To the only person I can't hug, besides in my heart.

I love you, Daddy.

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General Introduction

Skeletal Muscle Development

The skeletal muscles of the body and limbs are derived from myogenic progenitors of the somitic compartment in a developing embryo [1] [2]. The somites are initially formed as epithelial spheres that bud off the anterior end of the paraxial mesoderm. Somitic budding occurs sequentially in an anterior to posterior direction on either side of the neural tube, thus providing the basis for a bilateral segmental body pattern for musculoskeletal development (Fig.1). Within hours of somite epithelialization, dorsoventral orientation becomes established by the formation of the epithelial dermomyotome (dorsal) and the mesenchymal sclerotome (ventral).

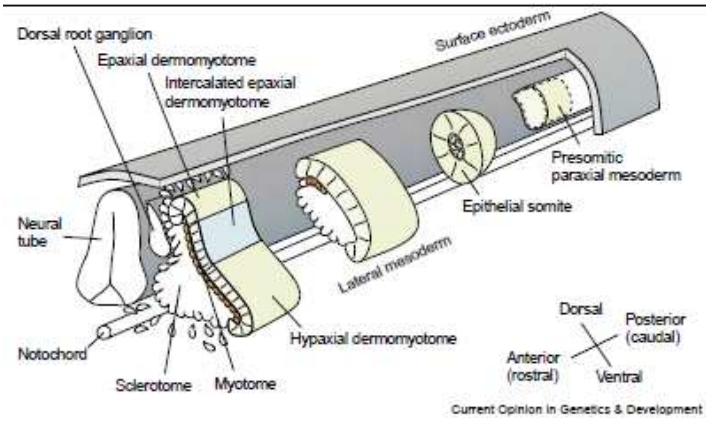

Figure 1 - Schematic representation of vertebrate somitogenesis as it occurs in the mouse embryo. Somites are formed and mature following a rostrocaudal gradient on either side of the axial structures.

The cells of the sclerotome give rise to the complete axial skeleton, while myogenic progenitor cells (MPC) of the somite originate from the dermomyotome. MPCs of the dermomyotome can be identified by the expression of markers such as *Pax3* [3-5], *Pax7* [6], *Dash2*, *Eya2*, and *Six1* [7]. The dermomyotome subdivides into three regions, defined by the dorsal medial lip (DML), the ventral lateral lip (VLL), and the intervening central dermomyotome region. Myocytes originating from the DML contribute to the formation of the deep back epaxial muscles, whereas the VLL provides the anterior hypaxial muscles and limb muscle progenitors. Developmental myogenesis is divided into the early embryonic and late fetal stages [8]: embryonic MPCs differentiate, forming the primary myofiber scaffolding; continual muscle growth into the later stages of development occurs through the addition of secondary myofibers from fetal myogenic progenitors. Satellite cells are the primary source of myogenic progenitors during the postnatal period [9].

Myogenic Signal Induction in the Developing Somite

The interplay of diffusible signals secreted by neighboring tissues induces the determination of the somitic myogenic compartment [10, 11]. In the early embryonic somite, the myogenic compartment is composed of the dorsally-positioned epithelial dermomyotome and the underlying mesenchymal myotome. This myogenic compartment is anatomically divided into the medial epaxial and the lateral hypaxial domains. Myogenic determination of these regional domains is largely influenced by the action of signaling molecules at these locales. Axial

structures such as the notochord and the neural tube [12-14] secrete signaling molecules such as Sonic Hedgehog (Shh) and Wnts, that act as positive effectors of epaxial MPC determination [15-20]. Hypaxial MPCs are subject to the effects of Wnt signaling from the dorsal ectoderm, in addition to negative bone morphogenetic protein (BMP) signaling from the nearby lateral mesoderm in limb-level somites [21-23].

The Shh signaling pathway is important in developmental patterning, proliferative control, cell survival, and growth in the embryo and the adult [24]. Shh is a secreted glycoprotein that binds to and activates a heterodimeric receptor complex of Patched (Ptc) and Smoothened (Smo) proteins. Shh functions as an inhibitor of Ptc, which in turn is an inhibitor of Smo. Shh signaling ensues when Smo is released from Ptc inhibition by Shh. Induction of Shh signaling mobilizes the Gli family of transcription factors into the nucleus, thereby activating target genes.

Wnt proteins are highly conserved secreted molecules that are involved in developmental signaling [11, 25, 26]. Wnt ligands initiate signaling by binding to their target receptors known as Frizzled (Fz). Downstream Wnt signaling cascades are currently grouped into two categories. First, the so-called canonical pathway, which involves the stabilization of cytoplasmic β-catenin and its subsequent nuclear localization and target gene activation via TCF/LEF interaction. Second, all other Wnt mediated signaling that is independent of β-catenin and TCF/LEF activation are grouped as non-canonical pathways.

BMPs are members of the TGF-β super-family of growth factors. BMPs are critical developmental factors involved in chondrogenesis and chondrocyte differentiation [27, 28]. BMP signaling is also known to inhibit adipocyte differentiation and myogenic differentiation [29, 30]. In the context of somitic myogenesis, BMPs provide important inhibitory signals that are coordinated with Shh and Wnt signaling [22].

Myogenic Transcription Factors

Skeletal muscle specific gene regulation in the embryonic somite requires a family of bHLH transcription factors known as the myogenic regulatory factors (MRFs) [2]. The members of the family include Myf5, Mrf4 (Myf6), MyoD, and myogenin.

Clear candidates for upstream regulators that coordinate lineage specification during myogenesis are the highly homologous paired-box transcription factors Pax3 and Pax7. *Pax3* expression can first be detected in the presomitic mesoderm prior to the formation of the epithelial somite [4, 5]. At the onset of dorsal ventral somite compartmentalization, *Pax3* is expressed throughout the entire epithelial dermomyotome. Expression of *Pax3* is subsequently down regulated in the DML, and becomes regionalized to the hypaxial VLL domain [4, 31]. Unlike Pax3, Pax7 deficiency does not affect embryonic myogenesis [32]. The *Pax7* expression domain does partially overlap that of *Pax3* during the early epithelial dermomyotome stage [6]. Pax7 is also capable of substituting for most of Pax3's function [33, 34]. In summary, myogenic transcription

factors interact in a developmental hierarchy with partially overlapping functions. Myogenic transcriptional activation results in a cascade of gene induction events that ultimately result in a physiologically functional myotube.

Myotubes are sustained by hundreds of postmitotic myonuclei and are efficiently repaired and regenerated by a pool of stem cells located beneath the basal lamina that surrounds each fiber, called satellite cells [35].

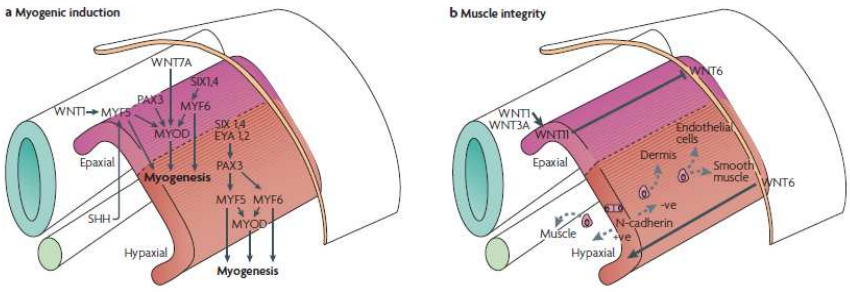

Figure 2 - Signalling pathways for myogenic induction and integrity. a | Myogenic induction in the mouse. In the epaxial muscle, paired box gene 3 (PAX3), myogenic factor 5 (MYF5) and MYF6 (also known as MRF4) are capable of inducing myogenic differentiation 1 (MYOD1; also known as MYOD) independently31. By contrast, in the hypaxial dermomyotome, PAX3 induces expression of MYF5 directly, which in turn activates MYOD1 expression. WNT1 signalling from the dorsal neural tube induces myogenesis through direct activation of MYF5, whereas WNT7A expression from the dorsal ectoderm preferentially activates MYOD1. Hedgehog signalling also regulates myogenesis through the maintenance of MYF5. The SIX protein family also have a role in myogenesis, sine oculisrelated homeobox 1 (SIX1) and SIX4 regulate MYF6 in the epaxial dermomyotome, and, together with the cofactors eyes absent 1 homologue (EYA1) and EYA2, induce PAX3 in the hypaxial dermomyotome. SHH, Sonic hedgehog. b | Muscle integrity in the chick. Canonical WNT1 and WNT3A signalling from the neural tube inhibits WNT6 expression in the medial surface ectoderm through the non-canonical WNT11 signalling that originates in the dermomyotome. WNT11 acts to maintain the epithelial nature of the dorsomedial lip and WNT6 the ventrolateral lip. Prior to the second myogenic phase cells of the disintegrating epithelium divide perpendicular to the plane of the epithelium, apical cells maintain N-cadherin expression and contribute to myogenesis, with the basal cell adopting a dermal fate.

Satellite cells

Skeletal muscle satellite cells were first described in frog muscle by Mauro [35] based on their morphology and position relative to mature myofibers and were later identified in adult avian and mammalian muscle [36]. Satellite cells adhere to the surface of myotubes prior to the formation of the basal lamina, such that the basal lamina surrounding the myofiber and satellite cells is continuous [36, 37]. Satellite cells mediate the postnatal growth of muscle and are the primary means by which the mass of adult muscle is formed [38, 39]. The overall population of satellite cells decreases with increasing age in rodents [40, 41]. At birth satellite cells account for about 32% of muscle nuclei followed by a drop to less than 5% in the adult (2 months for mice) [42]. This decline in satellite cell nuclei as the postnatal muscle develops is a direct reflection of satellite cell fusion into new or preexisting myofibers. Satellite cells in adult skeletal muscle are normally mitotically quiescent but are activated (i.e., initiate multiple rounds of proliferation) in response to stress induced by weight-bearing exercise or trauma [41, 43, 44]. The descendants of activated satellite cells, called myogenic precursor cells (mpcs), undergo multiple rounds of division prior to fusing with existing or new myofibers [42, 43, 45]. Satellite cells appear to form a population of stem cells that are distinct from their daughter mpcs as defined by biological and biochemical criteria [42, 45]. The number of quiescent satellite cells in adult muscle remains relatively constant over multiple cycles of degeneration and regeneration, demonstrating an inherent

capacity for self-renewal [40, 46]. However, the numbers and doubling potential of satellite cells become severely reduced in muscle diseases such as Duchenne muscular dystrophy presumably due to high levels of ongoing regeneration [46-49].

Satellite cells are believed to constitute a myogenic cell lineage distinct from the embryonic lineages and first appear in the limbs of mouse embryos at about 17.5 days postcoitum [50-52]. Adult satellite cells may also be further divided into subclasses based on the fiber type in which they take up residence in the mature muscle. It is clear from several studies that satellite cells form fibers genetically similar to the muscle from which they originate [53-55].

The MRF expression program during satellite cell activation, proliferation, and differentiation is analogous to the program manifested during the embryonic development of skeletal muscle (Fig. 5). Quiescent satellite cells express no detectable levels of MRFs. MyoD is rapidly up-regulated within 12h of experimentally induced muscle injury prior to expression of proliferating cell nuclear antigen (PCNA), a marker for cell proliferation. Myogenin is expressed last during the time associated with fusion and differentiation [56, 57]. Activated satellite cells (satellite cells entering the cell cycle) first express either *Myf5* or *MyoD* followed soon after by co-expression of *Myf5* and *MyoD*. Following proliferation, *myogenin* and *MRF4* are expressed in cells beginning their differentiation program.

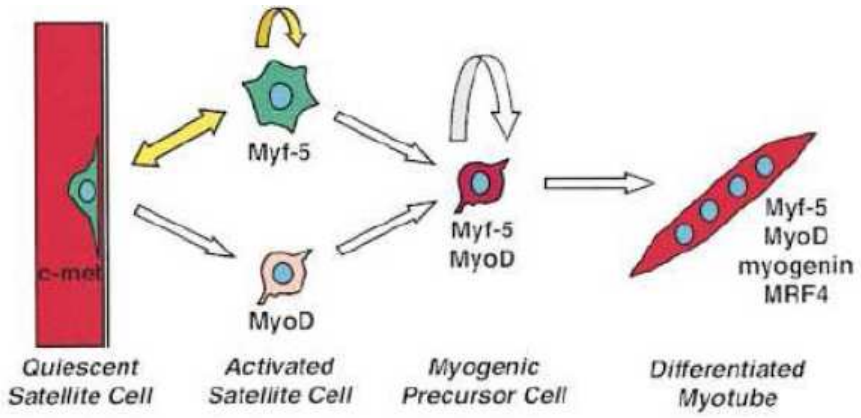

Figure 5 - Role of MRFs in satellite cells. When satellite cells are activated, they start to express Myf5 and MyoD, originating myogenic precursor cells that complete their differentiation program fusing in myotubes.

The main role of the satellite cell during the early postnatal period is to provide myonuclei for skeletal muscle growth. In adult muscle, its role changes to one of providing myonuclei for homeostasis and hypertrophy, or in response to the more sporadic demands for myofibre repair and regeneration. Moreover, satellite cells are comparatively numerous group – approximately 2-5% of nuclei in muscle of adult mouse belong to this population [58]. These features decide about considering satellite cells as a good material for autologous transplantations in cases of muscle dysfunction.

Morphological Characteristics of Skeletal Muscle Regeneration

Adult mammalian skeletal muscle is a stable tissue with little turnover of nuclei. Minor lesions inflicted by day-to-day wear and tear elicit only a slow turnover of its constituent multinucleated muscle fibers. It is estimated that in a normal adult rat muscle, no more than 1–2% of myonuclei are replaced every week. Nonetheless, mammalian skeletal muscle has the ability to complete a rapid and extensive regeneration in response to severe damage. Whether the muscle injury is inflicted by a direct trauma (i.e., extensive physical activity and especially resistance training) or innate genetic defects, muscle regeneration is characterized by two phases: a degenerative phase and a regenerative phase. The initial event of muscle degeneration is necrosis of the muscle fibers. This event is generally triggered by disruption of the myofiber sarcolemma resulting in increased myofiber permeability. Disruption of the myofiber integrity is reflected by increased serum levels of muscle proteins, such as creatine kinase, which are usually restricted to the myofiber cytosol. In human and animal models, increased serum creatine kinase is observed after mechanical stress (e.g., extensive physical exercises) and in the course of muscle degenerative diseases such as muscular dystrophies, all of which are characterized by the induction of a muscle regeneration process. It has been hypothesized that increased calcium influx after sarcolemmal or sarcoplasmic reticulum damage results in a loss of calcium

homeostasis and increased calcium-dependent proteolysis that drives tissue degeneration. Calpains are calcium-activated proteases that can cleave myofibrillar and cytoskeletal proteins and hence are implicated in the process. Thus disrupted myofibers undergo focal or total autolysis depending on the extent of the injury. The early phase of muscle injury is usually accompanied by the activation of mononucleated cells, principally inflammatory cells and myogenic cells. Neutrophils are the first inflammatory cells to invade the injured muscle, with a significant increase in their number being observed as early as 1–6 h after myotoxin or exercise induced muscle damage. After neutrophil infiltration and 48 h post-injury, macrophages become the predominant inflammatory cell type within the site of injury. Macrophages infiltrate the injured site to phagocytose cellular debris and may affect other aspects of muscle regeneration by activating myogenic cells. Moreover, studies demonstrating the stimulation of peritoneal macrophages after intensive physical exercise suggest that a systemic factor capable of inducing an inflammatory response throughout the body is released following muscle damage. Although several mediators involved in the activation of the inflammatory response have been characterized, further studies are necessary to demonstrate their potential role in the muscle regeneration process in vivo. Thus muscle fiber necrosis and increased number of non-muscle mononucleate cells within the damaged site are the main histopathological characteristics of the early event following muscle injury. Muscle degeneration is followed by the activation of a muscle repair process. Cellular proliferation is an important event

necessary for muscle regeneration as demonstrated by the reduced muscle regenerative capacity after exposure to colchicine (an inhibitor of mitotic division) or after irradiation. This process is carried out by myogenic stem cells. Myogenic stem cells are cells with the ability to differentiate into muscle fibers. There are still a lot of controversies concerning origin, potential and characteristic features of different subpopulations of this heterogenous group. The typical population of cells which are recognized as myogenic stem cells are satellite cells, even if in the last decade other cell types were postulated as potentially contributing to regeneration of skeletal muscles, including skeletal muscle side population cells, bone marrow-derived haematopoietic lineages, mesoangioblast and pericytes endothelial precursor cells of blood vessel walls and, quite recently, brown fat precursor cells [59-62].

Contribution of other stem cells to the muscle repair process: mesoangioblasts

Recent findings have demonstrated the presence of multipotential stem cells in various adult tissues and challenged the widely held view that tissue-specific stem cells are predetermined to a specific tissue lineage. In fact, adult stem cells isolated from various tissues appear to differentiate in vitro and in vivo into multiple lineages depending on environmental cues. In particular, the demonstration that postnatal murine bone marrow contains a transplantable, circulating progenitor that can differentiate into skeletal muscle stimulated the search for non-canonical progenitors of mesodermal tissues.

The notion that hematopoietic stem cells originate from a locally specified subset of endothelial progenitors in the floor of the dorsal aorta — within the aorta–gonad–mesonephros (AGM) region [63], suggested the somewhat speculative hypothesis that other progenitor/stem cells might be generated in the same embryonic region. Asymmetric cell division would generate luminal (hematopoietic) and abluminal (solid-phase mesoderm) progenitors as the spatially segregated offspring of multipotent angiopoietic progenitors. Retention of the ability to generate mesodermal progenitors in growing blood vessels of the fetus and post-natal organism would contribute to the growth and repair capacities of mesoderm-derived tissues.

The name ‘mesoangioblasts’ was thus chosen to denote a common progenitor for vascular and extravascular mesodermal derivatives,

suited for marking the conceptual resemblance of this novel entity with the hemoangioblast, a common progenitor for vascular and hematopoietic (intravascular) mesodermal derivatives. At variance with hemoangioblasts, the ultimate progenitors of all definitive, rapidly turning over hematopoietic cells, mesoangioblasts would be only an additional source of post-natal differentiated mesoderm-derived tissues — the bulk of which are established from canonical embryonic sources during development and turn over slowly — but possibly a main source of post-natal progenitors thereof.

