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Facoltà di Medicina e Chirurgia

Scuola di Dottorato in Scienze Biomediche Cliniche
e Sperimentali

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THE NEUROTROPHIN RECEPTOR p75NTR AND ITS LIGAND BDNF IN HUMAN SKELETAL MUSCLE: INVOLVEMENT IN MYOGENESIS AND MUSCLE REPAIR

Settore Disciplinare BIO/10

Emanuela COLOMBO

Relatore: Prof.ssa Paola VIANI

Correlatore: Dott.ssa Cinthia FARINA

Coordinatore: Prof. Enrico GINELLI

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1. INTRODUCTION

1.1 HUMAN SKELETAL MUSCLE TISSUE

1.1.1 Skeletal muscle structure

Skeletal muscle is one of the most highly organized structures, it is responsible for all voluntary movements and its structure is optimized for this function. Muscle is a unique tissue in that it arises from the fusion of mononuclear myoblasts to form a multinucleate syncytium. The entire muscle is surrounded by the epimysium, a dense layer of collagen fibers. The epymisium separates the muscle from other tissues and organs. The connective tissue fibers of the perimysium divide the skeletal muscle into a series of compartments, each containing a bundle of muscle fibers called fascicle. In addition to collagen and elastic fibers, the perimysium contains blood vessels and nerves that maintain blood flow and innervate the fascicles. Within a fascicle, the connective tissue of endomisyum surrounds the individual skeletal muscle fibers and interconnects adjacent muscle fibers. The sarcolemma, or cell membrane, surrounds the cytoplasm of muscle fibers, called sarcoplasm. The sarcolemma is constituted by a plasmatic membrane, a basal membrane (mucopolysaccarides, glycoproteins and collagen) and reticular fibers. Transverse tubules, or T tubules, are narrow tubes that are continuous with the sarcolemma and extend into the sarcoplasm at right angles to the cell surface. Inside the muscle fibers branches of T tubules encircle cylindrical structures called myofibrils. A myofibril is 1-2 μm in diameter and as long as the entire cells and is made up of contractile proteins. Each skeletal muscle fiber contains hundreds to thousands of myofibrils.

The major structural components of muscle fibers include:

1. the sarcomere, the unit of contraction. It is made up of actin, myosin, and associated proteins. These proteins directly convert chemical energy in the form of ATP to movement and force using molecular motors and a scaffold of networked thin filaments.
2. The membrane that overlies individual myofiber interacts directly with the underlying cortical cytoskeleton, the Z band, and the extracellular matrix to help transmit force.

3. Interwoven through the myofibers is a membrane-bound structure for the efficient delivery of calcium that regulates the timing of muscle contraction.
4. Myocyte nuclei are typically found in the periphery of the myocyte syncytium. Like the plasma membrane, underlying the nuclear membrane is a scaffold of proteins with both structural and signaling roles.

Myofibrils consist of bundles of myofilaments, protein filament composed primarily of action and myosin. The actin forms the bulk of thin filaments and the myosin forms thick filaments. Myofibrils, which can actively shorten, are responsible for skeletal muscle fiber contraction. At each end of the skeletal muscle fiber, the myofibrils are anchored to the inner surface of the sarcolemma. In turn, the outer surface of the sarcolemma is attached to collagen fibers of the tendon of the skeletal muscle. Then, when myofibrils contract, the entire cell shortens. Myofilaments are organized into repeating functional units called sarcomeres. Sarcomeres are the smallest functional units of the muscle fiber. A sarcomere contains (1) thick filaments, (2) thin filaments, (3) proteins that stabilize the positions of the thick and thin filaments and (4) proteins that regulate the interactions between thick and thin filaments.

Myosin is a hexamer with two heavy chains (220 kDa each) and four light chains (20–25 kDa each). The amino terminus of myosin is globular and the two pairs of light chains bind at the junction between the globular head and the carboxy-terminal region. Within the head domain are the sequences required for ATP hydrolysis and actin binding. Thick filaments assemble bidirectionally, so that heads from myosin orient in opposite directions. A typical native thick filament assembles to a length not greater than 1–1.5 μm. Thin filaments are made up of several different proteins, but filamentous actin is the major constituent. Monomeric, or G actin, is 43 kDa. Modulated by ionic strength and actin binding proteins, actin assembles to form a right-handed double helical filament (F actin). The pitch of the actin filament is 36 nm with seven actin monomers per strand. Along the grooves of the actin double helix are the thin filament regulatory proteins, troponins T, C, and I and tropomyosin. Tropomyosin is a rod-like molecule, and troponin T directly interacts with tropomyosin. Troponin C directly binds calcium providing calcium sensitivity to the actomyosin interaction.

Differences in the size, density, and distribution of thick filaments and thin filaments account for the banded appearance of each myofibril. There are dark bands (A bands) and light bands (I bands). The names of these bands are derived from anisotropic and

isotropic, which refer to the appearance of these bands when they are viewed under polarized light.

The thick filaments are located at the center of a sarcomere, in the A band, which is subdived in:

1.The M line. The central portion of each thick filament is connected to its neighbors by proteins of the M line. These dark-staining proteins help stabilize the positions of the thick filaments.

2.The H zone. Or H band, is a lighter region on either side of the M line. The H zone contains thick filaments but no thin filaments.

3.The zone of overlap. In the zone of overlap, thin filaments are situated between the thick filaments. In this region, each thin filament is surrounded by three thick filaments, and each thick filament is surrounded by six thin filaments.

