonstrate a return to quiescence, which is associated with a greater stem cell capability compared to the population of aRC that were unable to exit the cell cycle. The regenerative potential is yet to be determined).
The two approaches presented in this thesis have demonstrated that cells sharing common features with SC are maintained in long-term culture, demonstrates that it is possible to expand muscle-regenerative cells ex vivo, which is and had been the first and foremost obstacle in the development of cell-based therapies for muscle degenerative diseases.

Muscle damage can also be observed in systemic conditions such as ageing, endocrinological disorders or cancer. Rhabdomyosarcomas (RMS) are pediatric soft tissue tumors characterized by myogenic differentiation. RMS has an annual incidence of seven per million in children aged 14 years or younger and remains fatal to one third of the patients. There are indications that RMS, like a number of other malignancies, is composed of various cellular subsets with different tumorigenic potential. In this regard, cells with increased tumorigenic potential, often referred to as tumor-initiating cells (TIC), are thought to present a major obstacle for therapy. TICs are associated with increased therapy resistance and propensity to relapse the tumor. Much debate exists whether tumors harboring TICs follow the hierarchical tumor stem cell model or evolve by clonal evolution. However, irrespective of these models it is becoming clear that the identification and elimination of this highly tumorigenic population is clinically relevant.
Populations of cells with increased tumorigenic potential are defined by the expression of a specific cell surface molecule signature that allow purification of these TICs for further study. Here we discovered that TICs from both mouse and human RMS can be isolated using the same single marker CD24, that was used to purify normal muscle stem cells.

These CD24-ve RMS TICs displayed properties of self-renewal and enhanced clonogenicity, as well as efficient tumor initiation and increased drug resistance as compared to the CD24+ve fraction. Furthermore, in this study we established that CD24 was a functional marker as its re-expression reduced the clonogenic potential and drug resistance of RMS cells. The tumor suppressive function of CD24 explains the success of using CD24 as a (negative) marker for selecting RMS TICs. The function of CD24 was dependent on the extent of glycosylation, illustrated by the loss of clonogenic potential following treatment with N-acetylglucosamine (GlcNAc). GlcNAc-treatment increased CD24-glycosylation and extended CD24 cell surface residency and stability, suggesting a role for CD24 in cell adhesion and/or signaling. CD24 expression was specifically downregulated in tumors harboring oncogenic RAS mutations. Forced expression of CD24 reduced the TIC frequency in oncogenic RAS-positive RMS, indicating that loss of CD24 expression contributed to the tumorigenic activity of mutant RAS. Altogether, our study highlights the existence of tumor heterogeneity as well as TICs in RMS. More importantly, the use CD24 as a marker will facilitate the isolation of TICs, which represent a target of choice for the development of novel therapies.

In brief, in this thesis, the single marker CD24 and a preplating approach was used to enrich for stem-cell like subpopulation from malignant and healthy long-term muscle derived cultures. These approaches offer the possibility to purify a considerable amount of cells from long-term cultures to study and increase the understanding of the control of stem-cell fate in healthy and tumorigenic context. This might offer the opportunity of developing novel targeted therapies for these devastating conditions.

Samenvatting

Met meer dan 600 individuele eenheden (i.e. myofibrillen) en meer dan 40 procent van het totale lichaamsmassa, zijn skeletspieren het grootste weefsel in het menselijk lichaam. Skeletspieren trekken samen in reactie op zenuwimpulsen om een cruciale rol te spelen in fysiologische fenomenen zoals ademhaling, beweging, glucose homeostase en warmtehuishouding.