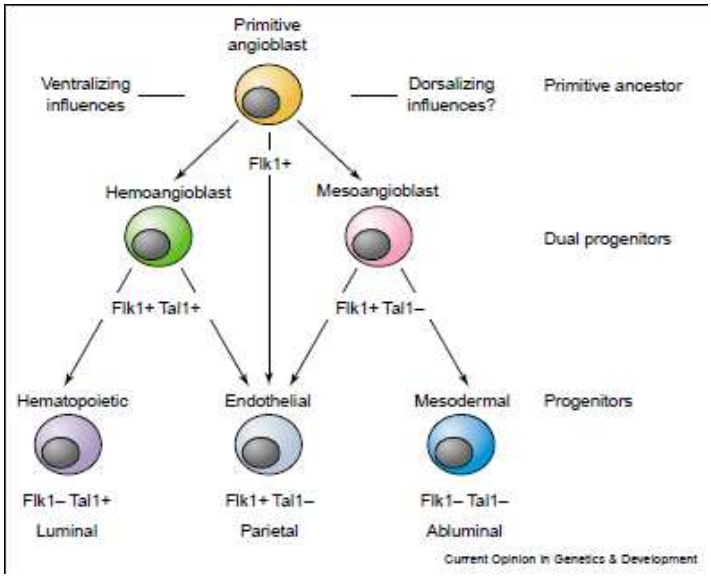

Figure 6 - A tentative scheme define a possible origin of mesoangioblasts cells. Enviromental cues impart a hematopoietic potential to a subset of angiopoietic progenitors.

Mesoangioblasts express angioblast markers such as Sca-1, Flk-1, and CD34, as well as genes typical of the mesoderm [64]. Among the family of membrane receptors, signal transducers and transcription factors, mesoangioblasts express at high level many members of the TGFβ/BMP pathway [65], including several receptors and SMADs: more than 50% and up to 80% of the mesoangioblasts promptly differentiate into smooth muscle cells in response to TGFβ1 and into osteoblasts in response to BMP2.

Many members of the Wnt signalling pathway are expressed at high level in mesoangioblasts, including several Frizzled and co-receptors, axin, GSK-3, beta catenin and Tcfs. Besides their role in cell growth and transformation, different Wnts have a positive role in the activation of differentiation in many cell types. However, simple withdrawal of mitogens did not induce spontaneous differentiation into any recognizable cell type, such as neurons or skeletal muscle. Indeed, mesoangioblasts mainly express Wnt5a that is involved in early angiogenesis and this is compatible with an angioblast phenotype.

Among genes selectively expressed by mesoangioblasts are many cytokines, chemokines and their receptors. This is consistent with a role of these cells in tissue regeneration and first inflammatory response to damage. In this context it is interesting to note that, when injected into the femoral artery, mesoangioblasts can adhere to the endothelium and extravasate but only in the presence of inflammation, as it occurs in muscular dystrophy or after local injection of a

myotoxic agent. Indeed, mesoangioblasts express many but not all of the proteins that leukocytes use to this purpose, and this may explain a lower efficiency (only 30% of injected mesoangioblasts end up in downstream skeletal muscle). To increase the efficiency of muscle repair by mesoangioblasts, it is essential to increase their migration to skeletal muscle, with the additional benefit of reducing unspecific trapping in the capillary filters of the body, such as liver and lung. For example, it was shown that expression of α4 integrin and exposure of cells to SDF-1 or TNF-α improve up to fivefold migration of wild-type (WT) mesoangioblasts to the dystrophic muscles and consequent production of new fibers that express the normal copy of the mutated gene [66].

Furthermore, mesoangioblasts respond to high mobility group box 1 (HMGB1), a nuclear protein released by necrotic and by inflammatory cells [67]. They proliferate, migrate through the endothelial layer and accumulate in vivo around beads soaked with HMGB1 and implanted into skeletal muscle [68]. Indeed, dystrophic muscle contains a high amount of HMGB-1, as it happens in the regenerating muscle. In agreement with the observation that the recruitment of mesoangioblast depends on the generation of specific chemoattractants at the site of damage and the generation of signals, which favor stem cell survival and differentiation, a model in which macrophages recruit stem cells was recently proposed [69]: they demonstrated that the polarization of macrophages dramatically skews the secretion of HMGB1, TNF-α, vascular endothelial growth factor, and metalloproteinase 9 (MMP-9), molecules involved in the regulation of cell migration.

Another factor that limits the effect of cell therapy with mesoangioblasts is the reduced ability of these cells to fuse and to resist to the cytotoxic environment existing in the damaged muscle, where several pro-inflammatory and pro-apoptotic stimuli may be present [70, 71]. A good candidate molecule to increase the efficiency of muscle repair by mesoangioblasts is nitric oxide (NO), a short-lived messenger generated by skeletal muscle to play important roles in regulating its own physiological function [72-74]. A brief ex-vivo treatment of mesoangioblasts with NO donors enhances their ability to migrate, resist death-inducing stimuli of the type known to be present in dystrophic muscles and fuse with regenerating myofibers.

A necessary prerequisite to optimizing stem cell-based therapeutic strategies is to increase stem cell survival. In this context, it was shown that TGFβ, a pleiotropic cytokine that plays a major role in development and specifically induces smooth muscle differentiation of mesoangioblasts, efficiently protects them from programmed cell death [75]. In particular, TGFβ exerts a marked anti-apoptotic action in mesoangioblasts with a mechanism involving regulation of sphingosine kinase 1 (SphK1), a key enzyme in the sphingolipid metabolic pathway. These effects can be conceivably exploited in strategies aimed at ameliorating the efficacy of those therapies on which mesoangioblast administration has been proposed.

Interestingly, more recently mesoangioblasts were isolated from post-natal mouse, dogs and also human muscle. In particular, cells derived from blood vessels of human skeletal muscle can regenerate skeletal muscle, similarly to embryonic mesoangioblasts [59]. In future

clinical protocols, systemic delivery seems to be an obligate choice, as intra-muscular delivery would require an excessive number of injections and pericyte-derived human cells express some of the proteins that leukocytes use to adhere to and cross the endothelium (that is, β2 and α4 integrins), thus can diffuse into the interstitium of skeletal muscle when delivered intra-arterially (a distinct advantage over resident satellite cells that cannot). Moreover, their extensive, but not indefinite, *in vitro* proliferation and the maintenance of normal karyotype and myogenic potency, indicates that human adult pericytes from a single biopsy may generate enough cells to treat a paediatric patient with minimal risk of malignant transformation. Because of these features, pericyte-derived cells are an ideal cell population for future cell therapy of muscular dystrophy.

Muscular Dystrophies

The muscular dystrophies are a group of inherited disorders characterized by progressive muscle wasting and weakness [48]. A unifying feature of the dystrophies is the histological analysis of muscle samples which typically includes variations in fiber size, areas of muscle necrosis, and, ultimately, increased amounts of fat and connective tissue. Dystrophin and other associated proteins form a link between the extracellular matrix (endomysium) and intracellular F-actin (fig 4). It seems that the absence of any one of these proteins (and dystrobrevin might be the final common pathway) would interfere with the integrity and the strength of the membrane and so result in muscle weakness.

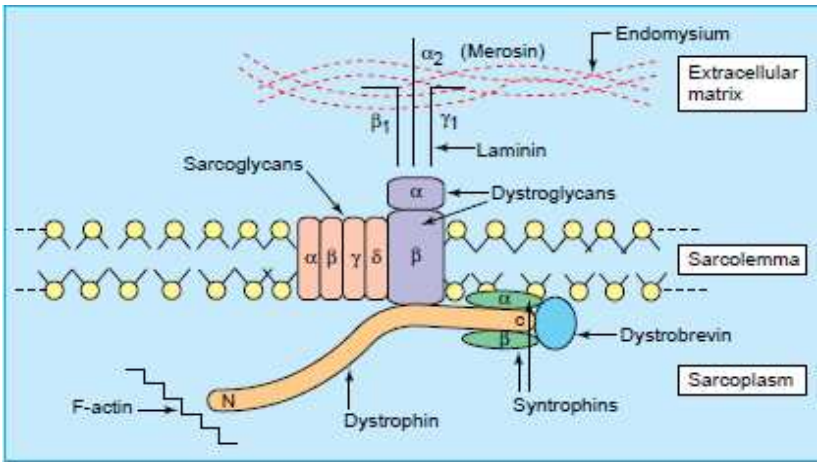

Figure 4 - Muscle membrane proteins. Specific muscular dystrophies have been found to be caused by deficiencies of dystrophin, or a particular sarcoglycan, or merosin.

The original classification scheme proposed by Walton and Nattrass depended on two considerations: the distribution of predominant muscle weakness (whether mainly proximal or distal and whether facial muscles were affected) (fig 1) and the mode of inheritance. They identified three principal groups of muscular dystrophies (Duchenne-type, facioscapulohumeral, and limb girdle) and three comparatively uncommon forms (distal, oculopharyngeal, and congenital).

Duchenne-type muscular dystrophy (Meryon's disease)

Duchenne-type muscular dystrophy (also known as Meryon's disease) is the commonest form of dystrophy; it is inherited as an X linked recessive trait and therefore predominantly affects boys. It is a serious condition with progressive muscle wasting and weakness which causes most boys to start using wheelchairs by age 12 and to die in their 20s. Becker-type muscular dystrophy is clinically similar but milder, with onset in the teenage years or early 20s. Loss of the ability to walk may occur later and many individuals with Becker-type dystrophy survive into middle age and beyond.

Facioscapulohumeral muscular dystrophy

Over the past few years the essential clinical features of weakness of the facial, scapulohumeral, anterior tibial, and pelvic girdle muscles have been extended to include retinal vascular disease, sensory hearing loss (usually asymptomatic) and, in severe cases, even abnormalities of the central nervous system.13 Many individuals are

only mildly affected by these dystrophies though some may later become dependent on wheelchairs.

Limb girdle muscular dystrophy

Limb girdle muscular dystrophy has turned out to be a clinically and genetically heterogeneous group of conditions. Less than 10% of cases are inherited as an autosomal dominant trait (type 1) and are relatively mild. One subtype (1B) may be allelic to autosomal Emery-Dreifuss dystrophy. All other cases are inherited as autosomal recessive traits (type 2) affecting both males and females; type 2 is often more severe and resembles Duchenne-type dystrophy. At least three dominant subtypes and eight recessive subtypes have been identified. Apart from limb girdle muscular dystrophy 2A, which is caused by a muscle specific protease (calpain 3) deficiency, four other recessive subtypes have been found to be caused by deficiencies of particular sarcoglycans (dystrophin associated glycoproteins) which form part of the dystrophin associated protein complex of muscle membrane.

Distal myopathies

These rare forms of dystrophy are associated with wasting and weakness of the distal muscles, usually without the noticeable involvement of other muscle groups. Many individuals with distal myopathies are only mildly affected although some may ultimately develop serious problems in walking and everyday life.

Oculopharyngeal muscular dystrophy

This autosomal dominant disorder is characterised by onset in late adulthood of progressive ptosis and dysphagia which is followed by involvement of other cranial and limb muscles.21 The gene associated with the disease is located on chromosome 14 not only among French Canadians but also in other populations; therefore it is likely to be genetically homogeneous but with different ancestral mutations in different populations.

Congenital muscular dystrophy

This relatively uncommon autosomal recessive form of dystrophy presents at birth or in early infancy with symptoms of hypotonia and generalised weakness which may be associated with joint contractures. Around 50% of cases are caused by a specific deficiency of the extracellular muscle protein known as laminin α2 chain, or merosin (on chromosome 6).

Strategies for muscular dystrophies therapy

Muscular dystrophies are among the most difficult diseases to treat, even though the underlying molecular defects are now known. This is due to the fact that skeletal muscle is the most abundant tissue of the body and is composed of large multinucleated fibers, the nuclei of which have permanently lost the ability to divide. Consequently, any cell or gene replacement must restore proper gene expression in hundreds of millions of post-mitotic nuclei, which are embedded in a highly structured cytoplasm and surrounded by a thick basal lamina. Similarly, most pharmacological trials must overcome the complex and partly unknown biochemical mechanism of fiber degeneration that involves pathways, such as calcium fluxes and protease activity, for which inhibitors are associated with high systemic toxicity.

Gene therapy

The task of replacing a missing gene in all, or at least in a good proportion, of the post-mitotic nuclei of skeletal muscle is daunting. Furthermore, for DMD, the form of dystrophy that most needs a therapy, the gene to be replaced is the largest known, with a cDNA of 14 Kb. Two successive waves of enthusiasm were generated by the use of adenoviral vectors that were successful in delivering dystrophin to a very large fraction of muscle fibers in newborn dystrophic mice. The first generation of treatments, however, caused a strong immune reaction against the vector (which was not apparent in newborn animals, which were tolerant). The second generation of treatment,

‘gutted’ vectors, can accommodate the full-length cDNA for dystrophin, but do not carry genes encoding viral proteins. These induce a much weaker immune reaction, but have to cross the basal lamina of muscle fibers and the efficiency of transduction is greatly reduced in juvenile and adult animals [76]. Adeno-associated viral (AAV) vectors are derived from a non-pathogenic replication-deficient virus with a small (w4.7-kb) single-stranded DNA genome. They appear to be more efficient for transducing adult fibers (owing to their smaller size) [77], especially if delivered systemically together with factors that increase vascular permeability; a clinical trial using these vectors is ongoing. They cannot accommodate the full-length dystrophin cDNA, but a truncated version (micro-dystrophin) that gives good functional rescue when replacing dystrophin in transgenic mdx mice [78]. Moreover, they can accommodate the full-length sarcoglycan cDNAs, the proteins that are mutated in several forms of limb girdle muscular dystrophies [79].

An alternative strategy for gene therapy involves ‘exon skipping’. This molecular strategy prevents the transcription of the exon containing the mutation. Skipping can be achieved through oligonucleotides or by small RNAs that hybridize with the donor and/or acceptor sites of the mutated exon, causing its exclusion from the otherwise intact transcript. Because the skipped exon usually does not encode a functionally essential domain, the resulting protein is shorter but functional. Despite the fact that the oligonucleotides appear to function for only a short period in vitro, they are much more stable in muscle fibers in vivo and, recently, long-term correction of

dystrophy in mdx mice was achieved by a single injection of oligonucleotides.

Cell therapy

The identification of satellite cells in 1961 offered the first hope for treating muscular dystrophy with cells that can make new muscle. Since the beginning, two alternatives appeared: (i) using cells obtained from a healthy donor, which express the normal copy of the mutated gene but induce an immune rejection unless the patient is permanently immune suppressed; or (ii) using cells obtained from the patient, which do not require immune suppression but must be 'genetically corrected' in vitro (to restore the expression of the mutated protein). This latter task was made possible (although far from easy) by the cloning of the genes that result in muscular dystrophy. Satellite cells and cell lines derived from them have been used since the late 1970s, mainly through intra-muscular injection. A first pivotal study involved the injection of wild-type myoblasts (from the immortal myogenic cell line C2C12) into mdx mice and resulted in the conversion of muscles from dystrophin-negative to dystrophin-positive [80]. This study led to several clinical trials in the early 1980 s that failed for several reasons, the most important of which were the poor survival and the very limited migratory capacity of injected donor cells, together with the immune response of the patient that was not suppressed at that time. The major problem still faced by this approach is the lack of dispersion of donor cells, which remain in the area of injection, making it difficult to reach an even distribution within the whole muscle. This might be overcome by using blood-borne stem or

progenitor cells. The possible systemic delivery of circulating cells was the obvious choice over satellite cells that cannot cross the endothelial layer.

New generations of viral vectors, improved methods for efficient and long-lasting exon skipping, and increasing knowledge about the various types of stem cells (resulting in more efficient ways to manipulate them) should be the basis for the next generation of trials, all of which might benefit from the design of combined pharmacological therapies.

Necdin

Necdin is a member of the melanoma antigen-encoding gene family [81], a large family of proteins initially isolated from melanomas. Most MAGE genes have a number of properties in common: they are specifically expressed in a number of different tumors and in the healthy testis, they have an open reading frame (ORF) with an approximately 200-aa-long MAGE-homology domain at the C-terminal end; and they are situated on the X chromosome. The 23 different human MAGE genes known today have been divided into four subfamilies, *A* to *D*, based on their sequence homology and chromosomal location. The most conserved parts of the MAGE proteins are located toward the C-terminal ends of the proteins and include a MAGE/NECDIN consensus sequence and a hydrophobic region (fig 7). It has been suggested that the hydrophobic region serves as a trans-membrane domain. However, the hydrophobic region is small and it may function only when associated with the trans-membrane region of other protein. Only one MAGE-like protein has been shown to associate with the plasma membrane, the neurotrophin receptor-interacting MAGE homologue (NRAGE) [82]. NRAGE most likely belongs to the MAGE-D subfamily since a major part of the NRAGE protein is 100% homologous to MAGE-D. NRAGE associates with the plasma membrane when bound to the p75 neurotrophin receptor [83].

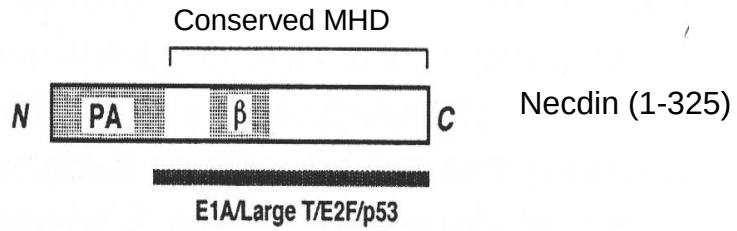

Figure7 - Schematic representation of Necdin protein. PA: prolin and aminoacid-rich domain. β is a portion of beta sheet. MHD: MAGE Homology Domain, conserved domain of MAGE protein family. It is shown the portion

Necdin is a 325-amino acid residue protein encoded in a cDNA sequence isolated from the library of neurally differentiated P19 embryonal carcinoma cells [84].The Necdin gene is expressed predominantly in postmitotic neurons [85] and this has led to the suggestion that it functions in growth suppression. Evidence that this is indeed the case comes from the observation that ectopic expression of Necdin in NIH3T3 cells suppresses cell growth without affecting cell viability [86]. Furthermore, Necdin has been shown to physically interact with cell-cycle-promoting proteins such as SV40 large T, adenovirus E1A, and the transcription factor E2F1 [87]. The E2F proteins act as important components of the cell cycle machinery, and

E2F1 specifically binds and trans-activates genes that are involved in cell cycle progression. This pathway is regulated by retinoblastoma protein (Rb), which represses E2F1 activation by binding to the trans-activating domain of E2F1 during the G1 phase. When cells move toward the S phase, Rb repression is released due to the phosphorylation of Rb, and the E2F1 complex becomes free to trans-activate cell cycle progression genes. Necdin acts in a way similar to that of Rb; by binding to E2F1 Necdin represses E2F1 function which leads to cell growth arrest [87].