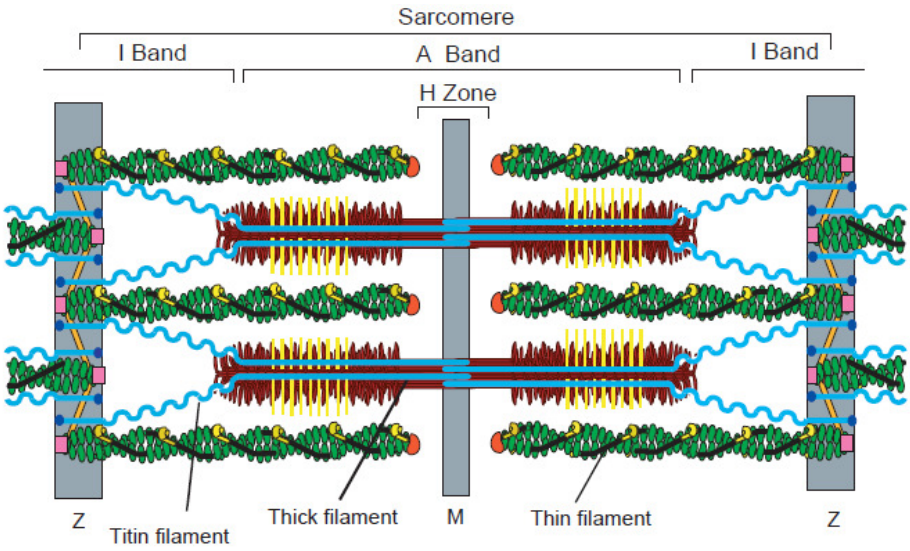


Figure 1.1. Major components of a muscle sarcomere. (Gregorio and Antin, trends in CELL BIOLOGY, 2000)

Each I band, contains thin filaments but not thick filaments, extends from the A band of one sarcomere to the A band of the next sarcomere. Z lines mark the boundary between adjacent sarcomeres. The Z lines consist of proteins called connectins, which interconnect thin filaments of adjacent sarcomeres. From the Z lines at either end of the sarcomere, thin filaments extend toward the M line and into the zone of overlap. Strands of the protein titin extend from the tips of the thick filaments to attachment sites at the Z line. Titin helps keep the thick and thin filaments in proper alignment; it also helps the muscle

fiber resist extreme stretching that would otherwise disrupt the contraction mechanism. In the sliding filament theory model, the heads of myosin protrude from the thick filaments and directly interact with actin thin filaments to increase the overlap of thick and thin filaments, reducing the I band and effectively shortening the sarcomere and the muscle fiber as a whole. Force is transmitted parallel to the long axis of muscle. As the Z band anchors thin filaments, force is also to some degree transmitted perpendicular to the long axis of myofibers.

Z bands anchor the sarcomere to the plasma membrane. Concentrated over the Z band at the plasma membrane is the dystrophin glycoprotein complex (DGC). The DGC is composed of extracellular, transmembrane and cytoplasmic proteins including sarcoglycans, dystroglycans, dystrophin, dystrobrevins, syntrophins, caveolins and calpains (Dalkilic and Kunkel, 2003). The major function of DGC is to link the extracellular matrix to the intracellular cytoskeleton, which provides mechanical stability to the muscle plasma membrane. Dystrophin, one of the largest genes known in human, bind filamentous actin in the muscle cytoskeleton and the transmembrane protein β-dystroglycan, which is linked to α-dystroglycan to form the dystroglycan subcomplex. The highly glycosylated α-dystroglycan binds laminin in the basal lamina (for review (Wallace and McNally, 2009)). In the absence of dystrophin, the transmembrane DGC elements are unstable and are reduced at the sarcolemma. Skeletal muscle membrane is abnormally susceptible to damage and myofibers rapidly lose peak force with rapid, successive contraction.

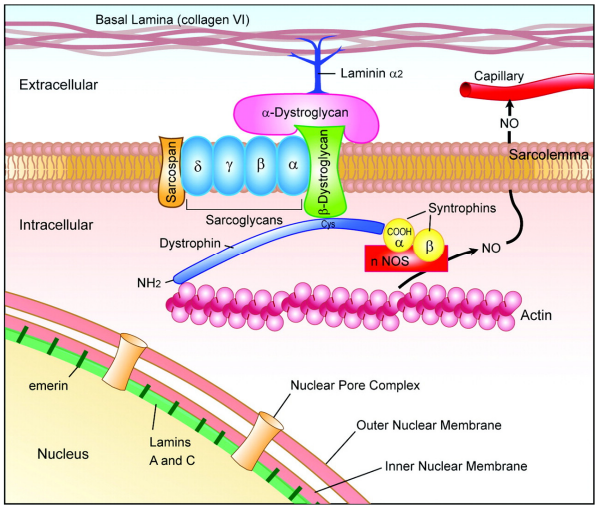


Figure 1.2. Structure of the dystrophin glycoprotein complex. (Verhaert et al, Circulation: Cardiovascular Imaging, 2011)

For coordinated activation of actomyosin interaction, intracellular calcium is tightly regulated. Such excitation contraction coupling occurs at regions where the intracellular sarcoplasmic reticulum forms junctions with the sarcolemma. The sarcoplasmic reticulum (SR) is an internal membrane system which stores Ca ions and releases them when muscle is activated, and it forms a tubular network around each individual myofibril. On either side of a T tubule, the tubules of the SR enlarge, fuse, and form expanded chambers called terminal cisternae. The combination of a pair of terminal cisternae plus a transverse tubule is known as a triad. Here the SR senses the action potential and releases Ca ions which activate the thin filaments and enables the interaction of myosin and actin to proceed. The longitudinal tubules actively "pump" the Ca ions back into the closed membrane system by using ATP. This removes Ca from the thin filaments and inhibits contraction so the muscle fibers relax (McNally et al., Principles of Molecular Medicine; Clark et al., 2002).