Een aanzienlijk aantal neuromusculaire aandoeningen hebben invloed op skeletspieren dat leidt tot de verstoring van de structurele organisatie van het weefsel, de inductie van spiervezel necrose en het binnendringen van inflammatoire, adipogene en fibrotische cellen. Spierdegeneratieve ziekten, of myopathieën, worden gekenmerkt door progressieve spieratrofie. Hun klinische en pathofysiologische spectrum is breed. Tot nu toe is er geen therapie in staat de waargenomen skeletspier pathologie om te keren. De toepassing van stamceltherapie wordt nagestreefd als een interessante nieuwe benadering voor myopathieën. Spier regeneratieve cellen wordt gedacht in staat te zijn om fors bij te dragen aan de regeneratie van de beschadigde vezels en ook om de stamcel populatie aan te vullen. Daardoor worden zij als veelbelovend beschouwd voor de ontwikkeling van een celgebaseerde therapie. Onder spier regeneratieve cellen vertegenwoordigen satellietcellen (SC) de belangrijkste stamcelpopulatie in de spieren, die een rol spelen bij het behoud van weefsel homeostase en ze zijn de enige mediator van spierherstel na ernstig trauma of tijdens ziekte. Bovendien hebben SC het vermogen om bij transplantatie te integreren en bij te dragen aan de regeneratie van beschadigde spieren. Op basis van deze eigenschappen, zijn ze een ideale kandidaat voor cel gebaseerde therapie. SC vertegenwoordigen echter slechts 2 tot 4 procent van de myonuclei in menselijke volwassen spieren, zodat slechts een beperkte hoeveelheid SC kan worden verkregen uit een biopsie. Een in vitro groei stap is daarom noodzakelijk om klinisch relevante aantallen te verkrijgen. Verder zijn SC populaties fenotypisch en functioneel heterogeen en bestaan uit zowel latente en actief delende cellen. Bij in vitro groei van SC verliest de meerderheid hun regenererend vermogen door differentiatie in meer gespecialiseerde stadia (geactiveerd SC of myoblasten). Dit beperkt het gebruik van SC bij de behandeling van degenererende spierziekten en vergt een aanpak voor verrijking van een sterk regenererende subpopulatie.

Hier tonen we aan dat CD24 heterogeen tot expressie komt in SC afkomstige en C2C12 myoblast kweken. Met een aanpak gebaseerd op analyse van kweekjes afkomstig van één cel (clonal analysis strategy) kunnen we een zeldzame populatie van cellen identificeren die, in tegenstelling tot de hoofd populatie, volledig CD24-negatief blijven bij passage. De functionele karakterisering van deze aanhoudend CD24-negatieve (CD24-ve) cellen laat zien dat ze de satelliet cel marker PAX7 in een hoge dosis tot expressie brengen en efficiënt kunnen differentiëren in vitro. CD24-ve cellen dragen fors bij tot de vorming van nieuwe spiervezels na transplantatie in beschadigde

spieren van immunodeficiënte muizen. Bij secundaire transplantatie hebben deze CD24-ve cellen sterk deelgenomen aan spierregeneratie, wat erop wijst dat CD24-ve cellen zelfvernieuwing ondergingen in vivo. De populatie van spiercellen die hoge expressie van CD24 behouden, hebben hun myogene potentieel in vitro en in vivo volledig verloren, wat suggereert op een functionele rol van CD24 in myogene differentiatie.

Parallel aan het gebruik van CD24 om een populatie te zuiveren op stamcel eigenschappen hebben wij een strategie ontwikkeld om SC te verrijken gebaseerd op de snelheid om aan de kweekschaal te hechten (preplating methode). Op deze manier hebben we aangetoond dat cellen met eigenschappen van latente satelliet cellen behouden worden en verrijkt kunnen worden vanuit C2C12 spier cellijn. Wij hebben vastgesteld dat deze zogenaamde reserve celpopulatie verkregen uit de niet-differentiërende fractie van differentiërende kweken heterogeen is. Deze bestaat uit een eerder beschreven reserve celpopulatie (aRC) die snel hecht na opnieuw platen en een traag hechtende reserve cel fractie (qRC). Verdere analyses hebben aangetoond dat de qRC werden verrijkt in PAX7-positieve cellen. Celcyclus karakterisering gaf aan dat de qRC in de G0-fase van de celcyclus waren, in tegenstelling tot de aRC die in de G1-fase stilstonden. Deze qRC toont een terugkeer naar de latente fase, die wordt geassocieerd met een groter stamcel vermogen vergeleken met de populatie van aRC die de celcyclus niet konden verlaten. Het regeneratieve potentieel moet nog worden vastgesteld.
De twee benaderingen beschreven in dit proefschrift hebben aangetoond dat cellen die gemeenschappelijke kenmerken vertonen met SC worden behouden gedurende lange termijn kweek. Dit toont aan dat het mogelijk is spierregeneratieve cellen ex vivo op te kweken, wat het eerste en belangrijkste obstakel is en was voor de ontwikkeling van celgebaseerde therapieën in spier degeneratieve ziekten.