Necdin also interacts with p53, a well-known tumor suppressor that trans-activates genes involved in growth arrest and apoptosis [88]. Necdin binds to the trans-activating domain of p53 and in this way inhibits p53-induced apoptosis and represses p53-dependent activation of the p21/WAF. In particular, necdin promotes p53 deacetylation by facilitating the interaction between the deacetylase Sirtuin-1(Sirt1) and p53 to suppress p53-dependent neuronal apoptosis [89]. Thus, it is likely that Necdin acts as a specific growth suppressor and anti-apoptotic protein in early neurons through interaction with p53 and E2F proteins.

The human *necdin* gene (*NDN*) is maternally imprinted and maps to 15q11–q12, a region known to be deleted in patients suffering from the Prader–Willi syndrome (PWS) [90] (Fig. 8). PWS is a neurobehavioral disorder characterized by respiratory distress and poor muscle tone in the newborn and by mental retardation, hypogonadism, and obesity later in childhood [91]. Several genes, including *NDN,* are present and paternally expressed from the 15q11–

q13 region, a fact which has led to the suggestion that PWS is a multigenic disorder.

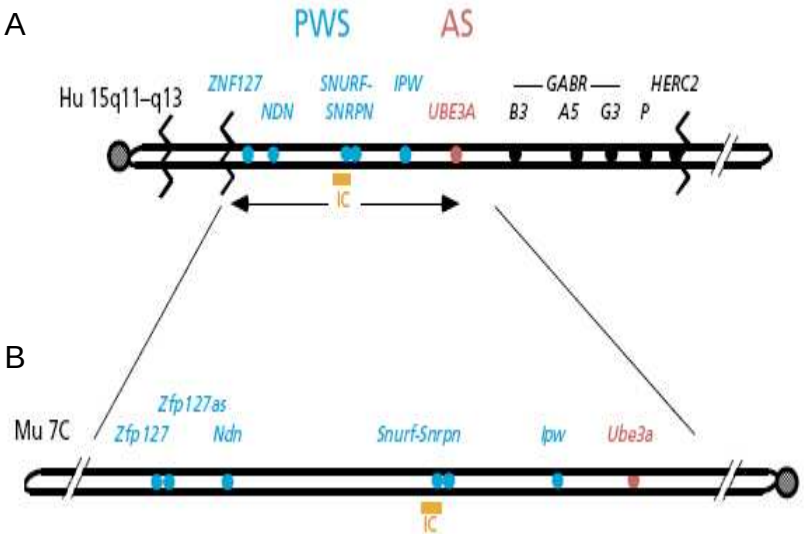

Figure 8 - **A**) Genes on 15q11-q13 human chromosomic region. Blue: maternally imprinted region, red: paternally imprinted region; black: non-impinted region. Zig-zag lines are breakpoint of typical deletion of Prader-Willi and Angelman syndromes. **B**) Corresponding mouse chomosomic region (7C). IC: imprinting centre.

Necdin knockout mice show a phenotype resembling Prader-Willi syndrome, a genomic imprinting-associated neurobehavioral disorder, suggesting that the absence of Necdin impairs neuronal differentiation or maturation. These findings suggest that Necdin facilitates terminal differentiation and prevents apoptosis in neurons and that Necdin has

a similar function in terminally differentiated non-neuronal cells. The precise cellular mechanism leading to cell cycle arrest is still unclear, although evidence points to interaction with proteins involved in cell cycle progression such as p53 and E2F [87, 88, 92, 93].

Recently, it was shown that Necdin promotes cell cycle exit of hematopoietic stem cells (HSCs) thus service to maintain their quiescence [94], but this function is restricted to the regenerative phase of hematopoiesis [95].

Necdin associates with the Msx homeodomain proteins via MAGE-D1 to modulate their function. In particular, it releases Msx2-induced repression of myogenic differentiation in C2C12 myoblasts. This supports the notion that necdin promotes terminal differentiation of postmitotic cells by repressing cell proliferation [93]. Our group have shown that Necdin cooperates with Msx2 during smooth muscle differentiation of mesoangioblast cells [96]. Whether Necdin acts as a transcriptional cofactor or as a direct transcriptional repressor or activator in these different scenarios is still unknown.

Necdin is also expressed in developing skeletal muscle, which suggests that it plays a relevant role in this tissue [93, 96, 97]. Recent studies in our laboratory demonstrated that Necdin is an important player in skeletal muscle differentiation and maintenance [98]. In particular, we identified Necdin as a novel, important factor required for proper myoblast differentiation in vitro and in vivo, for the first phase of muscle fiber growth, and for efficient repair upon muscle injury. We also showed that Necdin acts at different levels: it cooperates with MyoD to promote the transcriptional activation of

myogenin by direct binding to the promoter, and possibly by contributing to p53 action, and by accelerating myoblasts differentiation. In addition, it exerts a pro-survival, anti-apoptotic action in vitro and in vivo, counteracting the cytotoxic effect of several apoptotic agents, including the formation of oxidant species. A prosurvival role of Necdin consistent with this has also been described in neurons [90, 92], where caspase 3 was found to be activated. In this work, in addition to caspase-3 activation, it was established for the first time a link between Necdin and the extrinsic, caspase 9–dependent apoptosis pathway. Altogether, these data characterized at molecular level the function of Necdin in myogenic cells, showing its relevance in muscle growth and regeneration.

Scope of the thesis

The two chapters of results of my PhD thesis focus on the role of Necdin in skeletal muscle differentiation and regeneration.

In the first chapter, in which is described the main focus of my PhD project, I investigated the function of Necdin in mesoangioblasts-dependent skeletal muscle regeneration. Since we already knew that Necdin could enhance differentiation ability of satellite-derived myoblasts cells, we wondered if this role could be exploited also in mesoangioblasts cell system, because they are the best candidates for stem cell therapy for muscular dystrophy. What we found was that Necdin not only potentiates mesoangioblasts differentiation potential in vitro, but it increases dystrophic muscle reconstitution in vivo. This may be very important to widen our knowledge about molecular mechanisms through which mesoangioblasts act to finally optimize their therapeutic applications and make them successful for humans.

The second chapter regards the role of Necdin in another model of muscle wasting and degeneration, the muscle atrophy or cachexia, induced by tumour. We showed that Necdin protects from inhibition of myogenic differentiation and fibers regeneration interfering with TNFα signaling at different levels, in particular by controlling expression of TNFRI and p53, and also regulating caspases activity. These results identify Necdin as a candidate target to design new therapeutic approach to treat cachexia, for which no valid protocol is still available.

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Results

Necdin enhances muscle reconstitution of dystrophic muscle by mesoangioblast cells

Patrizia Pessina[1], Valentina Conti[1], Jorge Diaz Manera[2], Rossana Tonlorenzi[2], Thierry Touvier[2], Raffaella Meneveri[1], Enrico Tagliafico[3], Giulio Cossu[2,4] and Silvia Brunelli[1,2*]

[1]Dept. of Experimental, Environmental Medicine and Medical Biotechnology, University of Milano-Bicocca, 20052 Monza, Italy; Division of Regenerative Medicine, H. San Raffaele Scientific Institute, 20132, Milan, Italy; [3]Dept. of Biomedical Science, University of Modena and Reggio Emilia, 41100 Modena, Italy; [4]Dept. of Biology, University of Milano, 20130 Milan, Italy

*Corresponding author

Silvia Brunelli

[1]Dept. of Experimental, Environmental Medicine and Medical Biotechnology, University of Milano-Bicocca, 20052 Monza, Italy

Tel, +390264488308

silvia.brunelli@unimib.it

KEYWORDS

Necdin, muscular dystrophy, stem cells,, apoptosis, differentiation

ABSTRACT

Improving stem cell therapy is a major goal for the treatment of muscle diseases, where physiological muscle regeneration is progressively exhausted. Mesoangioblasts are vessel-associated stem cells that appear to be the most promising cell type for the cell therapy for muscular dystrophies because of their significant contribution to restoration of muscle structure and function in different muscular dystrophy model. Here we report that MAGE protein Necdin enhances muscle differentiation and regeneration by mesoangioblasts. Indeed, when Necdin is constitutively over-expressed, it accelerates their differentiation and fusion in vitro and it increases their efficacy to restore dystrophic phenotype of α-sarcoglycan mutant mouse. Moreover, Necdin confers a enhanced survival ability when mesoangioblasts are exposed to cytotoxic stimuli that mimic inflammatory dystrophic environment. Taken together, these data demonstrate the pivotal role of Necdin in muscle reconstitution from which we could take advantage to boost therapeutic applications of mesoangioblasts.

INTRODUCTION

Adult mammalian skeletal muscle is a stable tissue with little turnover of nuclei. Nonetheless, it has the ability to complete a rapid and extensive regeneration in response to severe damage inflicted by direct trauma or genetic defects, like muscular dystrophies. Anyway, in the most severe forms of muscular dystrophy, such as Duchenne muscular dystrophy, muscle regeneration is progressively exhausted leading the patient to complete paralysis and death. Therapeutic approaches based on exogenous stem cell administration have been proposed with success in animal models of the disease. In particular, the mesoangioblasts (MABs) are a population of vessel-associated stem cells that have been shown to contribute to muscle repair in dystrophic mice and dogs when injected intra-arterially [1].

Despite the identification of mesoangioblasts as potential sources of skeletal muscles, and the experimental evidence that they can be used to support in vivo skeletal myogenesis, the molecular mechanism regulating their growth and differentiation into skeletal muscle remain unexplored. Many genes and molecules are now being investigated in order to manipulate their fate and act as a pool of resident stem cells. We believe that Necdin may be an ideal candidate molecule. Necdin (Ndn) is a member of melanoma antigen-encoding gene (MAGE) protein family, a large family of proteins initially isolated from melanomas [2]. It is a maternally imprinted gene that maps on a chromosomal region known to be deleted in patients suffering from the Prader–Willi syndrome (PWS), a neurobehavioral disorder which occurs in 1 in 10000-15000 births [3]. Necdin null mice exhibit an high post-natal lethality due to a respiratory defect that resemble neonatal respiratory distress observed in humans with PWS. In particular, the absence of Necdin impairs differentiation and maturation of hypothalamic neurons [4, 5].

Necdin is also expressed in developing and regenerating skeletal muscle, where it plays a pivotal role in tissue differentiation and maintenance [6, 7]. In particular, our group provided the first evidence that this protein is required for proper myoblast differentiation in vitro and in vivo, for the first phase of muscle fiber growth, and for efficient repair upon muscle injury. We also showed that Necdin acts at different levels: it cooperates to promote the transcriptional activation of myogenin by direct binding to the promoter, and possibly by contributing to p53 action; in addition, it exerts a pro-survival, anti-apoptotic action in vitro and in vivo, counteracting the cytotoxic effect of several apoptotic agents, including the formation of oxidant species [6]. Moreover, we found that Necdin activity, when combined with that of the homeobox transcription factor Msx2, is required for smooth muscle differentiation of mesoangioblasts [8]. Finally, we recently showed in vivo that Necdin counteracts the muscle wasting and inhibition of differentiation specifically induced by cachexia, a pathology in which atrophy is associated with tumor load [9]. In this context, Necdin exerts its protecting effect by interfering with TNFα-activated signaling at various levels: it causes a reduction of TNFRI expression on the surface of myoblasts, modulating the extent of TNFα signaling, and it influences the activity and the expression of p53, which is the key downstream mediator of the effect of TNFα.

These data prompted us to investigate if this role of Necdin could be exploited also to enhance myogenic differentiation of mesoangioblasts and promote their survival. Understanding the molecular mechanism of the effect of Necdin on regeneration and designing tools to boost this process may be a crucial step towards the optimization of cell therapy by mesoangioblasts or other stem cells.

MATHERIALS AND METHODS

Mesoangioblasts cell culture and infection

Mesoangioblasts were isolated from explants of mouse muscle and cultured as previously described [10].

The necdin lentiviral vector (in pRRLsin.PPT.CMV.NTRiresGFPpre) and the GFP lentiviral vector (pRLLsin.PPT.CMV.GFP) were generated and prepared as described in Brunelli et al. (2004). The final MOI was 10^7 TU/ml. After 24 h in culture, mesoangioblasts were transduced with an MOI of 100 in proliferation medium overnight. The next day, the medium was changed and cells were maintained in proliferation medium. Viral titer was determined by GFP fluorescence, with serial dilution of the virus.

In vitro differentiation of mesoangioblasts

Mesoangioblasts were induced to differentiate to smooth muscle and osteogenic cells as described in Tagliafico et al. (2004). The proportion of cells differentiating in each different cell type was calculated by counting the cells expressing the appropriate differentiation markers against the total number of cells. An average value was determined by counting cells in at least ten microscopic fields, in at least three different experiments.

Mesoangioblasts derived from adult muscle can spontaneously differentiate into multinucleated skeletal myotubes when cultured onto matrigel coated plastic support in differentiation promoting medium (complete medium supplemented with 2% Horse Serum).

Immunofluorescence

Immunofluorescence on cell cultures and cryosections was performed according to Brunelli et al. (2004), using antibodies specific for sarcomeric myosin MF20, alpha-smooth muscle actin (α-

SMA, Sigma), laminin (Abcam), GFP (Chemicon International) and alpha-sarcoglycan. For fluorescent detection, we used appropriate secondary antibodies conjugated with either Alexa 488 (green; Invitrogen) or Alexa 594 (red; Invitrogen).

The fusion index of differentiating myoblasts was measured as the number of nuclei in sarcomeric myosin-expressing cells with more than two nuclei versus the total number of nuclei.

RT-PCR

RNA (1 μg) collected from cells or tissues using RNeasy Mini (or Micro) kit (Quiagen) or the TRIzol protocol (Invitrogen) was converted into double-stranded cDNA using the cDNA synthesis kit "High Capacity Reverse Transcription Kit" (Applied Biosystem), according to the manufacturer's instructions. Real-time quantitative PCR was carried out with a real-time PCR system (Mx3000P; Stratagene). Each cDNA sample was amplified in duplicate by using the SYBR Green Supermix (Bio-Rad Laboratories) for 28s (5' AAACTCTGGTGGAGGTCCGT 3'; 5' CTTACCAAAAGTGGCCCACTA 3'), cyclophillin A (5' CATACGGGTCCTGGCATCTTGTCC 3'; 5' TGGTGATCTTCTTGCTGGTCTTGC 3'), Necdin (5' GGTGAAGGACCAGAAGAGGA 3'; 5' TGGGCATACGGTTGTTGAG 3'), SCA1 (5' CTCTGAGGATGGACACTTCT 3'; 5' GGTCTGCAGGAGGACTGAGC 3'), CD34 (5' TTGACTTCTGCAACCACGGA 3'; 5' TAGATGGCAGGCTGGACTTC 3'); VE-CAD (5' GTACAGCATCATGCAGGGCG 3', 5' GTACAGCATCATGCAGGGCG 3'), PDGFR (5' TCATTGAGTCTGTGAGCTCTG 3'; 5' AACATGGGCACGTAATCTATA 3'); NG2 (5' ACAAGCGTGGCAACTTTATC 3'; 5' ATAGACCTCTTCTTCATATTCAT 3'), MHC (5' GGCCAAAATCAAAGAGGTGA 3'; 5' CGTGCTTCTCCTTCTCAACC 3'), Myogenin (5' GACATCCCCCTATTTCTACCA 3'; 5' GTCCCCAGTCCCTTTTCTTC 3'), MyoD (5' ACGGCTCTCTCTGCTCCTTT 3'; 5' GTAGGGAAGTGTGCGTGCT 3'), GFP (5' ACAAGCAGAAGAACGGCATC 3'; 5'

CGGTCACGAACTCCAGCA 3'), a-SG (5' CTTGTGGGTCGTGTGTTTGT 3'; 5' GGTGAGCGTGGTAGGTGAGT 3').

Cell resistance to cell death

Mesoangioblasts from the different genotypes were incubated with or without 20μM As_2O_3 for 24 h. Cells were detached and stained with propidium iodide (PI) according to the kit's manufacturer's instructions and analyzed by flow cytometry as described [11].

Protein extracts and immunoblot analysis

Cells were scraped in Tris HCl pH=7.4, 150mM NaCl, 1mM EDTA, 1% TRITON X-100, and protease inhibitor cocktail 1X (Roche), cells were centrifuged 10 minutes at 10000 rpm at 4°C to discard cellular debris.

After electrophoresis, polypeptides were electrophoretically transferred to nitro-cellulose filters (Schleicher & Schuell, Dassel, Germany) and antigens revealed by the respective primary Abs and the appropriate secondary Abs, as already described [6]. We used specific antibodies for MyHC and Myogenin; protein levels were normalized to GAPDH protein expression.

***In vivo* injection**

3-months-old α-SG null mice were injected by intra-muscular delivery with $5x10^5$ mouse mesoangioblasts. A set of animals was sacrificed after 10 days and the other ones after 20 days. Different muscles (quadriceps, gastrocnemius, and tibialis) were collected and real-time PCR for GFP and α-sarcoglycan were performed on all the samples as described (see Real-time PCR).

Image acquisition and manipulation

Images in fluorescence and phase contrast have been taken on the Nikon microscope Eclipse E600, (lenses Plan Fuor: 4x/013, 10x/0.33, 20x/0.50, 40x/0.75) or on the Leica AF6000. Images have been

acquired using the NIKON digital camera DXM1200, and the acquisition software NIKON ACT-1, or using the DFC350 FX digital camera and the Leica AF600 acquisition software, imaging medium, PBS buffer, room temperature. Images were assembled in panels using Adobe Photoshop 7.0. Images showing double fluorescence were first separately acquired using the different appropriate filters, the two layers then merged with Adobe Photoshop 7.0.

RESULTS

Isolation and characterization of mesoangioblasts from mouse muscle biopsies

Mesoangioblasts were first isolated from the dorsal aorta of mouse embryos, but more recently cells with similar properties have been isolated from biopsies of post-natal mouse and human skeletal muscle, where they express markers of pericytes.

This work has been carried out on mesoangioblasts isolated from muscles of adult (6 month old) C57 mice. Fragments of interstitial tissue containing vessels were dissected and plated on collagen-coated dishes. After the initial outgrowth of fibroblasts, small round and refractile cells were observed. These cells were collected and cultures for many passages in standard proliferation medium (Fig 1A-B).

To test the role of Necdin on proliferating cells, we generated a cell line that over-express Necdin constitutively: we cloned the coding sequence of Necdin in pIresEGFP mammalian expression vector and then we produced a lentiviral vector expressing NecdinIresEGFP to infect wt mesoangioblasts (Fig 1D). As a control, we transduced the same cells with a lentiviral vector expressing EGFP under a constitutive promoter (Fig.1C). As shown in Fig 1E, mesoangioblasts that were transduced with lentiviral vector get near 2,5 thousand times fold increased Necdin expression.

RT-PCR assay confirmed that the two populations retained mesoangioblasts stem cells markers (Fig 1F).