1.1.2 Skeletal muscle histogenesis

Skeletal muscle is mesodermal in origin. After gastrulation, the mesodermal cells that migrate from the primitive streak begin to form three condensations lateral to the notochord: the paraxial mesoderm, the intermediate mesoderm, and the lateral plate mesoderm. In the vertebrate embryo, the paraxial mesoderm begins to divide into adjacent, rounded somitomeres which consist of mesenchymal cells without differentiation. Somitomeres show further segmentation into somites, which are paired block-like condensation of primitive mesenchymal cells clearly seen in human embryos. These somites then undergo a number of morphological changes that eventually result in their dissolution and differentiation into a variety of tissues including bone, cartilage, dermis and muscle. During early embryogenesis, some primitive mesenchymal cells differentiate into "pre-myoblasts," so-named because these cells lack definitive histological features to distinguish them from the other mesenchymal cells, but have begun differentiation toward myogenesis. Once these cells achieve some phenotypic features of muscle such as presence of desmin and myofilaments in the cytoplasm, they are known as myoblasts. These are spindle-shaped, elongated cells with fine granular cytoplasm and ovoid nuclei with prominent nucleoli. Muscle fibers are formed by the

fusion of multiple muscle cells, and mature muscle fibers are recognized by their peripheral nuclei and organization of filaments into sarcomeres. The first step toward the formation of muscle fibers begins with the formation of primary myotubes, formed by the fusion of embryonic myoblasts. Primary myotubes have both immature-appearing cytoplasm and nuclei. They are characterized by chains of large central nuclei, each with prominent central nucleoli, surrounded by cytoplasm containing myofibrils, glycogen, and mitochondria. Secondary myotube development is characterized by the fusion of fetally derived myoblasts and begins to show contractility. Secondary myotubes mature into myofibers characterized by an increasing number of myofibrils with easily visible cross-striations, and more nuclei become peripherally located along the muscle fiber. Newborn muscle fibers have polygonal shapes, but appear more rounded than the typical angulated polygonal fibers seen in older children and adults. Usually muscle fibers are tightly packed together, but there is a variable amount of perimysial connective tissue. Postnatal growth of muscle fibers occurs by increasing the length of sarcomeres and by adding new sarcomeres (for review (Ernst et al., Skeletal Muscle; Buckingham et al., 2003).

1.1.3 Muscle precursor cells

During embryogenesis multiple signaling processes are required to determine presomitic mesoderm to form different muscle lineages. These signals initiate and maintain lineage-specific gene expression, regulate the spatial domain of gene expression, and control cell growth, survival and differentiation. Two transcription factors, Myf5 and MyoD, are essential for the determination of the presomitic mesoderm to skeletal muscle lineages. Myogenic precursors were first detected as sublaminar cells during late embryogenesis and are specified during development by signals emanating from neighboring cells of the notochord, neural tube, and dorsal ectoderm. This specification depends critically on the function of myogenic transcription factors, such as Pax-3 and Pax-7 (Borycki et al., 1999; Goulding et al., 1994; Cossu et al., 1996). Once committed, somite-derived cells migrate to multiple sites of embryonic myogenesis, begin to express the myogenic basic factors Myf-5 and MyoD (Birchmeier and Brohmann, 2000), and differentiate into muscle fibers. Myogenic progenitors that do not differentiate into myofibers at this time have been

suggested instead to be retained into adulthood as muscle satellite cells (Armand et al., 1983; Mauro, 1961). Adult skeletal muscle possesses remarkable regenerative capacity, and large numbers of new myotubes normally are formed in only a few days after acute muscle damage. This rapid repair occurs through the differentiation and subsequent cell fusion of satellite cells, positioned between the plasma membrane and the surrounding basal lamina of mature, differentiated muscle fibers (Mauro, 1961; Snow, 1978). Satellite cells represent "dormant myoblasts" left over from embryonic muscle development and capable of recapitulating the developmental program of skeletal myogenesis in response to muscle damage (Mauro, 1961). Pulse-chase experiments using a single dose of tritiated thymidine to label dividing cells indicated that DNA synthesis among sublaminar nuclei was limited to satellite cell nuclei (Moss and Leblond, 1970). Moreover these methods labeled satellite cells infrequently, indicating the relative quiescence of satellite cells (Schultz et al., 1978). Muscle satellite cells are the primary mediators of postnatal muscle growth and repair. These cells respond to regenerative cues, such as injury or exercise, by proliferating to form myoblasts, which divide a limited number of times before terminally differentiating and fusing to form multinucleated myotubes (reviewed in (Morgan and Partridge, 2003). Satellite cell number and regenerative capacity normally remain nearly constant through multiple cycles of injury and repair, suggesting satellite cell self-renewal. Accumulating evidence suggests that the satellite cell compartment contains cells of distinct ontogeny and function. Although several markers have been associated with satellite cells, no single marker defines all satellite cells. For example while CD34 and Pax-7 identify quiescent satellite cells, M-Cadherin, MyoD, and Myf-5 are actually upregulated with differentiation of satellite cells into myoblasts (Morgan and Partridge, 2003; Seale et al., 2000). This heterogeneity of marker expression may reflect functional differences among satellite cells or may distinguish myogenic from nonmyogenic cell types within myofiber compartment. Intrinsic differences in proliferation, differentiation and fusogenic capacity among individual satellite cells have been reported (Beauchamp et al., 1999; Rouger et al., 2004; Rantanen et al., 1995; Zammit et al., 2004).

1.1.4 Skeletal muscle regeneration after injury

Muscle tissue has an in born repair mechanism whereby muscle fibers can regenerate from satellite cells. Skeletal muscle regeneration includes three distinct stages: satellite cell proliferation, terminal myofiber differentiation and muscle remodeling. If the muscle repair mechanisms are inadequate, the consequence might be reduced muscle function and muscle wasting (Bodine-Fowler, 1994). Necrosis of muscle fibers, which is the initial event in the degeneration of muscle, is triggered by disruption of the sarcolemma followed by increased serum levels of proteins such as creatine kinase and myoglobin (Charge and Rudnicki, 2004). In the early phase of muscle damage, the injured muscle activates an inflammatory response driven by T helper cytokines, such as IFN-γ and TNF-α. After the proinflammatory phase, quiescent Pax7-expressing muscle satellite cells are exposed to signals such as IGF-1, fibroblast growth factor 2, and hepatocyte growth factor which promote activation, proliferation and migration to the site of injury. During this stage, the cells become MyoD- and Myf5-expressing myoblasts. Following the proliferation stage, expression of myogenin and myogenic regulatory factor 4 is upregulated, and the myoblasts become terminally differentiated and exit the cell cycle. These muscle progenitor cells then fuse together or with the existing fibers to replace the damaged muscle cells (Charge and Rudnicki, 2004).