Spierbeschadiging kan ook waargenomen worden in systematische aandoeningen zoals veroudering, endocrinologische stoornissen of kanker. Rhabdomyosarcomen (RMS) zijn tumoren bij kinderen in zachte weefsels gekenmerkt door myogene differentiatie. RMS heeft een jaarlijkse incidentie van zeven per miljoen bij kinderen van 14 jaar of jonger en blijft fataal voor een derde van de patiënten. Er zijn aanwijzingen dat RMS, net als een aantal andere maligniteiten, bestaat uit verscheidene cellulaire subsets met verschillend tumorigeen potentieel. In dit opzicht wordt gedacht dat cellen met verhoogde tumorgeniciteit, vaak aangeduid als tumor-initiërende cellen (TIC), een groot obstakel voor therapie vormen. TIC worden geassocieerd met verhoogde weerstand tegen de therapie en neiging tot terugval van de tumor. Er is veel discussie gaande of tumoren die TIC bevatten, het hiërarchische tumor stamcel model volgen of evolueren door klonale evolutie. Echter, los van deze modellen wordt duidelijk dat de identificatie en eliminatie van deze uiterst tumorgene populatie klinisch relevant is. Populaties van cellen met een verhoogd tumorigeen potentieel worden gedefinieerd door de expressie van een specifiek celoppervlak molecuul

profiel dat zuivering van deze TIC mogelijk maakt voor verdere studie. Hier ontdekten we dat TIC van zowel muis en mens RMS kunnen worden geïsoleerd met een en dezelfde merker CD24, die werd gebruikt om normale spierstamcellen te zuiveren.

Deze CD24-ve RMS TIC vertoonden eigenschappen van zelfvernieuwing en verbeterde, alsmede efficiënte tumor initiatie en verhoogde resistentie tegen geneesmiddelen ten opzichte van de CD24+ve fractie. Verder hebben we in deze studie aangetoond dat CD24 een functionele marker was omdat de herexpressie de mogelijkheid om klonen te vormen verminderde en de resistentie tegen geneesmiddelen van RMS cellen verminderde. De tumor onderdrukkende functie van CD24 verklaart het succes van het gebruik van CD24 als een (negatieve) marker voor de selectie van RMS TIC. De functie van CD24 is afhankelijk van de mate van glycosylatie, dat blijkt uit het verlies van het klonogene potentieel na behandeling met N-acetyl glucosamine (GlcNAc). GlcNAc-behandeling verhoogde CD24-glycosylering en zorgde ervoor dat CD24 langer op het celoppervlak verbleef en de stabiliteit verbeterde, wat erop wijst dat CD24 een rol speelt in cel adhesie en/of communicatie. CD24 expressie was specifiek downgereguleerd in tumoren die oncogene RAS mutaties bevatten. Geforceerde expressie van CD24 verminderde de TIC frequentie in oncogene RAS-positieve RMS, wat aangeeft dat het verlies van CD24 expressie heeft bijgedragen tot de tumorogene activiteit van mutante RAS. Onze studie wijst op het bestaan van de tumor heterogeniteit evenals TIC in RMS. Belangrijker is dat het gebruik CD24 als een marker de isolatie van TIC bevorderd, die doelwit zijn voor de ontwikkeling van nieuwe therapieën.

Kortom, in dit proefschrift, was de enkele marker CD24 en de preplating methode gebruikt voor de verrijking van een stamcelachtige subpopulatie van maligne en gezonde lange termijn spier afgeleide kweken. Deze benaderingen bieden de mogelijkheid om een aanzienlijke hoeveelheid cellen te zuiveren van lange termijn kweken om ze beter te bestuderen en om beter inzicht te krijgen in de controle van de ontwikkeling van stamcellen in gezonde en tumorgene context. Dit zou de mogelijkheid bieden voor het ontwikkelen van nieuwe gerichte therapieën voor deze verschrikkelijke aandoeningen.