As the other stem cells, mesoangioblasts are able to differentiate into cell types of the tissue from which they derive. We tested the ability of GFPMabs (Fig 1H) and NdnMabs (Fig 1I) to respond to differentiation programs induction by appropriate stimuli. TGFβ treatment, usually used to induce

smooth muscle differentiation, led to an high and comparable differentiation frequency in the two populations (fig 1L). Likewise, after BMP2 treatment (Fig 1M-P), known to promote osteogenic differentiation, NdnMabs showed a number of AP-positive cells similar to GFPMabs (Fig 1Q).

NEcdin enhance s Skeletal muscle differentiation of mesoangiobalst stem cells

Unlike their embryonic counterpart, mesoangioblasts derived from adult muscle are able to differentiate in skeletal muscle cells spontaneously.

Thanks to this property, skeletal muscle differentiation was induced only by culturing Ndnmabs and GFPmabs, as control, in muscle-differentiation medium (Fig 2A-B) and fusion index was calculated to compare their differentiation potential (Fig 2C): over-expressing necdin mesoangioblasts showed an higher fusion degree.

Increased differentiation potential of over-expressing necdin mesoangioblasts may depend on changed expression of myogenic regulatory factors (MRFs) and/or decreased cell death. To investigate the first hypothesis we first analyzed myogenic genes expression during a time course skeletal muscle differentiation. Myogenic markers are not expressed by proliferating cell, but they start to be activated when skeletal muscle differentiation is induced.

GFPmabs and Ndnmabs were cultured for six days in differentiating medium (DMEM with 2%HS). Protein extracts from cells was collected two (T2), three (T3), five (T5) and six (T6) days after differentiation to perform a Western Blotting analysis (Fig 2 D). The increased fusion index we observed in over-expressing Necdin mesoangioblasts was accompanied by increased expression of MyHC and Myogenin proteins (Fig 2E).

Necdin protects mesoangiobalsts from cell death

An important aspect of damaged muscle is that it originates a pro-apoptotic microenvironment in which cytokines such as TNFα oxidative stress and immune-competent cells play a role, and that may contribute the the limited effect of myogenic cells transplantation.

Our previous studies revealed that necdin acts as a pro-survival factor in muscle cells. In particular, myogenic precursor cells from both necdin-over expressing MlcNec2 mice and C2C12 cells were protected from apoptosis induced by three different stimuli (radiation, hydrogen peroxide, and staurosporine), whereas cell death of Ndn−/− myoblasts was greatly increased, indicating that necdin acts as an anti-apoptotic agent.

In view of these results, we studied whether Necdin has the same role also on mesoangioblasts cells. We performed a cell death experiment in which we treated cells with a cytotoxic stimulus, arsenic trioxide (As_2O_3). GFPmabs and Ndnmabs were exposed for 12 hours to As_2O_3 (20 μM). After treatment, we evaluated cell death by measuring Propidium Iodide (PI) incorporation by FACS (Fig 2F). We can say that over-expressing Necdin mesoangioblasts are more resistant to cell death.

Necdin promotes mesoangioblasts mediated muscle reconstitution in vivo.

Given the promising results obtained *in vitro*, we decided to move to *in vivo* analysis, to check if Necdin could optimize mesoangioblasts contribution to muscle repair in a murine model of muscular dystrophy, α-sarcoglycan (α-SG) null mice.

As a preliminary experiment, we performed intra-muscular injections of GFPMabs mesoangioblasts and NdnMabs mesoangioblasts in dystrophic mice. We treated 3 mice with each cell line, and we injected four muscle for mouse (right and left anterior tibialis, right and left quadriceps). We injected 5 x 10^5 cells (50 μl of physiological solution) for muscle.

Ten and twenty days after injections, mice were sacrified and muscles were collected to check if mesoangioblasts were able to survive and give their contribution to muscle regeneration: some muscles were used to have RNA extract and the other ones to perform immunofluorescence analysis. In both the experiments, we measured survival level of mesoangioblasts by testing the presence of GFP protein (Fig 4A), while we determined their ability to regenerate new muscle fibers by evaluating restoration of a-SG expression (Fig 4B). We normalized our results with those obtained with intra-muscular injections of D16 mesoangioblasts, a control mesoangioblasts line already characterized in our lab.

As shown in the charts, after ten days from injection, over-expressing Necdin mesoangioblasts showed a greater GFP expression, marker of an higher survival level of these cells. This date fits with the *in vitro* one, in which Necdin conferred an higher resistance to cell death to mesoangioblasts. More interestingly, while muscle injected with GFPMABs exhibited a modest α-SG expression, comparable with injections with D16 line, muscles treated with NdnMABs showed an outstanding level of α-SG expression. This result is very encouraging, because it means that Necdin enhances mesoangioblasts ability to restore dystrophic phenotype. Anyway, twenty days after injection, both GFP and α-SG expression decreased also in muscle treated with over-expressing necdin mesoangioblasts, probably because cells could not survive so long. For this reason, we will try to optimize efficacy of cell injection, by performing multiple intra-muscular injections, three for each mouse.

Results obtained with RT-PCR analysis was then confirmed by immunofluorescence analysis on muscle sections. We performed immunofluorescence staining for GFP and α-SG on cryo-sections of tibialis muscle of α-SG KO mice treated with intra-muscular injections of GFPMabs (Fig 4C) and NdnMabs (Fig 4D). GFP expression was higher in muscles from α-SG mice treated with NdnMABs, that means that these cells could survive more that the other ones . More interestingly, only sections from mice treated with NdnMabs showed α-SG positive fibers (Fig 4M-O), that

means that over-expressing Necdin mesoangioblasts are more efficient to contribute to muscle regeneration.

DISCUSSION

In this work we demonstrate that the efficacy of mesoangioblasts stem cells to reconstitute dystrophic muscle is improved by the MAGE protein Necdin. In particular, when Necdin is over-expressed, mesoangioblasts differentiation and survival is enhanced both in vitro and in vivo. This may be a powerful tool to optimize stem cell therapy protocols. Indeed, even if they have been shown to give a significant contribution to restore muscle structure and function in mouse and dog models of muscular dystrophy [1, 14], their effect is still modest. Since we already showed that Necdin has an important role in skeletal muscle differentiation and maintenance by satellite-derived myoblasts [6], we thought we could take advantage of these properties to developed a new strategy to improve mesoangioblasts therapeutic potential.

Mesoangioblasts usually don't express detectable level of Necdin, but when it is over-expressed together with the homeobox transcription factor Msx2 it promotes their smooth muscle differentiation [8]. In order to investigate a possible role of Necdin in mesoangioblasts skeletal muscle differentiation, we constitutively over-expressed it in mesoangioblasts from adult muscle of C57 mouse. In contrast to their embryonic counterpart, these cells are able to spontaneously differentiate in skeletal muscle, even if their efficacy is partial. Anyway, we found that in presence of a constitutive over-expression of Necdin, mesoangioblasts show an higher fusion rate in vitro. This suggests that Necdin is required to increase their differentiation potential by promoting new myofibers growth and fusion. To understand the molecular mechanisms that regulate this process, we first investigated if Necdin could act on transcriptional pathways, as we already demonstrated for satellite-derived myoblasts. The gain-of-function effect in vitro in myoblast cultures correlates with an increased fusion index and with changes in the expression of different myogenic markers, including myogenin and sarcomeric myosin. In particular, Necdin acts through a transcriptional regulation of myogenin, in cooperation with MyoD. In addition, when muscle regeneration is

induced in adult muscle after acute injury the reconstitution of new fibers is accelerated. But in the case of mesoangioblasts, when skeletal muscle differentiation is induced, over-expression of Necdin doesn't affect transcriptional levels of myogenic markers, even if Western Blot analysis demonstrate an higher level of myogenic proteins respect to wild type cells. Maybe some post-transcriptional mechanisms are altered, like protein degradation pathways.

Given this result about a transcriptional role of Necdin, we hypothesized that the enhanced differentiation of over-expressing Necdin mesoangioblasts could be due to an higher survival ability of these cells. Indeed, Necdin has been described also as pro-apoptotic protein, both in neurons and in satellite cells. In particular, we demonstrated that Necdin interacts both with caspase-3 and caspase-9 pathways in satellite cells. Since the reduced ability of mesoangioblasts to resist the cytotoxic environment existing in the damaged muscle is one of the main reasons for their partial effect, it would be of great interest if Necdin could act as pro-survival factor also in this cells. To investigate this aspect, mesoangioblasts were exposed to a cytotoxic stimulus, As_2O_3. We found that Necdin protects mesoangioblasts from cell death. We are now further investigate this aspect, to clarify what are the mechanisms that drive this observation, in order to take advantage of this property to improve their clinical application. Indeed, as already said, the latest goal of mesoangioblasts is to restore the dystrophic phenotype in vivo. We found that, when Necdin is over-expressed, mesoangioblasts delivered in α-sarcoglycan null mice are able to better resist to the inflammatory environment and, more interestingly, provide a greater contribution to muscle repair, as demonstrated by the restoration of α-sarcoglycan protein. Next step will be to deliver mesoangioblasts intra-arterially in the same murine model, to allow them migrate to several dystrophic muscles and make their efficacy more powerful.

We think our results are very encouraging and open a new way to manipulate mesoangioblasts fate.

In conclusion, we proved that Necdin plays an active role in growth and differentiation of mesoangioblasts, as well as in their ability to contribute to skeletal muscle reconstitution. Detailed

studies about its way of action in this cell system are needed in view of the chance to further enhance the benefic effect of Necdin on therapeutic potential of mesoangioblasts.

Acknoledgements

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Figure 1

(A-E) Mesoangioblasts were transduced with lentiviral vector pRLL-CMV-Necdin-Ires-EGFP to over-express Necdin constitutively (NdnMABs) (A, C) and with lentiviral vector pRLL-CMV-EGFP (GFPMABs) (B, D) as control. After 24h, transduction efficacy was evaluated by GFP expression by fluorescence microscopy. Necdin over-expression was tested by qPCR (E): NdnMABs express more than two thousand fold than wild-type mesoangioblasts.

(G-H) FACS analysis of GFPMABs and NdnMABs using SCA1 and CD34 antibodies.

(I-L) Smooth muscle differentiation was induced by treating mesoangioblasts with 5ng/ml of TGFb. Immunofluorescence with a specific antibody against aSMA was performed on GFPMABs (I) and NdnMABs (L) to quantify differentiation rate. Percentage of differentiated cells was calculated as number of positive cells to the staining. Results were expessed as mean + SEM of three independent exprerients.

(M-P) Osteogenic differentiation as induced by treating mesoangioblasts with BMP2. Alkaline Phosphatase staining was peformed on GFPMABs and NdnMABs to quantify differentiation rate. Percentage of differentiated cells was calculated as number of positive cells to the staining.

Figure 2

(A-B) Skeletal muscle differentiation assay. GFPMABs (A) and NdnMABs (B) were cultured for six days in differentiating medium (DMEM with 2% Horse Serum). Immunofluorescence was peformed with specific antibodies (MF20, red; GFP, green). Nuclei were stained with HOECHST.

(C) Fusion index was determined by counting the number of nuclei in myotubes: over-expressing mesoangioblasts showed an higher number of cells fused to myofibers. Results were expressed as the mean + SEM of three independent experiments.

(D-E) time course skeletal muscle differentiation. Western Blotting as performed at two (T2), four (T4) and six (T6) days after inducing skeletal muscle differentiation with specific antibodies for

Myosin Heavy Chain (MHC) and Myogenin. Results were compared with those obtained in proliferating cells (T0). MHC and Myogenin expressions were normalized with GAPDH expression.

(F) Cell death assay. Cell death of GFPMABs and NdnMABs was induced by a cytotoxic stimulus, As2O3 (20mM). Percentage of dead cells was evaluated 24h after treatment by measuring Propidium Iodide (PI) incorporation by FACS analysis.

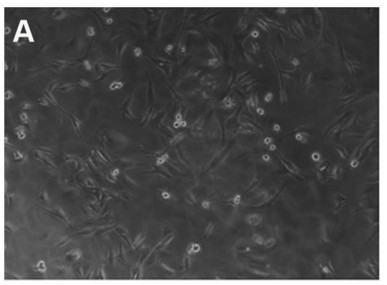
Figure 3

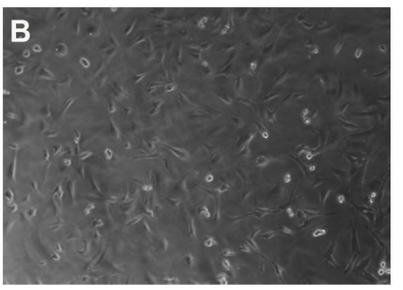
(A-O) In vivo assay. GFP (A) and a-SG (B) expression in a-SG null mice treated with mesoangioblasts intra-muscular delivery evaluated by qPCR. D16 = mesoangioblasts control line; WT NT = negative (for GFP expression) and positive (for a-SG expression) control mouse. 10d and 20d are the days after which mice were sacrified. The values indicated in the chart are the results of a mean between the results obtained from all the analyzed muscle.

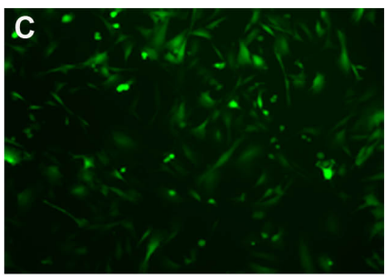
Immunofluorescence with specific antibodies against GFP (green) and Laminin (violet) on sections of tibialis of a-SG null mice treated with intra-muscular delivery of GFPMABs (C) and NdnMABs (D). Nuclei were stained with HOECHST.

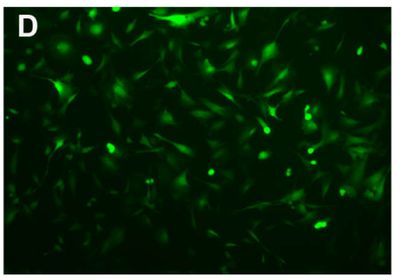
Immunofluorescence with a specific antibodies for a-SG (red) and Laminin (green) on sections of tibialis of a-SG null mice treated with intra-muscular delivery of GFPMABs (H-I) and NdnMABs (M-N). Nuclei were stained with HOECHST. Results were compared with immunofluorescence on tibialis of wt mouse (E-F). G, L, O are merges of a-SG and Laminin stainings.

Figure 1









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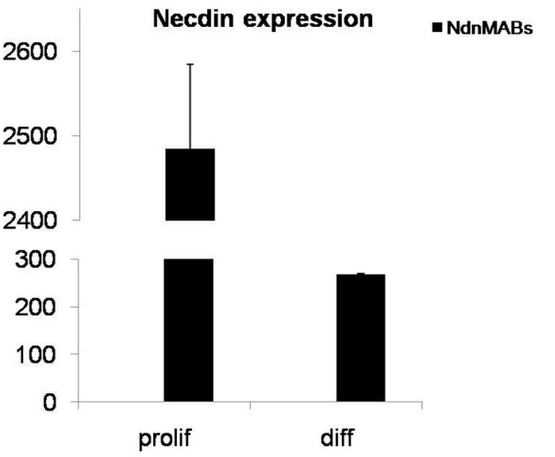
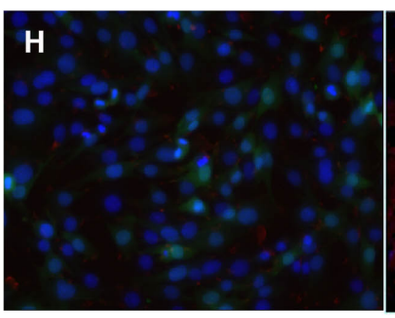
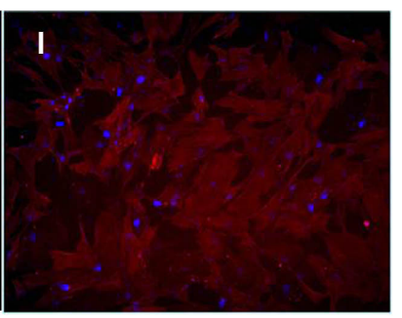
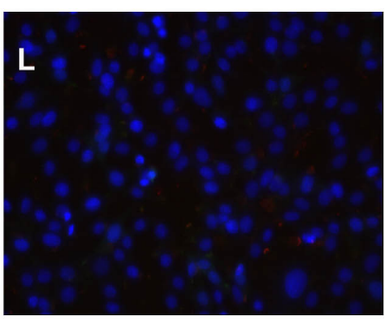


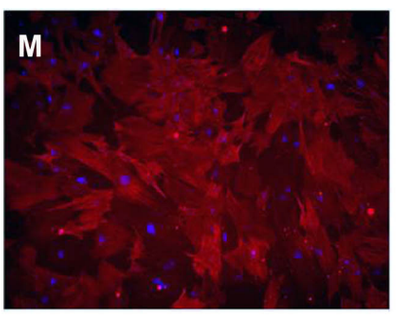
F

	SCA1	Ve-CAD	CD34	PDGFR	NG2	CD45
GFPMABs	+	+	+	+	+	-
NdnMABs	+	+	+	+	+	-









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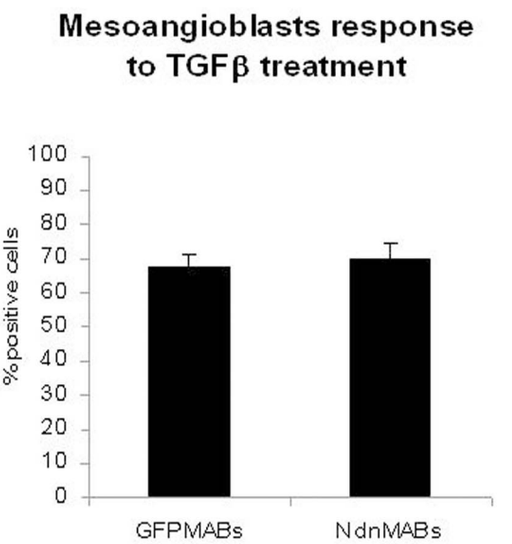
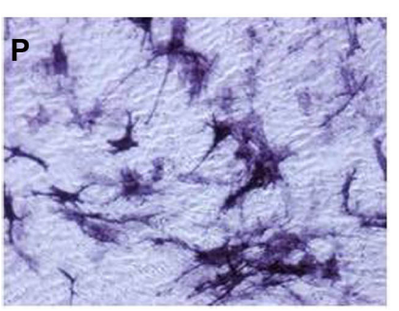
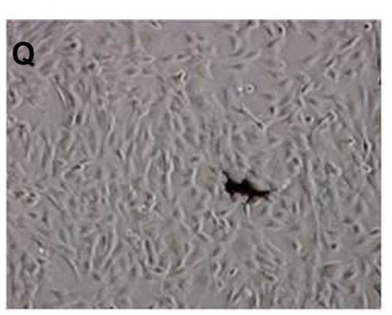
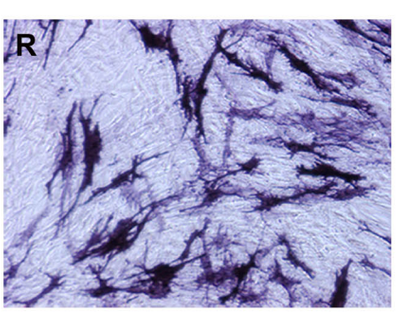