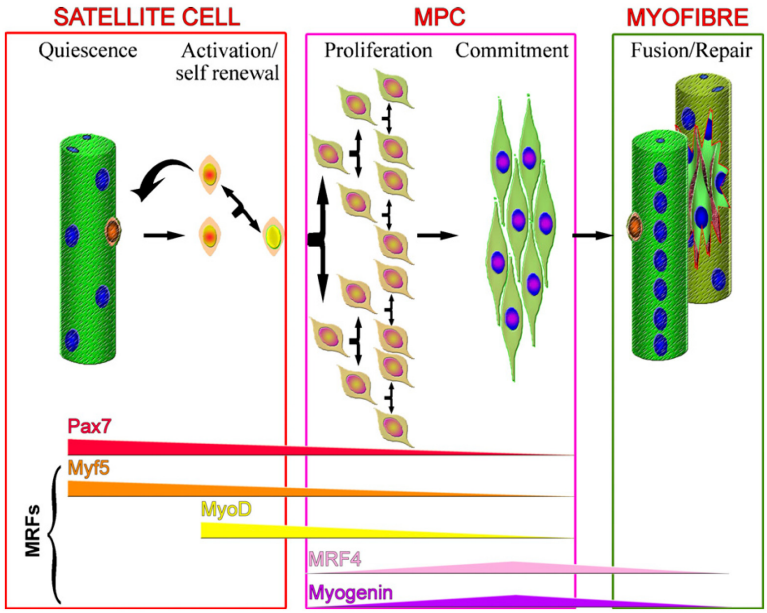


Figure 1.3. Model of satellite cell activation and progression through the myogenic program. (Boldrin et al.,Journal of Histochemistry & Cytochemistry, 2010)

Several studies report that the number or the functions of satellite cells are altered in diseased muscle (Kottlors and Kirschner, 2010; Laule and Bornemann, 2001; Maier and Bornemann, 1999). Myofiber degeneration stimulates the endogenous regenerative mechanisms mediated by satellite cells, however the repeated cycles of degeneration and repair exhaust the satellite cell pool, leading to the progressive fall in the replicative ability of the precursor muscle cells (Luz et al., 2002). This phenomenon may be due to telomere shortening upon repeated replication (Sacco et al., 2010). A few reports show an elevated number of satellite cells in DMD muscle biopsies (Kottlors and Kirschner, 2010; Maier and Bornemann, 1999; Ishimoto et al., 1983), however in vitro studies suggest a deficit in proliferation and differentiation of DMD and mdx satellite cells (Luz et al., 2002; Jasmin et al., 1984; Iannaccone et al., 1987; Delaporte et al., 1984; Webster and Blau, 1990; Decary et al., 2000).

Also aging may have an effect in satellite cells functionality. In fact in aged muscle the number of satellite cells is decreased and cellular senescence limits their proliferative potential (Wright and Shay, 2002). Proliferation rate and clonogenicity of myoblasts from patients with Inclusion Body Myositis (IBM) are significantly lower and doubling time significantly longer than normal controls, indicating that proliferative capacity of IBM muscles becomes exhausted (Morosetti et al., 2010).

1.5 Role of inflammation in muscle repair

Muscle injury typically initiates a rapid and sequential invasion of muscle by inflammatory cell populations that can persist for days to weeks, while muscle repair, regeneration, and growth occur. Recent discoveries have shown that the response of the immune system to muscle injury and disease is a complex regulated process, in which multiple cell populations are involved not only in the pathogenic process but they also regulate the regeneration of muscle.

Skeletal muscle, as other tissues, initially responds to injury with an innate immune response. Neutrophils are rapid responders within the first hours. Morphological observations indicate that the invading neutrophils may be phagocytic (Lowe et al., 1995), but they also have the ability to release proteases that can help degrade cellular debris that may be produced by muscle injury. Th1 cytokines, especially interferon-γ (IFN-γ) and

TNF-α, drive the classical activation of M1 phenotype macrophages, a proinflammatory population. Macrophages damage target muscle cells by a nitric oxide (NO)-dependent mechanism and their cytolytic capacity is increased by the presence of neutrophils (Nguyen and Tidball, 2003). Moreover these two cell types contribute to further muscle membrane lysis by production of free radicals (Tidball, 2005). M1 macrophages seem to contribute to muscle repair processes by participating with the neutrophils in the removal of debris, but it is not clearly understood whether this removal is effectively required for repair or whether macrophages are important contributors to the removal of debris. After M1 macrophages reach their peak concentration in injured and regenerative muscle, they switch their phenotype to M2 macrophages that can attenuate the inflammatory response and promote tissue repair. M2 macrophages are activated by Th2 cytokines; interleukin-4 (IL-4), IL-10, and IL-13 (Tidball and Villalta, 2010; Arnold et al., 2007). M2 macrophages release cytokines that deactivate the M1 phenotype and can promote proliferation of nonmyeloid cells. CD206 is the mannose receptor that binds and internalizes sugar moieties on molecules present at high levels in inflamed tissue. CD206 expression by M2 macrophages is promoted by anti-inflammatory cytokines, and its binding increases the expression of anti-inflammatory cytokines, leading to a positive feedback that can enable M2 macrophages to more rapidly deactivate Th1 cells that are capable of free radical-mediated damage of muscle cells. Muscle can also release factors that promote inflammatory cell invasion, particularly macrophages, which may help muscle repair (Tidball and Villalta, 2010). Recently, human satellite cells were shown to release factors that attracted macrophages/monocytes through an endothelial layer in vitro (Chazaud et al., 2003). Interestingly, chemoattractiveness of these myogenic cells is highest immediately after they are activated to proliferate and then declines as the muscle cells exit the cell cycle and begin to differentiate.