Abbreviations

ARMS: Alveolar RMS

BMDC: Bone Marrow Derived Cell

BMP: Bone Morphogenic Protein

COPD: Chronic Obstructive Pulmonary Disease

CSC: Cancer Stem Cell

DM: Differentiation Medium

DMD: Duchenne Muscular Dystrophy
DMEM: Dulbecco's modified Eagle's medium

DNA: Desoxyribonucleoside Acid
DOX: Doxycycline
DRB: Doxorubicin
ECM: Extra Cellular Matrix
EdU: 5-ethynyl-2'-deoxyuridine

eMHC: Embryonic MyHC

ERMS: Embryonal RMS

ERT: Enzym Replacement Therapy

ES: Embryonic Stem Cell

FACS: Fluorescence Activated Cell Sorting
FCS: Fetal Calf Serum
FGF: Fibroblast Growth Factor

FKHR: Forkhead

FSHD: Facioscapulohumeral dystrophy

GAA: Fcid α-glucosidase

GFP: Green Fluorescent Protein
GlcNAc: N-acetylglucosamine
GM: Growth Medium

GMP: Good Manufacturing Practice

GRMD: Golden Retriever Muscular Dystrophy

HGF: Hepatocyte Growth Factor

HSC: Hematopoietic Stem Cell

igG: Immunoglobulin G

iPS: Induced Pluripotent Stem Cell
IRES: Internal Regulatory Enhancer Sequence
MAPK: Mitogen Activated Protein Kinase
Mb: Myoblast

MDSC: Muscle Derived Stem Cell

Mgn: Myogenin
Mir: MicroRNA
mRNA: messenger RNA

MRF: Myogenice Regulatory Factor

mTR: telomerase RNA component Terc

MyHC: Myosin Heavy Chain
NDC: Non Differentiating Cell

NO: Nitric oxide

NT: Neural Tube
PBS: Phosphate Buffer Saline
PCR: Polymerase Chain Reaction

PDGFa: Platelet Derived Growth Factor a

PEG: Polyethylene Glycol
PFA: Paraformaldehyde
PS: Penicillin Streptomycin

RB: Retinoblastoma protein

(a,q)RC: (activated, quiescent) Reserve Cell

RMS: Rhabdomyosarcoma
RNA: Ribonucleosidic Acid
RT-PCR: Retro Transcriptase PCR

SC: Satellite cell

SDF1: Stromal Derived Factor 1

SF: Scatter Factor

Shh: Sonic Hedgehog
ShRNA: Short Hairpin RNA

SUI: Stress Urinary Incontinency
TA: Tibialis Anterior

TGF-beta: Transforming Growth Factor -beta

TIC: Tumor Initiating Cell
TSA: Trichostatin A
UT: Untreated
WT: Wild-Type

Curriculum Vitae

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EDUCATION

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TRAINING

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PUBLICATIONS

Schaaf, G*, Sage F*, Stok M, Brusse E, Pijnappel Wwm, Reuser Aj, and Ploeg At. 2012. "Ex-vivo Expansion of Muscle-Regenerative Cells for the Treatment of Muscle Disorders."J Stem Cell Res Ther 2012, S11. *Equal contribution

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F. Sage, S. Bhattarai, A. Chan, F. Chen, S. Boutet, L. Boon, R. Van den Linden, T.A. Rando and G. Schaaf. « Cultured muscle stem cells characterized by persistent lack of CD24 expression contribute to muscle regeneration and self-renew in vivo ». Submitted

ORAL PRESENTATIONS

2011	Dutch Stem Cells Research meeting, Leiden, NL.
2011	MGC PhD Student Workshop, Maastricht, NL.

POSTER PRESENTATIONS

2012	International Society of Differentiation stem cell, development and regulation conference, Amsterdam, NL
2010	Stem cells in development and disease, Amsterdam, NL. MGC PhD Student Workshop, Cologn, DE. Erasmus Stem Cell institute inauguration meeting, Rotterdam, NL.

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Workshops	
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Conferences	
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