S

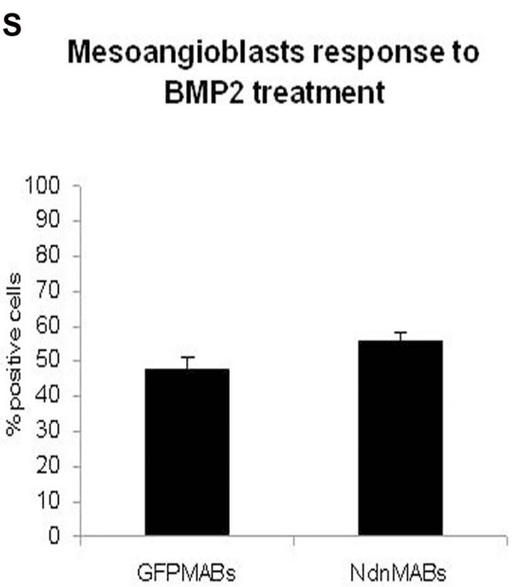
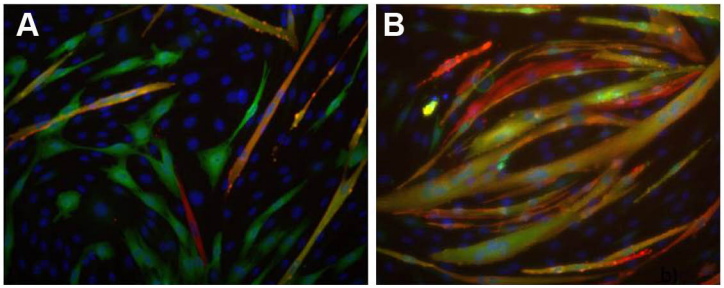
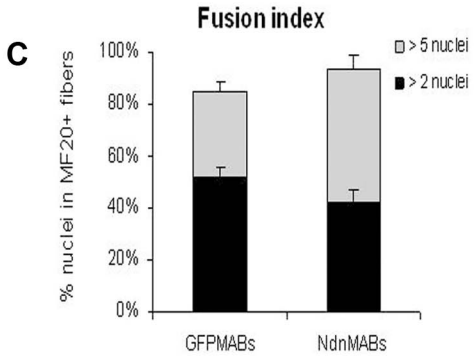
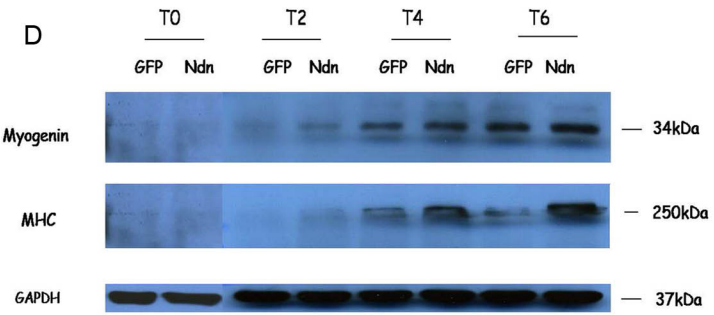
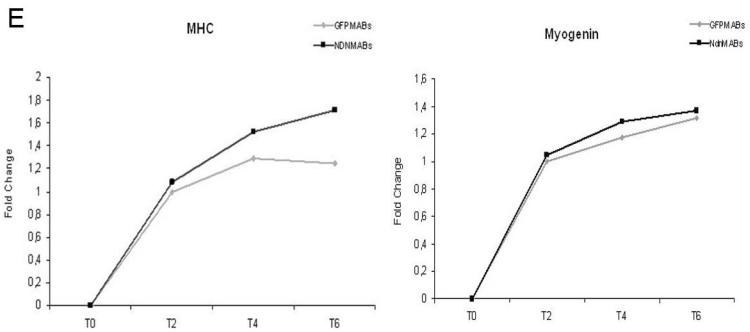


Figure 2


A
B


C
Fusion index
% nuclei in MF20+ fibers
100%
80%
60%
40%
20%
0%
> 5 nuclei
> 2 nuclei
GFPMABs
NdnMABs


D
T0
T2
T4
T6
GFP Ndn
GFP Ndn
GFP Ndn
GFP Ndn
Myogenin
MHC
GAPDH
34kDa
250kDa
37kDa


E
MHC
GFPMABs
NDNMABs
Fold Change
T0
T2
T4
T6
Myogenin
GFPMABs
NdnMABs
Fold Change
T0
T2
T4
T6

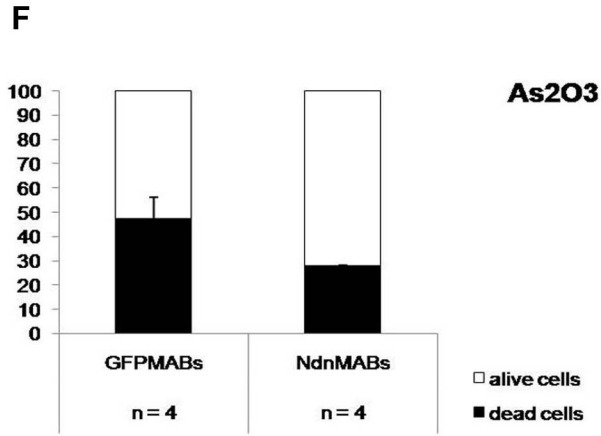
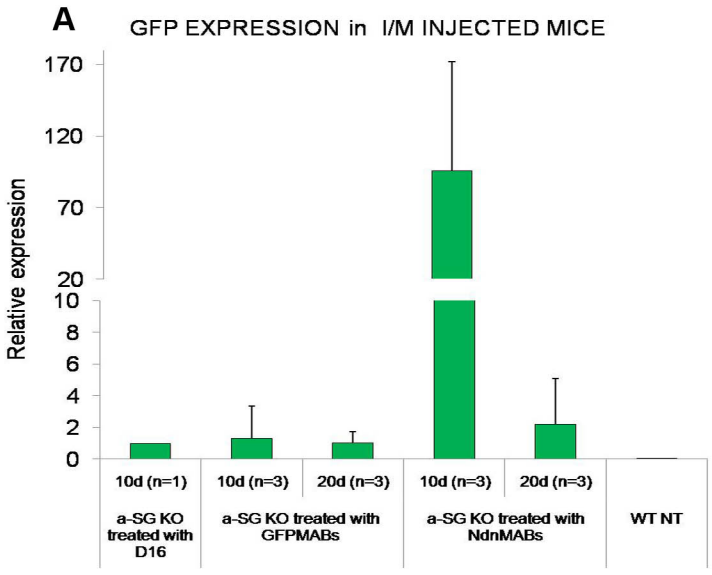
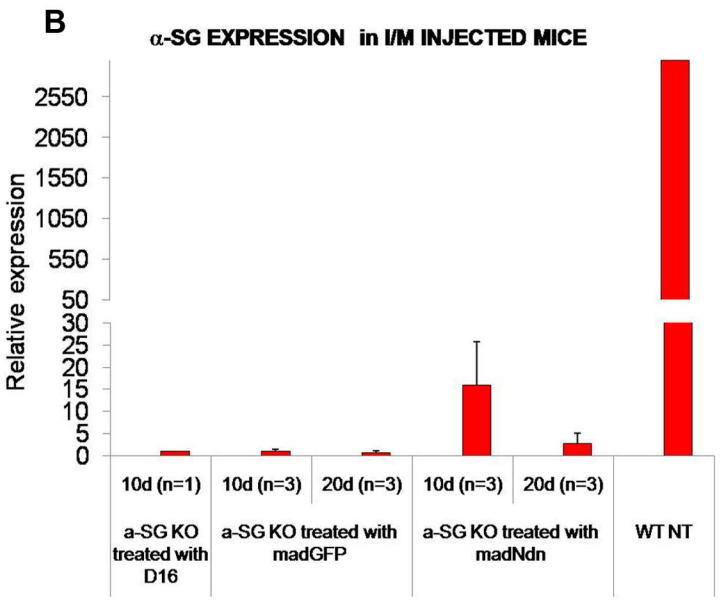
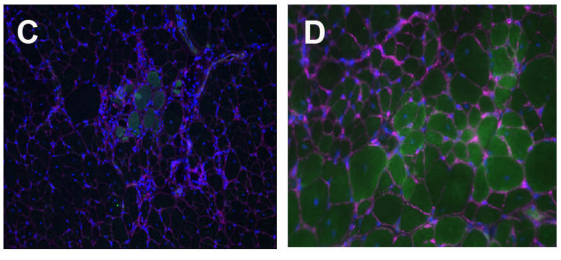
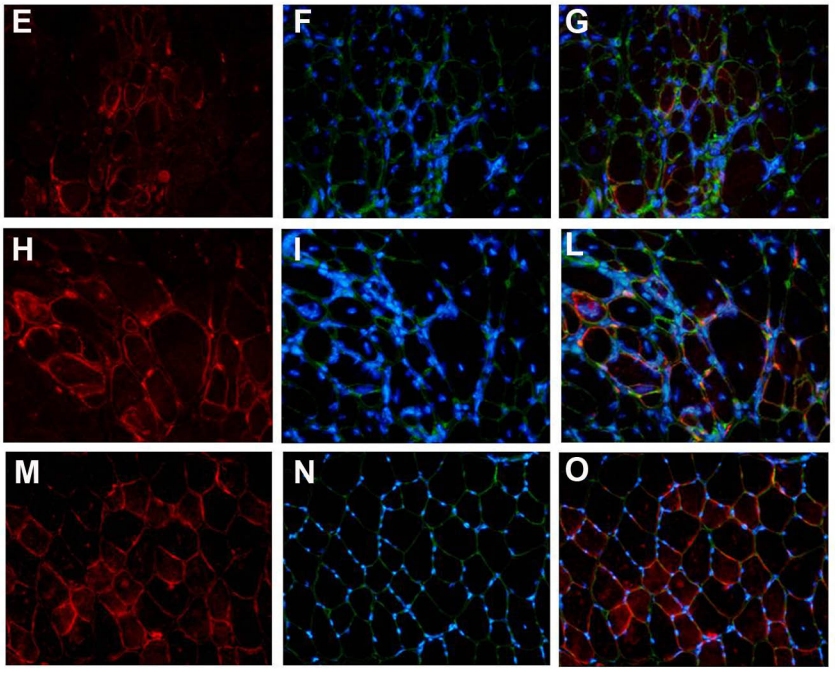

F
As2O3
100
90
80
70
60
50
40
30
20
10
0
GFPMABs
n = 4
NdnMABs
n = 4
alive cells
dead cells

Figure 3


A
GFP EXPRESSION in I/M INJECTED MICE
Relative expression
170
120
70
20
10
8
6
4
2
0
10d (n=1)
10d (n=3)
20d (n=3)
10d (n=3)
20d (n=3)
a-SG KO treated with D16
a-SG KO treated with GFPMABs
a-SG KO treated with NdnMABs
WT NT


B
α-SG EXPRESSION in I/M INJECTED MICE
Relative expression
2550
2050
1550
1050
550
50
30
25
20
15
10
5
0
10d (n=1)
10d (n=3)
20d (n=3)
10d (n=3)
20d (n=3)
a-SG KO treated with D16
a-SG KO treated with madGFP
a-SG KO treated with madNdn
WT NT


C
D


E
F
G
H
I
L
M
N
O

Necdin is expressed in cachectic skeletal muscle to physiologically protect fibers from tumor-induced wasting

Clara Sciorati[1], Thierry Touvier[1], Roberta Buono[1], Patrizia Pessina[2], Stephanie François[2], Cristiana Perrotta[1], Raffaella Meneveri[2], Emilio Clementi[3,4] and Silvia Brunelli[1,2]

[1]Stem Cell Research Institute, DIBIT-HSR, 20132 Milan, Italy; [2]Dept. of Experimental Medicine, University of Milano-Bicocca, 20052 Monza, Italy. [3]Dept. of Preclinical Sciences, LITA-Vialba, University of Milano, 20157 Milan, Italy; [4]E. Medea Scientific Institute, 23842 Bosisio Parini, Italy.

Corresponding authors:

Silvia Brunelli, DIMS-University of Milano-Bicocca and SCRI, DIBIT-HSR, via Olgetttina 58, 20132 Milano. Tel.+390226435066, Fax. +39-0226434621, brunelli.silvia@hsr.it

Emilio Clementi, Dept. of Preclinical Sciences, LITA-Vialba, University of Milano, and E. Medea Scientific Institute, via GB Grassi 74, 20157 Milan, Italy; Tel. +390250319683; Fax: +390250319682, emilio.clementi@unimi.it.

Running title: Necdin protects muscles from tumor-induced wasting

Keywords: necdin, cachexia, muscle regeneration, TNF-α

Abstract

Skeletal muscles of subjects with advanced cancer undergo progressive wasting, referred to as cachexia. Cachexia is an unmet medical need: strategies proposed until now have yielded little benefit. We have recently identified necdin as a key player in physiological myogenesis and muscle regeneration. Here we show that necdin is selectively expressed in muscles of cachetic mice and formally prove that its expression is causally linked to a protective response of the tissue against tumor-induced wasting, inhibition of myogenic differentiation and fiber regeneration. Necdin plays this role mainly via interference with TNF-α signaling at various levels, including regulation of expression of TNFR1, of p53 and of the activity of caspase 3 and 9. The data suggest that inhibition of muscle wasting via necdin is a feasible approach to treat cachexia in neoplastic patients.

INTRODUCTION

Cachexia consists of marked muscle wasting and atrophy associated with tumor load (Morley et al., 2006; Tisdale, 2002). This condition lowers responsiveness to treatment, contributing to poor prognosis and quality of life.

Several mediators of muscle wasting have been identified. These include immune and tumor-derived cytokines such as tumor necrosis factor (TNF-α), interleukin (IL)-1, IFNγ, and IL-6 (Argiles et al., 2006); in addition angiotensin-mediated activation of caspases and p53 transcription factors have all been shown to play a role (Tisdale, 2005). Most of these pathways mediate their effects by reducing the rate of protein synthesis at the level of protein translation or RNA content and by stimulating protein catabolism through the activation of the ubiquitin-proteasome pathway accompanied by induction of the ubiquitin E3 atrophy markers, muscle RING finger-1 (MuRF1) and muscle atrophy F-Box (MAFBx or atrogin-1) (Tisdale, 2005). The heterogeneity of these factors and their potential synergistic mode of action have made their targeting challenging and yielded little clinical benefit: therapeutic options against the cachectic syndrome are in fact still lacking.

A defective skeletal muscle regeneration also substantially contributes to muscle wasting (Coletti et al., 2005; Moresi et al., 2008). The key cells in muscle regeneration are satellite cells, that are activated and undergo myogenic differentiation and fusion with damaged muscle fibers or with themselves to produce new fibers (Charge and Rudnicki, 2004). A balance between cell proliferation, differentiation and fusion is required for the correct muscle regeneration to occur. The mechanisms leading to impaired muscle regeneration in cachexia have not been fully investigated.

We recently found that necdin, a member of the MAGE family of proteins, plays key

roles in regeneration (Brunelli et al., 2004; Deponti et al., 2007). Here we show that necdin is central to the strategy operated by the muscle to physiologically counteract tumor-induced muscle wasting and identify the mechanisms of necdin action that suggests it as a suitable target for therapeutic intervention in cachexia.

RESULTS

Necdin is a physiological inhibitor of colon carcinoma-induced muscle wasting

To investigate whether necdin plays a role in tumor induced muscle atrophy we initially measured its expression in muscles of cachectic mice. 2 months old wt mice, (F1C57xBalbC) were inoculated with 10^6 colon-26 carcinoma cells (C26), a well described model of cachexia inducing tumor cells (Tanaka et al., 1990). Muscles were collected 6, 8, 10, 12 days after the injection. Necdin expression, measured by Real Time PCR, was significantly upregulated during the first phases of cancer induced cachexia, followed by a decrease from day 10 onwards (Fig. 1A), suggesting that upregulation of necdin is a physiological response of muscle to wasting.

To verify this hypothesis, MlcNec mice overexpressing the protein in the muscle (Deponti et al., 2007), $Ndn^{-/-}$ (Muscatelli et al., 2000) and wt mice were inoculated with C26 cells as above. Treated mice underwent hypoglycemia and a substantial weight loss at least partially due to loss of skeletal muscle mass (see below) (Fig.1B,C). The lack of necdin in the muscle of $Ndn^{-/-}$ mice undergoing cachexia resulted in a substantial and significant increase of these events. In contrast, overexpression of necdin in MlcNec mice clearly protected against the systemic effects related to the growing tumor (Fig.1B,C).

The mean muscle weight in tumor-bearing $Ndn^{-/-}$ mice was significantly lower than in wt mice, while it was significantly higher in MlcNec mice. (Fig.1D). This was also true, though at a lesser extent, for the fat and liver weight (Fig.1D).

The muscles of $Ndn^{-/-}$ mice appeared less preserved and showed a lower density of fibers respect to wt and even more to MlcNec mice (Fig.2A). Reduction in muscle

weight was accompanied by a reduction of the fiber cross-section area, more marked in $Ndn^{-/-}$ mice than in wt and less severe in MlcNec mice (Fig.2B).

Changes in muscle weight and morphology were accompanied by decreased levels of the muscle specific proteins Myosin heavy chain, Myogenin and MyoD in C26-inoculated wt mice (Fig.2C, Suppl.Fig.1), consistent with the increased protein catabolism that occurs during tumor load (Acharyya et al., 2004; Langen et al., 2004; Tisdale, 2005) and associated with expression of atrogin1 and MuRF1, specific markers of muscle wasting (Fig.2D). In C26-inoculated $Ndn^{-/-}$ mice loss of muscle-specific proteins was increased, as increased were the levels of the atrogin1 and MuRF1 transcripts. Protein catabolism and expression of atrogin1 and MuRF1 were reduced in C26-inoculated MlcNec mice (Fig.2C,D, Suppl.Fig.1).

Real time PCR analysis showed expression in C26-inoculated mice of embryonic myosin, a molecular hallmark of fiber regeneration. Embryonic myosin levels were increased in MlcNec mice respect to wt and reduced in $Ndn^{-/-}$ mice (Fig.2D), indicating that regeneration occurs at some extent in all mice but that it is significantly enhanced when necdin is overexpressed.