1.2 INFLAMMATORY MYOPATHIES

Idiopathic inflammatory myopathies (IIM) constitute a heterogeneous group of subacute, chronic or sometimes acute acquired muscle diseases, which have in common the presence of moderate to severe muscle weakness and inflammation on muscle biopsy. Based on distinct clinical, immunopathological, histological and prognostic criteria, the most common IIM can be separated into three distinct subsets: polymyositis (PM), dermatomyositis (DM), and sporadic inclusion body myositis (sIBM). The disorders have primarily an autoimmune pathogenesis, mediated either by cytotoxic T cells, as in PM and sIBM or by a complement-mediated microangiopathy as in DM. All forms of inflammatory myopathies are characterized by proximal and often symmetric muscle weakness that develops subacutely, over weeks to months as in PM and DM, or insidiously over months to years as in IBM. The weakness in IBM affects also the distal muscles and can be asymmetric accompanied by muscle wasting. DM affects both children and adults, and women more than men. PM is seen after the second decade of life. IBM is more common in men over the age of 50 than in other population groups. Genetic factors may have a role, as suggested by rare familial occurrences and association with certain HLA genes, such as DRB1*0301 alleles for PM and IBM, HLA DQA10501 for juvenile DM, or tumour necrosis factor 308A polymorphism for photosensitivity in DM. Patients report difficulty with everyday tasks, such as rising from a chair, climbing steps, lifting objects, or combing their hair. Fine motor movements that depend on the strength of distal muscles, such as holding or manipulating objects, are affected late in the course of DM and PM, but fairly early in sporadic IBM owing to prominent involvement of distal muscles, especially wrist and finger flexors. In advanced cases, and in rare acute cases, dysphagia with choking episodes and respiratory muscle weakness occurs. DM differs from the others because of a distinct red or heliotrope (blue-purple discoloration) rash accompanying or, more often, preceding muscle weakness. The weakness varies from mild to severe, leading to quadriparesis. DM can be seen in association with cancer or scleroderma.

PM does not have any distinct features and mimics other acquired or hereditary myopathies, necessitating the need to exclude all conditions that cause a subacute myopathy. The diagnosis of PM is one of exclusion. The diagnosis of IIMs is established

or confirmed by elevated levels of serum muscle enzymes, electromyographic findings and, definitively, by the muscle biopsy. In certain cases of DM, skin biopsy can be helpful. The serum CK, in the presence of active disease, can be elevated by as much as 50 times above normal and usually parallels disease activity; it can be however normal in active DM and sIBM. Needle electromyography shows myopathic motor unit potentials characterized by short-duration, low-amplitude polyphasic units on voluntary activation, and increased spontaneous activity with fibrillations complex repetitive discharges and positive sharp waves (Dalakas and Hohlfeld, 2003; Dalakas, 2011a; Dalakas, 2011b; Dalakas, 2006).

1.2.1 Histological findings

The muscle biopsy shows features distinct for each subset and remains the most sensitive diagnostic tool. Inflammation is the histologic hallmark for all the IIMs; however the site of inflammation and the cells type involved with some additional features are characteristic for each subtype. The most characteristic finding in patients with DM is perifascicular atrophy which sometimes involves grouped or scattered fibers inside fascicles. Necrosis of muscle fibers is also seen around the periphery of the fascicles or involves large groups of the center of a fascicle in a wedge-like distribution suggestive of microinfarcts. Thrombosed vessels may sometimes be seen in the connective tissue septa. Along with necrotic fibers, regenerating fibers are often seen isolated, or in large groups. Inflammatory infiltrates are present in the septa, around blood vessels, or inside fascicles in most of the cases, although in some biopsies, inflammation may not be prominent. In such cases, the diagnosis is based on the presence of perifascicular atrophy and reduced capillary density. The inflammatory cells include B and T lymphocytes, macrophages, and plasma cells, predominantly located in the perimysial and perivascular regions. The MHC class I antigen, as well as intercellular adhesion molecule (ICAM), neural cell adhesion molecule (NCAM) are expressed in the perifascicular regions, or on cell surfaces, in areas where fibers are damaged. The damage to the muscle fibers in patients with DM seems to be caused by the microvascular injury and the associated ischemia. The most characteristic sign is the progressive destruction of the muscle capillaries that precedes structural changes in the muscle, leading to capillary depletion and muscle

ischemia. Even before depletion of the capillaries, there are prominent alterations of endothelial cells morphology. Specifically, the cytoplasm may be swollen and filled with small dark granules, whereas the lumen is not visible. Further, the endothelial cells, and sometimes lymphocytes or macrophages, but never the muscle cells, contain tubuloreticular structures, or undulating tubules with enlarged cytoplasmic and dense bodies, in their smooth endoplasmic reticulum (Dalakas and Hohlfeld, 2003; Dalakas, 2002).

The main features of sIBM are primary endomysial inflammation, vacuolization, amyloid deposits, and loss of muscle fibers. Endomysial inflammatory infiltrates are variable in degree, but in most cases are extremely prominent, even late in the disease. The inflammation is more intense in patients with IBM than in patients with PM, and it is always associated with partial invasion of non-necrotic fibers by activated CD8+ T cells and macrophages. The invaded, as well as the non invaded muscle fibers express class I MHC antigen. B lymphocytes form a minor fraction of perivascular infiltrates and are almost absent from the endomysium. Natural killer cells are also rare. A few, scattered necrotic and regenerating fibers occur in most biopsies. Scattered muscle fibers contain single or multiple vacuoles; the number of vacuolated fibers seems to increase as the disease progresses. The vacuoles contain small, basophilic granules in their center or against their walls, hence their designation as ‘‘red-rimmed vacuoles’’. The granules that are located in, or along the wall of, the vacuoles correspond to whorls of cytomembranes or myelin figures, detectable by electron microscopy. The fibers that harbor the rimmed vacuoles may be normal, larger than normal, or small and atrophic. Deposits of amyloid are present in vacuolated fibers. The hypertrophic fibers are frequent in patients with IBM and they have a round or polygonal configuration. Endomysial connective tissue is increased in areas where there are numerous atrophic fibers (Dalakas and Hohlfeld, 2003; Dalakas, 2011a; Dalakas, 2011b; Dalakas, 2006).

PMs is often seen in association with other collagen vascular diseases, infections, or immune processes. The presence of primary inflammation (the MHC-I/CD8 complex) consisting of inflammatory infiltrates within the fascicles, is essential for the diagnosis of PM. The infiltrates are predominantly endomysial and spotty, although they can be present at perimysial or perivascular sites. T cells account for most of the endomysial infiltrates, and among them the CD8+ T cells predominate. Most of these T cells are

autoinvasive, but some are perivascular. In contrast, 80% of the cells that invade necrotic fibers are macrophages. As in s-IBM, the most important feature in PM is the partial invasion of non-necrotic muscle fibers by inflammatory cells seen in multiple foci. The invaded fibers show very strong expression of MHC class I molecules. The necrotic fibers are usually randomly scattered throughout the biopsy. Along with necrosis, all stages of regeneration of muscle fibers may be present. Small, rounded fibers of either histochemical type are scattered randomly in most biopsies. Hypertrophied fibers are less common in PM compared with IBM (Dalakas and Hohlfeld, 2003; Dalakas, 2011a; Dalakas, 2011b; Dalakas, 2006).

1.2.2 Immunopathologic mechanisms in Dermatomyositis

The primary antigenic target in dermatomyositis is the endothelium of the endomysial capillaries. The disease begins when antibodies directed against endothelial cells activate complement C3. Activated C3 leads to formation of C3b, C3bNEO, and C4b fragments and C5b–9 membranolytic attack complex (MAC), the lytic component of the complement pathway. Sequentially, the complement deposits induce swollen endothelial cells, vacuolization, capillary necrosis, perivascular inflammation, ischaemia, and destruction of muscle fibers. The characteristic perifascicular atrophy reflects endofascicular hypoperfusion, which is prominent distally. Finally, there is striking reduction in the number of capillaries per muscle fiber with compensatory dilatation of the lumen of the remaining capillaries. Cytokines and chemokines related to complement activation are released; they upregulate vascular-cell adhesion molecule (VCAM-1) and intercellular adhesion molecule (ICAM-1) on the endothelial cells and facilitate the egress of activated T cells to the perimysial and endomysial spaces. T cells and macrophages through their integrins bind to the adhesion molecules and pass into the muscle through the endothelial cell wall. The predominant lymphocytes are B cells and CD4-positive T cells, consistent with a humorally mediated process (Dalakas and Hohlfeld, 2003; Dalakas, 2011a; Dalakas, 2011b; Dalakas, 2006).

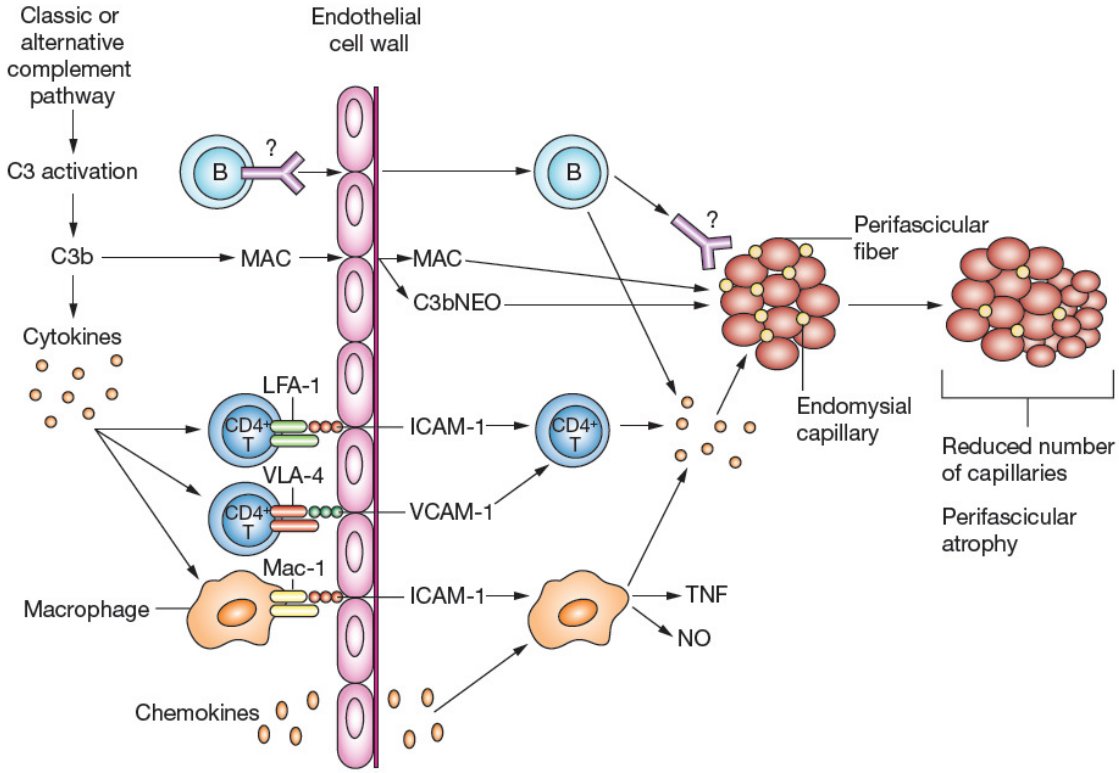


Figure 1.4. Sequence of immunopathologic events in dermatomyositis. (Dalakas, NATURE CLINICAL PRACTICE RHEUMATOLOGY, 2006)