Thus necdin overexpression in muscle is *per se* sufficient to counteract tumor-induced muscle wasting and its absence leads to an exacerbated phenotype.

The protective action of necdin is mediated through inhibition of TNFα dependent cachectogenic signaling

We next investigated the molecular mechanism underlying the effect of necdin in tumors. To this end we designed an *in vitro* approach mimicking the situation observed *in vivo*.

We exposed differentiating primary myoblasts (wt, MlcNec and $Ndn^{-/-}$) and C2C12 cells (transfected with either pIRESGFPNecdin or pIRESGFP plasmid) (Deponti et al., 2007) to the supernatant of confluent C26 cells, or 3T3 fibroblast as control. The C26-conditioned medium inhibited myogenic differentiation, decreased the expression of the differentiation markers MyoD, myogenin and MyHC (Fig.3A, Suppl.Fig.2A) and the number of MyHC-positive myotubes (Fig. 3B). This effect was counteracted by the overexpression of necdin in C2C12 and MlcNec myoblasts and enhanced by necdin ablation ($Ndn^{-/-}$ myoblasts).

TNF-α significantly contributes to cachexia (Zhou et al., 2003) (Coletti et al., 2005); in addition, low concentrations of TNF-α inhibit myogenic differentiation without causing apoptosis, which ensues at high concentration of the cytokine (Alter et al., 2008; Coletti et al., 2002; Moresi et al., 2008). Oxidative stress (Reactive Oxygen Species, ROS) accompanies and sustains the action of TNF-α (Barreiro et al., 2005; Li et al., 2003)

Myoblasts from MlcNec, $Ndn^{-/-}$ and wt mice were differentiated in the presence or absence of TNFα (5-20 ng/ml) or the ROS-generating As_2O_3 (2-5 μM). TNF-α inhibited myogenic differentiation and decreased expression of MyoD, Myogenin and MyHC. The effect of the cytokine was significantly enhanced in $Ndn^{-/-}$ cells (Fig.3B,C, Suppl.Fig2B) and inhibited in MlcNec cells. These results were confirmed in C2C12 cells where necdin overexpression overcame the inhibition of differentiation induced by TNF-α and As_2O_3 and maintained the expression of MyoD, Myogenin and MyHC (Fig. 3B,C, Suppl.Fig.2B).

We decided next to investigate how necdin interacts with the TNFα pathway. We examined whether the lower effect of TNFα in necdin-overexpressing cells was related to a differential expression of its p55kD receptor (TNFRI). The amount of

TNFRI transcript measured by Real Time PCR in myoblasts from the three mouse genotypes was comparable (Fig.4A). By contrast, the amount of TNFRI exposed at the plasma membrane of myoblasts cells measured by FACS analysis, was decreased in MlcNec cells, and increased in Ndn-/- mice with respect to wt (Fig.4B). The role of necdin was confirmed by overexpression of the protein in C2C12 (Fig.4B).

A key protein mediating the TNF-α-induced cachexia and its inhibition of myogenesis is p53 (Schwarzkopf et al., 2006). Exposure of myoblasts to TNF-α during differentiation increased the expression of p53 (Fig.4C, Suppl.Fig.2C). Overexpression of necdin in C2C12 and MlcNec myoblasts counterbalanced p53 overexpression, while significantly higher levels of p53 induced by TNF-α were observed in $Ndn^{-/-}$ myoblasts (Fig.4C, Suppl.Fig.2C). Higher expression of p53 transcripts was also observed in muscles of $Ndn^{-/-}$ mice respect to wt and a lower one in MlcNec (Fig. 4D). Of importance, we observed higher levels of p53 transcripts also in $Ndn^{-/-}$ untreated myoblasts (Fig. 4D) indicating that necdin acts on p53 also independently of its action on TNFRI.

TNF-α activates caspases that can mediate proteolysis of muscle proteins (Moresi et al., 2008). In both myoblasts and C2C12 exposure to TNF-α led to the activation of caspases 3 and 9. Overexpression of necdin significantly reduced this activation, while in its absence we observed an increased activation of caspases 3 and 9 (Fig.4C, Suppl.Fig.2C).

Thus, necdin protects skeletal muscle from tumor-induced cachexia by inhibiting the action of TNF-α; such inhibition occurs on the TNFα-activated cachectogenic signaling pathways at various levels.

Discussion

The aim of this study was to identify new candidate targets for cachexia, for which still no valid therapy has been proposed (Morley et al., 2006; Tisdale, 2006). Our results clearly indicate that such a candidate molecule is the MAGE protein necdin.

We previously demonstrated that *in vivo* necdin increases the ability of myoblasts to survive in presence of toxic stimuli and improves their myogenic differentiation, acting both at the transcriptional level, regulating the expression of myogenin, and by inhibiting apoptosis (Deponti et al., 2007). Now we show *in vivo* that necdin counteracts the muscle wasting and inhibition of differentiation specifically induced by the cachectogenic C26 cells. Necdin overexpression inhibited the drastic weight reduction, the loss of muscle mass and the decrease in cross section fiber area, and the reduction of the Myosin, Myogenin and MyoD protein levels. These effect of necdin were not an artifact due to its overexpression: necdin expression was physiologically upregulated in the muscle by the tumor *in vivo* and when such increase was prevented, such as in $Ndn^{-/-}$ mice, the cachectic phenotype was exacerbated. Thus, necdin expression is a response by muscles to tumor load of biological significance.

An important characteristic of necdin valuable in therapeutic perspective is that it exerts positive effects systemically even if overexpressed by skeletal muscle only. When we overexpress necdin in the muscle, it reduced the wasting effects of tumor also on epididymal fat and liver. It remains to be investigated whether necdin-expressing muscles maintain a higher metabolic state, resulting in reduced need for fat and liver energetic reserve, thus limiting wasting of these tissues, or act by releasing some anti-wasting factors for other tissues.

The beneficial effect of necdin appears to reside in the enhancement of both satellite cells resistance to tumor-generated toxic cues and their ability to differentiate. Indeed muscles of cachectic $Ndn^{-/-}$ mice were not only smaller, but appeared less preserved,

and showed a diminished expression of embryonic myosin, a molecular marker of regeneration.

The mechanism of the beneficial effect of necdin includes a specific action on the TNF-α- activated signaling, since necdin interferes with it at various levels. We found a decrease of TNFRI expression on the surface of necdin-overexpressing myoblasts, and an increase in $Ndn^{-/-}$ cells. Since the relative abundance of TNFRI mediates the extent of TNF-α signaling (Neznanov et al., 2001, Paland, 2008 #35), this may in part explain the protective effect of necdin. TNFRI belongs to the TNF/NGF death domain family and necdin has been described to bind to the death domain of p75NTR in endosomes and to modulate its signaling (Bronfman et al., 2003; Kuwako et al., 2005). Other proteins have been shown to interact with multiple death domains and mediate or inhibit signaling of different receptors (Yazidi-Belkoura et al., 2003). It is conceivable that necdin binds the death domain of TNFR1 to modulate TNF-α signalling pathway. Further investigation will be required to clarify this issue.

A second level of interaction between necdin and the TNF-α signaling occurs on the key downstream mediator of the effect of TNF-α, i.e. p53. Several studies suggest a role for p53 in regulating muscle homeostasis. Increased p53 levels are found in skeletal muscle during unloading-induced muscle atrophy, as well as in aging skeletal muscle (Chung and Ng, 2006; Siu et al., 2006). Importantly, tumor bearing p53-null mice are resistant to cachexia (Schwarzkopf et al., 2006). We have previously shown that necdin binds p53 (Deponti et al., 2007). We found that p53 transcript and protein levels were increased by TNF-α and that such increases were lower in necdin overexpressing myoblasts, and higher in $Ndn^{-/-}$ myoblasts, suggesting that necdin reduced the TNF-α dependent transcription/translation of p53, but may also influence its abundance or activity by directly binding it. Of importance, we also found that p53

transcript levels were regulated by necdin independently of TNF-α, since untreatd $Ndn^{-/-}$ myoblasts express higher basal levels of p53; thus regulation of this protein is not entirely a consequence of the effect of necdin on TNFRI expression. These results indicate that p53 is a major target of necdin and that necdin has a dual action on it, decreasing not only its activity but also its expression.

Important effectors downstream to p53 in skeletal muscle are caspases (Moresi et al., 2008). We detected an increased activation of caspases in $Ndn^{-/-}$ myoblasts treated with TNFα and the opposite in MlcNec mice. Consistent with such protective role of necdin is also the observation that this protein counteracts the deleterious role of ROS, that lead to reduced antioxidant gene expression associated with muscle catabolism (Li et al., 2003).

In conclusion, our data show that necdin is part of the biological response of muscle to tumor-induced cachexia *in vivo,* and that it acts through a multitargeted inhibition of a relevant cachectogenic pathway, that of TNFα. Muscle specific overexpression of necdin showed that this protein not only has a high degree of efficacy in preventing tumor-induced muscle wasting but exerts beneficial effects also on other tissues affected by the disease. We conclude that necdin can be considered a valid candidate molecule to design new approaches to cachexia.

Materials and Methods

Mice.

Necdin gain of funcion mice (MlcNec) and necdin loss of function mice $Ndn^{-/-}$ were described in (Deponti et al., 2007) and (Muscatelli et al., 2000). Male BALB/c x C57Bl/6 F_1 and BALB/c x MlcNec F_1 or BALB/c x $Ndn^{-/-}$ F_1 mice were used at the age of 6 weeks, maintained in a temperature-controlled room under a 12 h light/dark cycle and fed on water and food ad libitum. In the BALB/c x $Ndn^{-/-}$ F_1, male homozygote mice were used to obtain $Ndn^{-p/+m}$ offspring, that do not express necdin due to maternal imprinting of the gene (Deponti et al., 2007).

C26 colon carcinoma cells and injection.

Murine colon 26 adenocarcinoma cells (C26) (Tanaka et al., 1990) were cultured in DMEM containing 10% foetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. For in vivo inoculation a single suspension of cells, 10^6 cells in 100 μl of PBS, were injected subcutaneously into the inguinal flank of mice. The same volume of PBS were injected into the control groups.

Body weight measurements.

Mice body weight was measured the day of C26 cells injection and every 48h thereafter. The amount of food consumed by one mouse per cage was calculated from the weight of the food pellet that remains every 48h.

Glucose level measurements.

Serum glucose level was measured in both PBS and C26-treated animals 8 days after the injection using peripheral blood obtain by tail and employing the Ascensia Breeze 2 Glucose Monitoring System from Bayer (Mishawaka, IN USA).

Tumors and organs isolation.

Mice were sacrificed 6-8-10-12 days after cancer cells injection by cervical dislocation and gastrocnemius, tibialis anterior, quadriceps muscles collected and wighted. Liver, epididymal fat pads, and tumor were also isolated and weighed while wet at 12d. Tumor weights did not show any statistical significance between the mouse lines.

Real Time PCR analysis.

Total RNA from TA and gastrocnemius muscles dissected from PBS- or C26 injected mice (or myoblasts) were extracted using TRIzol protocol (Invitrogen, Carlsbad, CA). RNA (2 µg) was reverse-transcribed using SuperScript III First-strand synthesis system (Invitrogen) according to the manufacturer's instructions (Random Hexamers and dNTPs were from Invitrogen). Each cDNA sample was amplified in duplicate using the iQ SYBR Green SuperMix (Bio-Rad, Hercules, CA) on a real time PCR system (Mx3000P, Stratagene, La Jolla, CA). All results were normalized to levels of the GAPDH or 28S ribosomal RNA

GAPDH forward: 5′-TGAAGGTCGGTGTGAACGGATTTG-3′;

GAPDH reverse: 5′-CATGTGGGCCATGAGGTCCACCAC-3′;

NecdinF1: 5′-GTCCTGCTCTGATCCGAAGG-3′;

NecdinR1A: 5′-GGTCAACATCTTCTATCCGTTC-3′;

TNFR1aF, 5′ - TCAAAGAGGAGAAGGCTGGA-3′ ;

TNFR1R, 5′ - GCAGAGTGATTCGTAGAGCAGA-3′ ;

Atrogin1 forward, 5'- CAGAGAGGCAGATTCGCAAG-3′;;

Atrogin1 reverse, 5′-GGGAAAGTGAGACGGAGCA-3′;

MurF1 forward, 5′-GTTAAACCAGAGGTT CGG-3′;

MurF1 reverse 5′-ATGGTTCGCAACATTTCGG-3′;

p53 forward 5′-ACCTCACTGCATGGACGATCT-3′;

p53 reverse 5′-GACACTCGGAGGGCTTCACTT-3′;

embMHC forward 5′-TGAAGAAGGAGCAGGACACCAG-3′;

embMHC reverse 5′-CACTTGGAGTTTATCCACCAGATCC-3′;

Western Blot analysis

TA and gastrocnemius muscles dissected from PBS- or C26 injected mice or cells (primary myoblaswts and C2C12) were homogenized in 50 mM Tris/HCl, pH 7.4, 1 mM EGTA, 1 mM EDTA, 1% Triton X-100 or in 100 mM $NaHCO_3$, 1 mM EDTA, 2% sodium dodecyl sulfate and protease inhibitor cocktail (SIGMA) and centrifuged at 13,000 *g* for 5 min at 4°C to discard cellular debris.

Sample preparation and Western blot analyses were performed as described in (Deponti et al., 2007). After electrophoresis, polypeptides were transferred to nitrocellulose filters and antigens were revealed by incubation with primary antibodies and followed by the appropriate secondary antibodies. The antibodies used are the following: anti–glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mAb from Biogenesis, anti-MyoD mAb from DakoCytomation, anti-sarcomeric myosin MF20 and anti-myogenin from Developmental Studies Hybridoma Bank, anti-p53 antibody from Calbiochem, anti-activated caspase 3 pAb and anti-caspase 9 mAb (recognizing both activated and non-activated forms) from Cell Signaling.

Histology and Morphometric analysis

TA and gastrocnemius muscles were dissected from PBS- or C26 injected mice sacrificed 12 days after inoculation and frozen in liquid N_2–cooled isopentane. 8-μm serial muscle sections were either stained with haematoxyline/eosine (H&E) as described in (Deponti et al., 2007). Morphometric analyses were performed on sections of tibialis anterior and gastrocnemius using and the Image 1.63 program (Scion Corporation) to determine the cross sectional area. Ten sections and 300 fibers

were analyzed for each group.

Cell culture and tranfection.

C2C12 and fibroblast 3T3 cell line (3T3) cell lines were obtained from American Type Culture Collection and cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100μg/ml streptomycin (proliferation medium). C2C12 cells were differentiated in DMEM supplemented with 2% horse serum, 100 U/ml penicillin, and 100 μg/ml streptomycin (differentiation medium) as described in (Deponti et al., 2007).

C2C12 cells were transiently transfected with Lipofectamine Plus reagent (Invitrogen). as described in (Deponti et al., 2007), using pIRESEGFPNecdin plasmid or pIRESEGPF plasmid. Cells were grown for 24 h in proliferation medium and than incubated for additional 72 h in differentiation medium in presence of medium obtained from confluent culture of 3T3 or C26 cells (1:2 dilution), 5ng/ml of TNFα or 2μM of As_2O_3. The doses of TNFα or As_2O_3 were previously determined as the one resulting in no cell death (seen by Tunel assay, not shown). In the case of caspases or p53 experiments, the incubation in differentiation medium was reduced to 24 h.

Primary myoblasts culture.

Primary myoblasts from newborn mice of the different strains were isolated as described in (Deponti et al., 2007) and plated at clonal density using collagen-coated dishes. Cells were grown in proliferation medium (DMEM supplemented with 20% FBS, 3% chick embryo, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μg/ml gentamycin). To induce myotube formation cells were cultured in differentiation medium (DMEM supplemented with 2% horse serum, 100 U/ml penicillin, and 100 μg/ml streptomycin) for 24 h in presence of medium obtained from confluent culture of 3T3 or C26 cells (1:2 dilution), 20ng/ml of TNFα or 5μM of As_2O_3. The doses of

TNFα or As_2O_3 were previously determined as the one resulting in no cell death (seen by Tunel assay, not shown). In the case of caspases or p53 experiments, the incubation in differentiation medium was reduced to 16h.

Immunofluorescence

Immunofluorescence on cells were performed as in (Deponti et al., 2007) using the antibody specific for sarcomeric myosin MF20 (Developmental Studies Hybridoma Bank).

Flow cytometry

The cells were scraped of the petri dish and resuspended in PBS containing 1% bovine serum albumin and 0,05% NaN_3. Then, cells were stained at 4°C for 45 minutes with the TNF receptor 1 antibody (#19139, Abcam). After two washes with PBS, the cells were stained at 4°C for 30 minutes with an Alexa Fluor 488 dye-conjugated donkey anti-rabbit antibody (#A-21206, Molecular Probes). After three washes, analysis of the fluorescence was performed using a FACScan flow cytometer (Becton Dickinson). Cells stained with the secondary antibody alone were used as control.

Image acquisition and manipulation

Fluorescent and phase contrast images were taken on the Nikon microscope Eclipse E600, (lenses Plan Fuor: 10x/0.33, 20x/0.50). Images were acquired using the NIKON digital camera DXM1200, and the acquisition software NIKON ACT-1. The imaging medium was PBS buffer, images were taken at room temperature. Images and scanned films were assembled in panels using Adobe Photoshop 7.0. Images showing double fluorescence were separately acquired using the appropriate filters, and the different layers were then merged with Adobe Photoshop 7.0.

Statistical analysis

The results are expressed as means ± s.e.m.; *n* represents the number of individual experiments. Statistical analysis was carried out using the ANOVA test. Asterisks in the figure panels refer to statistical probabilities vs. wt controls respectively. Statistical probability values (*P*) of less than 0.05 were considered significant.

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FIGURE LEGENDS

Fig.1. Necdin inhibits colon carcinoma-induced muscle wasting

A) Necdin expression was measured by Real Time-PCR on TA muscle from PBS or C26-treated animals sacrificed 6, 8, 10 and 12 days after tumor injection. Five animals were analyzed in parallel for each time. Results were normalized to levels of the GAPDH RNA.

B**)** Body weight of both PBS- and C26- treated animals was measured the day of tumor injection and every two days. Ten animals were analyzed in parallel for each group: wild type mice (wt), MlcNec mice and necdin $Ndn^{-/-}$ mice. Results are expressed as % ± SEM of weight obtained for PBS-treated animals of each group (PBS) (n=10 per group). The mean weight ± SD of the PBS treated animals was 21.60± 0.42 g the day of injection and 24.95 ±0.68 g 10 days later.

C) Glycemia measurement. Serum glucose level of PBS- and C26-treated animals was measured eight days after tumor injection. Ten animal were analyzed in parallel for each group: wild type mice (WT), MlcNec mice and $Ndn^{-/-}$ mice. Results are expressed as % ± s.e.m. of the glycemia obtained for PBS-treated animals of each group (PBS). The mean glycemia level ± s.e.m. The glicemia level of PBS treated mice was 144.33 ±17.8 mg/dl, $P < 0.01$.

D) Muscles (TA, gastrocnemius, Gst, and quadriceps, Quad), liver and epydidimal fat (Ep.fat) were isolated from both PBS- and C26-treated animals 12 day after tumor injection and weighted. Ten animals were analyzed in parallel for each group: wild type mice (wt), MlcNec mice and $Ndn^{-/-}$ mice. Results are expressed as % ± SEM of the weight of PBS-treated animals for each group (PBS). The mean weigh ± SD for liver, epididymal fat pads, gastrocnemius, TA, quadriceps muscles of PBS treated

animals were respectively 1235,6 ±85,65, 385,5±77,8, 126,8±12,3, 54,5±7,1, 139,4± 5,7, 476,5±98,75 mg.

Fig.2 Necdin inhibits colon carcinoma-induced muscle protein catabolism

A) Hystology of tibialis anterior muscle**.** Rapresentative hystological images of H&E stained sections of TA muscles of mice sacrificed 12 days after PBS or tumor injection.

B) Distribution of cross sectional area of tibialis anterior fibers was analyzed on section obtained from PBS- (red, bleu and green lines) and C26- treated (yellow, light blue and light green lines) animals, 12 days after tumor injection (n= 5). Ten H&E stained sections and a total of 300 fibers were measured for different group (wt mice: red and yellow lines, MlcNec: blue and light blue lines, $Ndn^{-/-}$ mice: green and light green lines).

C) Expression of myosin heavy chain (MHC), Myogenin, MyoD or glyeraldehyde phosphate dehydrogenase (GAPDH) in TA of animals sacrificed 12 days after PBS or C26 tumor cells injection. Images are representative of 3 different experiments with muscles isolated from five animals for each group of wild type (WT), MlcNec or $Ndn^{-/-}$ mice. Quantification are shown in Supplementary Fig. 1B.

D) Embryonic myosin, (eMyHC), Atrogin1 (Atg1) and Murf1 expression was measured by Real Time PCR on TA muscle from C26-treated animals sacrificed 10 days after tumor injection. Five animals were analyzed in parallel for each group: wild type mice (wt), MlcNec mice and $Ndn^{-/-}$ mice. Results are normalized to levels of the GAPDH RNA and expressed as % of the wt.

Bar errors represent s.e.m. Single and double asterisks indicate $P < 0.01$ and $P < 0.001$ respectively vs. wt, single cross indicate $P < 0.05$ vs PBS.

Fig3. Necdin counteracts TNF-α and ROS induced inhibition of myogenic differentiation

A) Expression of myosin heavy chain (MHC), Myogenin (Myog.), MyoD or glyeraldehyde phosphate deidrogenase (GAPDH) in primary myoblasts isolated from wild type (WT), transgenic (MlcNec) or null ($Ndn^{-/-}$) newborn mice (myoblasts) or C2C12 cells transfected with a plasmid containing pIRES2-EGFP (EGFP) or pIRESEGF-necdin (EGFPNdn) (C2C12), and differentiated in presence of culture medium of the fibroblast 3T3 cell line (3T3) or the C26 colon carcinoma cells (C26). Images are representative of 3 reproducible experiments. Quantifications are shown in Supplementary Fig. 2A.

B) Expression of sarcomeric Myosin Heavy Chain by immunofluorescence on C2C12 cells transfected with pIRES2-EGFP (EGFP) or pIRESEGF-necdin (EGFPNdn) and differentiated in presence of culture medium of the fibroblast 3T3 cell line (3T3), C26 colon carcinoma cells, or of 5 ng/ml TNF-α (TNFα) or 2 μM of As_2O_3 (As_2O_3); nuclei are stained with Hoechst. Scale bar: 200 μm.

C) Expression of myosin heavy chain (MHC), Myogenin (Myog.), MyoD or glyeraldehyde phosphate deidrogenase (GAPDH) in primary myoblasts isolated from wild type (WT), transgenic (MlcNec) or null ($Ndn^{-/-}$) newborn mice (myoblasts) or C2C12 cells transfected with a plasmid containing pIRES2-EGFP (EGFP) or pIRESEGF-necdin (EGFPNdn) (C2C12), and differentiated in presence of TNFα (TNF) (20 ng/ml myoblasts, 5 ng/ml, C2C12) or of As_2O_3 (As_2O_3) (5 μM myoblasts, 2 μM C2C12). Images are representative of 3 reproducible experiments. Quantifications are shown in Supplementary Fig. 2B.

Fig.4 Necdin inhibits TNFα chachectogenic signalling at different levels

A) Expression of TNFR1 in primary myoblasts from wild type (wt), MlcNec or $Ndn^{-/-}$ newborn mice by Real Time PCR. Results are normalized to levels of the GAPDH RNA and expressed as % of the wt value (n=3)

B) Flow cytometry analysis of TNFR1 receptor in primary myoblasts (left panel) or trasfected C2C12 (right panel). Satellite cells, isolated from wild type (wt), transgenic (MlcNec) or null ($Ndn^{-/-}$) newborn mice, were analysed for TNFR1 expression (light blue, orange and light green line respectively). Controls were made using FITC conjugated secondary antibody alone (blue, red and green line dotted respectively) (RFI: MlcNec, 29.9 ± 0.4; wt 44.23 ± 1.1; $Ndn^{-/-}$ 55.7 ± 0.73 $P<0.001$ *vs*. wt, n=3). C2C12 was transiently transfected with a plasmid containing pIRES2-EGFP (EGFP) or pIRESEGF-necdin (EGFPNdn) and analysed for TNFR1 expression after gaiting of transfected cells (light blue and red line respectively). Control sample were made using secondary antibody alone (blue and orange respectively) (RFI: pIRESGFP, 19,4966 ± 1.15; pIRESEGFP-necdin, 9,136 ± 1.03, $P<0.001$ *vs.* pIRESEGFP, n=3)

C) Expression of p53 and activate caspases 3 and 9. Primary myoblasts (myoblasts) from wild type (wt), MlcNec or $Ndn^{-/-}$ newborn mice or C2C12 transiently transfected with a plasmid containing pIRES2-EGFP (EGFP) or pIRESEGF-necdin (EGFPNdn) were differentiated in absence (PBS) or in presence of 5-20 ng/ml of TNF-α (TNF). Cells were analyzed for p53 expression (p53) and for the activation of caspase 9 (Casp. 9, lower bands in the western blot images) and caspase 3 (Casp. 3). Images are representative of 3 reproducible experiments. Quantification are shown in Supplementary Fig. 2C.

D) p53 expression was measured by Real Time PCR in TA muscle from C26-treated wild type (wt), MlcNec and $Ndn^{-/-}$ mice sacrificed 6, 8, 10 and 12 days after tumor

injection (n=5) (left graph) or in primary myoblasts from wild type (wt), MlcNec or $Ndn^{/-}$ newborn mice (myoblasts) (n=3) (right graph). Results are normalized to levels of the GAPDH RNA and expressed as % of the wt value.

Bar errors represent s.e.m. Single and double asterisks indicate $P < 0.01$ and $P < 0.001$ respectively vs. wt.

Figure 1

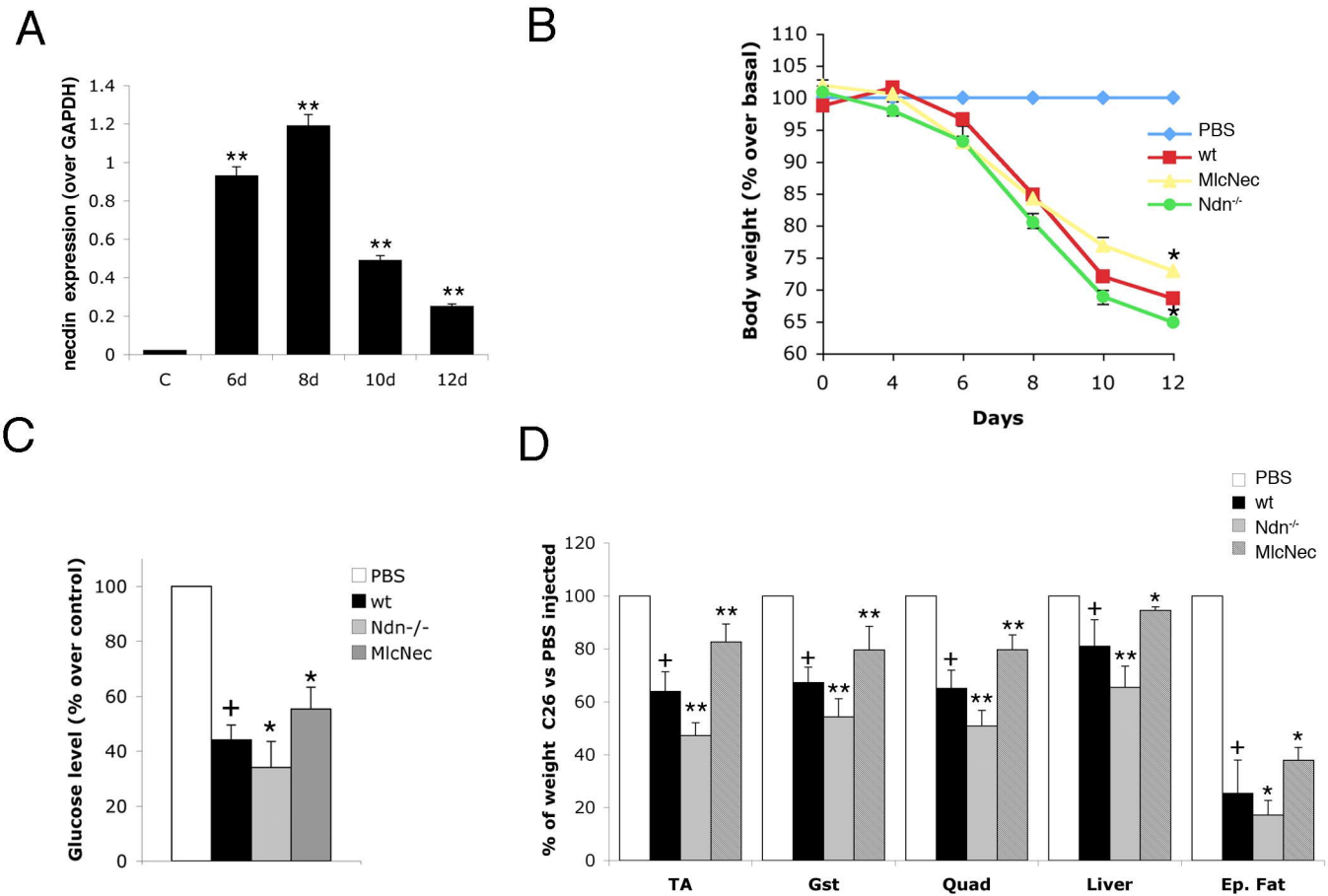

A
necdin expression (over GAPDH)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
C
6d
8d
10d
12d
B
Body weight (% over basal)
110
105
100
95
90
85
80
75
70
65
60
0
4
6
8
10
12
Days
PBS
wt
MlcNec
Ndn-/-
C
Glucose level (% over control)
100
80
60
40
20
0
PBS
wt
Ndn-/-
MlcNec
D
% of weight C26 vs PBS injected
120
100
80
60
40
20
0
TA
Gst
Quad
Liver
Ep. Fat
PBS
wt
Ndn-/-
MlcNec

Figure 2

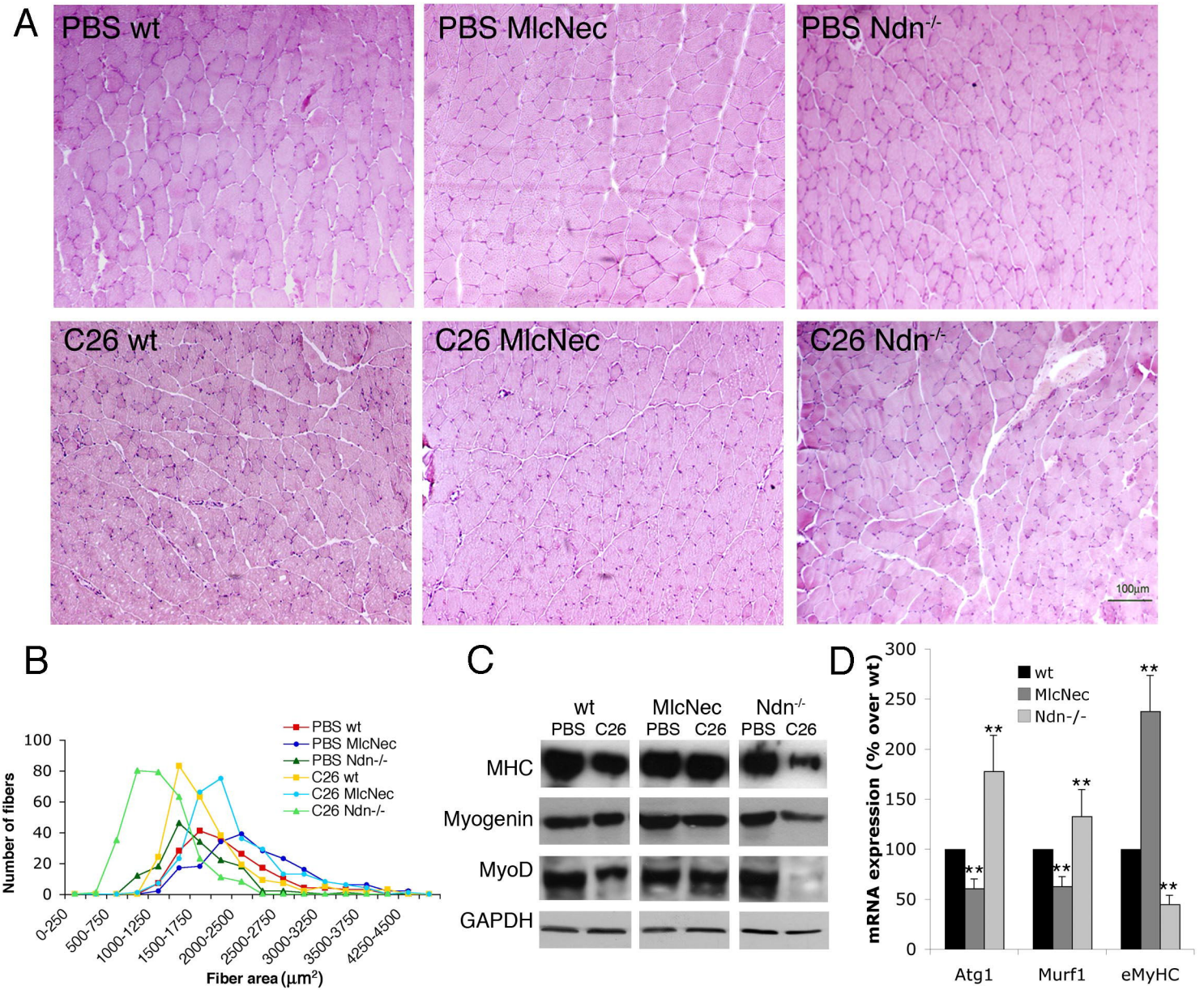

A
PBS wt
PBS MlcNec
PBS Ndn-/-
C26 wt
C26 MlcNec
C26 Ndn-/-
100µm
B
PBS wt
PBS MlcNec
PBS Ndn-/-
C26 wt
C26 MlcNec
C26 Ndn-/-
Number of fibers
0
20
40
60
80
100
0-250
500-750
1000-1250
1500-1750
2000-2500
2500-2750
3000-3250
3500-3750
4250-4500
Fiber area (µm²)
C
wt
MlcNec
Ndn-/-
PBS C26 PBS C26 PBS C26
MHC
Myogenin
MyoD
GAPDH
D
wt
MlcNec
Ndn-/-
mRNA expression (% over wt)
0
50
100
150
200
250
300
**
Atg1
Murf1
eMyHC