1.2.3 Immunopathologic mechanisms in Polymyositis and Inclusion Body Myositis

In PM and IBM, there is evidence of an antigen-directed and MHC-I-restricted cytotoxicity mediated by CD8 T cells, as supported by the cytotoxicity of endomysial T cells to autologous myotubes, the clonal expansion of autoinvasive T cells and the restricted usage of T cell receptor gene families, the upregulation of co-stimulatory molecules and the release of perforin granules by autoinvasive CD8 cells to lyse muscle fibers. In vivo, the CD8-positive cells send spike-like processes into non-necrotic muscle fibers, traverse the basal lamina, and focally invade the muscle cell. The autoinvasive cells express the memory and activation markers CD45RO and ICAM-1 and contain perforin and granzyme granules that are directed towards the surface of the fibers. Thus, the perforin pathway seems to be the major cytotoxic effector mechanism. By contrast, the Fas-Fas-L-dependent apoptotic process is not functionally involved, despite expression of Fas antigen on muscle fibers and Fas-L on the autoinvasive CD8-positive

cells. The co-expression of the anti-apoptotic molecules BCL2, FLICE (Fas associated death domain-like interleukin-1-converting enzyme inhibitory protein [FLIP]), and human IAP-like protein (hILP), may confer resistance of muscle to Fas mediated apoptosis. Upregulated cytokines, chemokines and adhesion molecules enhance the transmigration of T cells from the circulation to the muscle. Some of them, such as IFN-γ and IL-1β, may have a myocytotoxic effect whereas others, such as TGF-β, may promote chronic inflammation and fibrosis. Muscle-fiber necrosis occurs via the perforin granules released by the autoaggressive T cells. Death of the muscle fiber is mediated by a form of necrosis rather than apoptosis. The upregulated NCAM on degenerating muscle fibers may enhance regeneration. After successful immunotherapy, there is downregulation of cytokines with reduction of inflammation and fibrosis (Dalakas and Hohlfeld, 2003; Dalakas, 2011a; Dalakas, 2011b; Dalakas, 2006).

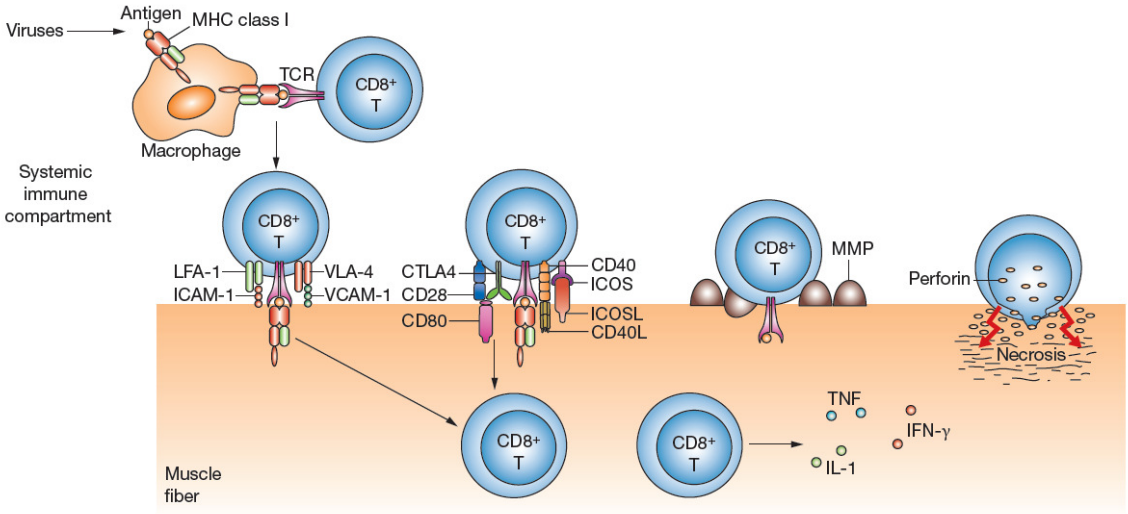


Figure 1.5. Immunopathogenesis of polymyositis and inclusion-body myositis. (Dalakas, NATURE CLINICAL PRACTICE RHEUMATOLOGY, 2006)

3. NEUROTROPHINS AND NEUROTROPHIN RECEPTORS

Neurotrophins (NT) are growth factors implicated in several different functions in the nervous system, including survival, proliferation, differentiation, myelination, apoptosis, axonal growth, and synaptic plasticity. The first neurotrophin identified was originally designated "the nerve growth factor" (NGF; (Levi-Montalcini, 1966)). The other neurotrophins, brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4 (NT-4), were identified 30 years later. One of the most extensively studied property of the neurotrophins is their ability to keep alive subpopulations of sensory neurons. Neurotrophins control the survival and growth of neurons and of their branches. Given the very large numbers of neurons that characterize the nervous system of higher vertebrates, it may have proved useful to generate an intercellular signaling system to modulate intrinsic programs of differentiation. Also, there is increasing evidence for an involvement of neurotrophins in activity dependent structural plasticity as part of a long-lasting response to environmental stimuli. Neurotrophins mediate their effects through binding to two classes of transmembrane receptor proteins, the Trks and the neurotrophin receptor p75.