Figure 3

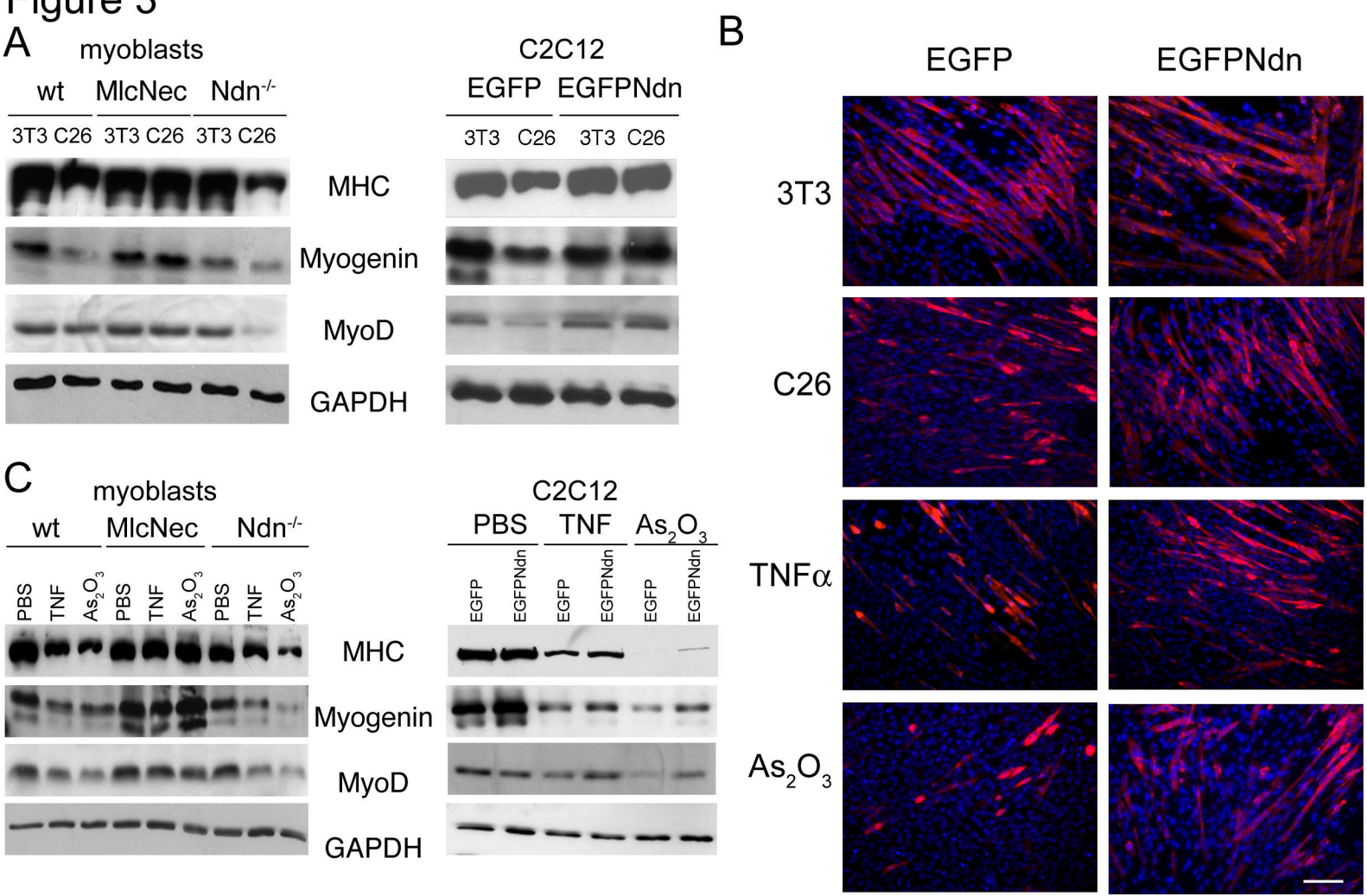
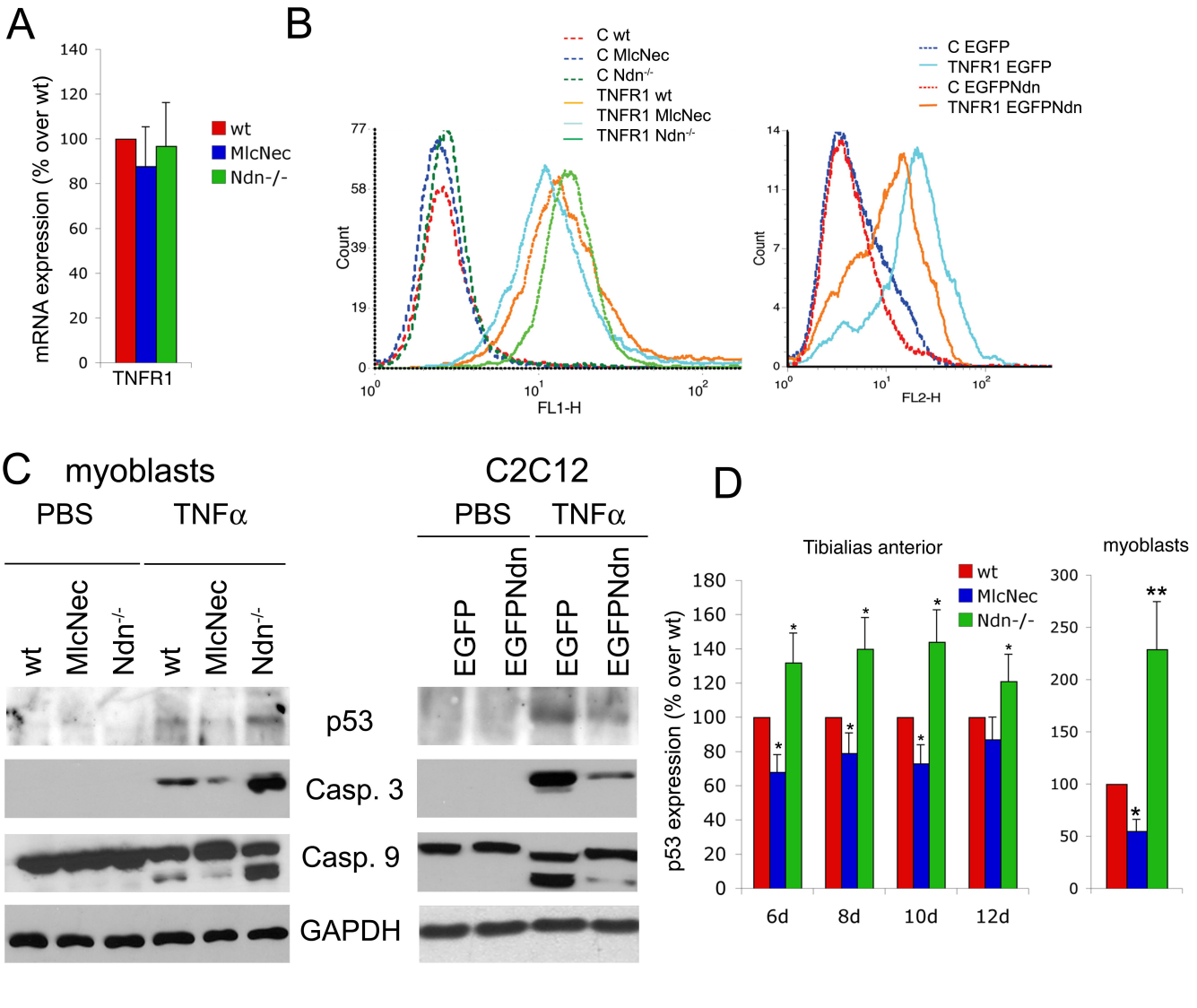

A
myoblasts
wt
MlcNec
Ndn-/-
3T3 C26 3T3 C26 3T3 C26
C2C12
EGFP EGFPNdn
3T3 C26 3T3 C26
MHC
Myogenin
MyoD
GAPDH
B
EGFP
EGFPNdn
3T3
C26
TNFα
As2O3
C
myoblasts
wt
MlcNec
Ndn-/-
PBS TNF As2O3 PBS TNF As2O3 PBS TNF As2O3
C2C12
PBS
TNF
As2O3
EGFP EGFPNdn EGFP EGFPNdn EGFP EGFPNdn
MHC
Myogenin
MyoD
GAPDH

Figure 4


A
mRNA expression (% over wt)
140
120
100
80
60
40
20
0
TNFR1
wt
MlcNec
Ndn-/-
B
C wt
C MlcNec
C Ndn-/-
TNFR1 wt
TNFR1 MlcNec
TNFR1 Ndn-/-
Count
77
58
39
19
0
10^0
10^1
10^2
FL1-H
C EGFP
TNFR1 EGFP
C EGFPNdn
TNFR1 EGFPNdn
Count
14
11
7
4
0
10^0
10^1
10^2
FL2-H
C myoblasts
PBS
TNFα
wt
MlcNec
Ndn-/-
wt
MlcNec
Ndn-/-
C2C12
PBS
TNFα
EGFP
EGFPNdn
EGFP
EGFPNdn
p53
Casp. 3
Casp. 9
GAPDH
D
Tibialias anterior
myoblasts
wt
MlcNec
Ndn-/-
p53 expression (% over wt)
180
160
140
120
100
80
60
40
20
0
300
250
200
150
100
50
0
6d
8d
10d
12d
*
**

Chapter 4

Discussion and future perspective

The aim of my PhD project was to study the role of the MAGE protein Necdin in increasing dystrophic muscle repair by mesoangioblasts stem cells. They are vessel-associated stem cells that have been shown to contribute to muscle repair and ameliorate the dystrophic phenotype of the alpha sarcoglycan (SG) null mouse and in dystrophic dogs when delivered intra−arterially [1, 2]. However, the capacity of mesoangioblasts to repopulate diseased muscles and restore their function still needs to be optimized. This task is complicated by the limited knowledge of the signals and the pathways that regulate their proliferation and commitment toward the myogenic lineage, as well as their survival and muscle homing upon transplantation. In particular, few information is available on their behavior when they reach the damaged muscle.

One of the main reasons for the partial effect of mesoangioblasts is likely to be ascribed to the limited ability of these cells to reach and colonize the muscle, and their recruitment depends on the generation of specific chemoattractants at the site of damage and the generation of signals, which favor stem cell survival and differentiation, such as HMGB1, TNF-α, vascular endothelial growth factor, and metalloproteinase 9 (MMP-9), molecules involved in the regulation of cell migration [3].

Nowadays, there are a lot of genes and molecules that are being investigating to improve mesoangioblasts activity. For instance, Nitric Oxide (NO) and TGFβ have been shown to be good candidate stem cell survival and the ability of these cells to resist to the inflammatory environment in the damaged muscle [4, 5]. We decided to build our

strategy to this intent on the MAGE protein Necdin. Necdin was first described as a protein expressed by post mitotic neurons, but subsequently it has been shown to be expressed also in developing and regenerating muscle, suggesting a critical role in this tissue. We provided the first evidence that Necdin is required for normal muscle growth and efficient muscle regeneration in vivo, by promoting satellite-derived myoblasts survival and accelerating their differentiation [6]. Indeed, Necdin acts at two different levels: it regulates myogenin transcription, in cooperation with MyoD and it protects myoblasts from cell death, probably by interacting with 53 activity.

We also recently showed in vivo that Necdin counteracts the muscle wasting and inhibition of differentiation specifically induced by cachexia, a pathology in which atrophy is associated with tumor load [7]. Necdin over-expression inhibited the drastic weight reduction, the loss of muscle mass and the decrease in cross-sectional fiber area, and the reduction of myosin, myogenin and MyoD protein levels. In this context, Necdin exerts its protecting effect by interfering with TNFα-activated signaling at various levels. First of all, it causes a reduction of TNFRI expression on the surface of myoblasts, probably binding its death receptor to modulate the extent of TNFα signaling. Secondary, it influences the activity and the expression of p53, not only by direct binding as previously shown, but also reducing its reduce TNFα-dependent transcription and translation. Muscle-specific over-expression of Necdin showed that this protein not only has a high degree of efficacy in

preventing tumor-induced muscle wasting, but also exerts beneficial effects on other tissues affected by the disease.

Given these previous results and since we found that Necdin, when it is over-expressed together with the homeobox transcription factor Msx2, promotes smooth muscle differentiation of mesoangioblasts [8], we wondered if it could play an active role also in skeletal muscle differentiation and regeneration by them. To test this hypothesis, we over-expressed Necdin under a constitutive lentiviral vector in mesoangioblasts from C57 adult mouse and we compared their behavior with wild type cells both in vitro and in vivo. Osteogenic and smooth muscle differentiation potential of mesoangioblasts is not affect by the presence of Necdin, as well as their phenotypic expression, as shown by microarray analysis. Anyway, when skeletal muscle differentiation is induced, over-expressing cells display an higher fusion index. This results is similar to that observed in satellite –derived myoblasts, but is more interesting if we think of the therapeutic application of mesoangioblasts. In order to understand the molecular mechanisms that regulate this process, we first investigated if Necdin could act on transcriptional pathways, as in myoblasts. The gain-of-function effect in vitro in myoblast cultures correlates with an increased fusion index and with changes in the expression of different myogenic markers, including myogenin and sarcomeric myosin. It doesn't happen in mesoangioblasts. Indeed, when skeletal muscle differentiation is induced, over-expression of Necdin doesn't affect transcriptional levels of myogenic markers, even

if these cells displays an higher level of myogenic proteins respect to wild type cells. A possible reason of the different behavior of Necdin in the two cell system may be its differentially cellular localization. Necdin was initially identified as a nuclear protein of post-mitotic neurons [9]; anyway it was found to be present also in the cytoplasm of these cells [10], as well as in muscle [8], and in brown adipocyte tissue [11]. Furthermore, it is demonstrated that a myelin-associated protein NogoA inhibits necdin-accelerated neuronal neurite outgrowth by sequestering necdin in the cytoplasm [12]. Here we provided the evidence that in wild-type satellite-derived myoblasts, Necdin is expressed in the cytoplasm, both in proliferation and in differentiation condition; anyway, cells from transgenic mice, express Necdin only in the cytoplasm when they are in proliferation stage, but when they are induced to differentiate it is expressed both from cytoplasm and the nucleus. Interestingly, over-expressing Necdin mesoangioblasts display a different pattern of expression: both in proliferation and in differentiation condition, Necdin is expressed only in the cytoplasm. This may explain why we don't appreciate any effect on transcription processes. It would be useful to target Necdin specifically to the nucleus, to let it act as transcriptional factor and investigate the effect on myogenic genes expression.

The other aspect that we investigated is a possible role of Necdin as pro-survival factor, as already demonstrated in neural cells [13-15] but also in muscle cells [6, 7]. It is known that the cytotoxic environment existing in the damaged muscle, that produces several pro-inflammatory and pro-

apoptotic stimuli, is one of the main reasons for partial effect of mesoangioblasts [5]. So it would be of great interest if Necdin could act as anti-apoptotic factor also in this cells. To this end, mesoangioblasts were exposed to a cytotoxic stimulus, As_2O_3. We found that Necdin protects mesoangioblasts from cell death. More studies will need to clarify what are the mechanisms underlying this observation, in order to take advantage of this property to improve their clinical application. We are focusing on the intrinsic death pathway. It is activated by intracellular events, factors such as DNA damage, resulting in mitochondrial-mediated cell death [16], where a cascade of events ultimately induce caspase 9 cleavage and activation; activated caspase 9 in turn activates caspase 3 [17]. Since we already shown in satellite-derived myoblasts that Necdin acts by limiting events in the common pathway to apoptosis converging on caspase 9 activation, we are investigating if it can interact with the apoptosis program also in mesoangioblasts.

Given the promising results obtained *in vitro*, we decided to move to *in vivo* analysis, to check if Necdin could also optimize mesoangioblasts contribution to muscle repair in a murine model of muscular dystrophy, α-sarcoglycan (α-SG) null mice. We delivered over-expressing Necdin mesoangioblasts intra-muscularly in the α-sarcoglycan null mouse and we compare their effect with that obtained with delivery of wild type cells. After ten days from the treatment, we found that Necdin confers an higher survival ability to mesoangioblasts, data that correlates with the one obtained with satellite-derived cells. But the most interestingly results

is that Necdin enhances mesoangioblasts ability to repair dystrophic phenotype, as showed by the higher restoration of α-sarcoglycan protein after treatment. This is a very encouraging result, in view of new possible strategy to improve mesoangioblasts activity and manipulate their fate. Nevertheless, intramuscular release of these cells leads to a partial rescue of dystrophic damage, localized to the area of the injection. For this reason, we are now delivering over-expressing Necdin and wild type mesoangioblasts intra-arterially, a more powerful protocol for mesoangioblasts treatment. Preliminary results demonstrated a positive effect of Necdin also in this system, even if there is a great variability among the samples. Moreover, we found a remarkable number of cells trapped inside filter organs, due to incomplete adhesion and extravasation. A possible strategy to limit this effect will be a pre-treatment of mesoangioblasts with cytokines and surface expression of certain adhesion molecules, that is already shown to dramatically improve their migration to the affected muscles [18].

In conclusion, we proved that Necdin plays an active role in growth and differentiation of mesoangioblasts, as well as in their ability to contribute to skeletal muscle reconstitution. It is not yet well understood what are the pathways involved in this process, but probably the effect of Necdin is mainly due to its pro-survival role. Anyway, detailed studies about its way of action are needed in view of the chance to further enhance the benefic effect of Necdin on therapeutic potential of stem cells. To this purpose, we are also investigating the signaling pathways leading to

enhanced satellite cells activation and proliferation and muscle regeneration in the transgenic mice Mlc2Nec, where Necdin is controlled by a muscle-specific promoter (Myosin Light Chain) in comparison with the wild type and Necdin null mice. We are focusing on two molecular partners of Necdin in myoblasts and during skeletal muscle differentiation, Cell Cycle and Apoptosis Regulator protein 1 (CCAR1/CARP1) and Sirtuin1 (Sirt1), not only to clarify what function has Necdin in these cells but also looking for a possible role of Necdin in stabilizing transcriptional complexes. CCAR1 is a perinuclear protein that we identified as Necdin interactor by using the Two Hybrid system in yeast; it mediates apoptosis signalling by diverse agents, leading to the activation of caspase 9 and 3, members of Jun N-terminal kinase (JNK) and p38 MAPK family of proteins. We were able also to identify p53 in the complex Necdin-CCAR1 and we postulated that this complex could be responsible of the anti apoptotic mechanism of action of Necdin, by blocking p53 and CCAR1 cell death induction (Francois et al., unpublished data).

Sirt1 is a mammalian nicotinamide adenine dinucleotide (NAD)-dependent histone deacetylase (HDAC) that down-regulates the acetylation levels of many regulatory proteins involved in energy homeostasis, DNA repair, cell survival, and lifespan extension [19]. In particular, it deacetylates p53 and represses its activity to protect various cells from DNA damage-induced apoptosis [20]. Recently, it was shown that necdin promotes p53 deacetylation by facilitating the interaction

between Sirt1 and p53 to suppress p53-dependent neuronal apoptosis [21]. Moreover, it is known that Sirt1 retards muscle differentiation [22], increases satellite cells proliferation [23] and controls the transcription of the PGC1a gene [24]. Now we are investigating if Necdin and Sirt1 interact also in muscle cells and, if so, if it alter Sirt1 activity or the expression of its target genes.

In satellite cells fine−tuned regulation of cell cycle is critical for activation in response to appropriate stimuli, for proliferation to increase the cell population and maintenance of the pool, and finally for return to quiescence, a reversible G0 phase. Since it was recently shown that Necdin promotes cell cycle exit of hematopoietic stem cells (HSCs) maintaining their quiescence [25] and restricts their proliferation [26], we are wondering if it may affect also satellite cells quiescence and/or proliferation. Actually, Necdin over-expression seems to increase the number of quiescent satellite cells, probably by up-regulating Pax7 expression. Getting more insight into the pathways of myogenic commitment and differentiation of satellite cells would be of great importance, not only in terms of a general understanding of the biology of muscle differentiation and homeostasis, but also to deepen our knowledge on the mechanisms controlling commitment and terminal differentiation of skeletal muscle cells during regeneration.

In parallel, in the next future we will be interested in further characterizing the involvement of Necdin in mesoangioblasts cell fate by analyzing two different models: the first one is the gain-of-function

mouse Mlc2Nec, in which it will be over-expressed only upon muscle differentiation. The second one is the loss-of-function model, Necdin null mouse. We are isolating mesoangioblasts from the two mouse models and we will characterize them both in vitro and in vivo. Preliminary data show that these cells retain stem cells properties, that is the expression of typical surface markers and the ability to take different mesodermic tissues fate. Next step will be to investigate whether muscle-specific expression or, on the contrary, the lack of expression of Necdin could anyway affect the contribution of mesoangioblasts to skeletal muscle regeneration. In this way, we will fully outline the role of Necdin in controlling mesoangioblasts stemness, with a particular focus on their skeletal muscle fate. This approach may lead us to clarify molecular mechanisms underlying their behavior in the perspective of making mesoangioblasts stem cells therapy successful for humans.

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Here I am. It seemed so close the day I begun my PhD and I'm already at the end. I think it was a great experience, with lights and shadows of course, but that made me definitively understand what is my way, even if I realized that it will be always climbing!

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