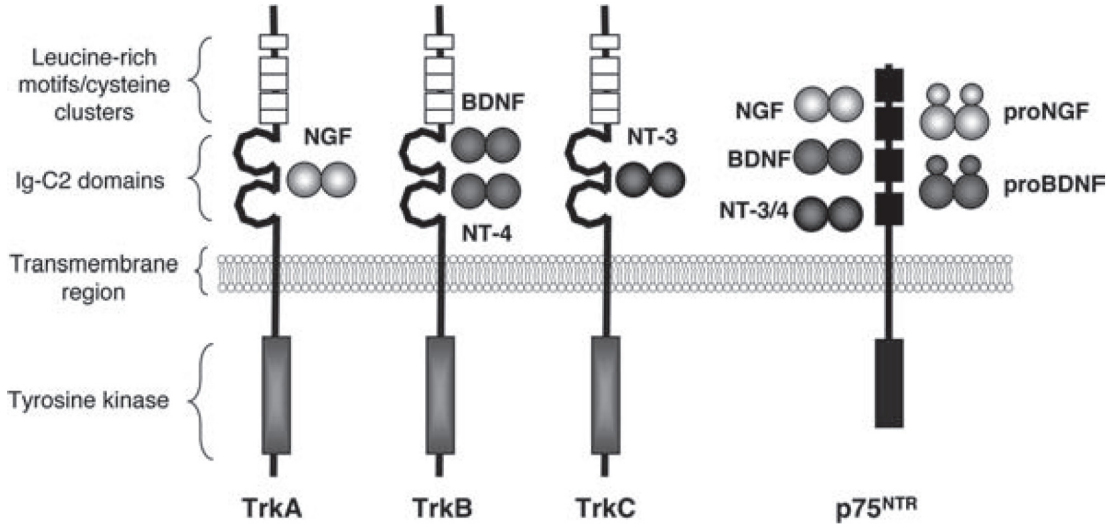


Figure 1.6. Neurotrophin and neurotrophin receptors. (Arevalo and Wu, Cellular and Molecular Life Sciences, 2006)

This dual system allows the transduction of very different signals following ligand binding, which can be as contrasted as signaling cell death through p75NTR or cell survival through the Trk receptors. These two classes of receptors also directly interact, allowing fine tuning and cross talk. The Trk (tropomyosin receptor kinase) receptors belong to the family of receptor tyrosine kinases, and three trk genes have been identified in mammals (Bibel and Barde, 2000; Arevalo and Wu, 2006). NGF is the preferred ligand for TrkA, BDNF and NT4/5 are preferred for TrkB, and NT3 for TrkC (Barbacid, 1994). These specificities are not absolute, and NT3 is also a ligand for TrkA and TrkB. Whereas the tyrosine kinase domains are highly related (~80% aminoacid identity), the extracellular domains are more divergent (~30%). The p75NTR receptor belongs to the tumor necrosis factor (TNF) superfamily and all neurotrophins bind to this receptor with equal affinity (Arevalo and Wu, 2006).

1.3.1 Trks receptors

Trk receptors are receptor tyrosine kinases that contain an extracellular domain composed of three leucine-rich motifs flanked by two cysteine clusters, two immunoglobulin-like C2 type domains (Ig-C2), a single transmembrane domain, and a cytoplasmic region with a kinase domain. Binding of neurotrophins to Trk receptors occurs mainly through the Ig-C2 domains. In addition to ligand binding, the Ig-C2 domains can also stabilize the monomeric form of the Trk receptor to prevent spontaneous dimerization and activation in the absence of neurotrophins. The signaling pathways activated by neurotrophins through Trk receptors result in many neuronal functions, such as cell survival, differentiation, dendritic arborization, synapse formation, plasticity, axonal growth, and axonal guidance. Binding of neurotrophins to Trk receptors leads to dimerization, phosphorylation in trans of the receptors, recruitment of different adaptors and enzymes, and activation of several signaling pathways. Among the signaling pathways activated by Trk receptors in response to neurotrophins, the Shc-Ras-MAPK, Rap-MAPK, PI3K-Akt, and PLCγ-protein kinase C (PKC) pathways are the most studied. Phosphorylation of MAPK leads to activation of several downstream targets that mediate gene transcription to control expression of genes essential for the survival and differentiation of neurons. Signaling through the PI3K-Akt pathway regulates pro-apoptotic and pro-survival

effectors that can mediate the survival actions of neurotrophins. PLCγ activation has been implicated in growth cone chemotropism and in the potentiation of thermal sensitivity on sensory neurons. PLCγ signaling may be important for TrkB signaling in response to BDNF that is involved in synaptic plasticity in the hippocampus (Arevalo and Wu, 2006).
Splice variants have been described for all three Trk receptors. These receptor molecules have either deletions in the extracellular domain or intracellular truncations including the tyrosine kinase domain (Shelton et al., 1995). Insert variants of the extracellular domain were found to influence ligand specificity. However, their biological roles remain unclear. The truncated TrkB receptor isoforms, designated as T1 and T2, contain short intracellular domains (23 and 21 amino acids) (Klein et al., 1990; Middlemas et al., 1991). They are up-regulated during early postnatal development and predominate over full-length TrkB in the adult brain (Fryer et al., 1996). They internalize BDNF and may restrict its availability (Biffo et al., 1995). However, too little is known about the cellular and subcellular localization of these receptors.
p75NTR can act as an accessory receptor modulating the signalling of the trk receptors (Chao and Hempstead, 1995). It is conceivable that the association of p75NTR with the trk receptors changes their conformation leading to increased ligand-binding specificity, as in the case of TrkA and TrkB with their ligands NGF and BDNF. At the same time, p75NTR reduces the ability of NT-3 to activate TrkA, and NT-3 and NT-4 to activate TrkB.

1.3.2 p75NTR

p75NTR was the first member to be molecularly cloned (Johnson et al., 1986; Radeke et al., 1987) of the large family of TNF receptors. It is formed by extracellular domain consists of cysteine repeats, typical motifs of this receptor family, which form the ligand-binding domain, and by a cytoplasmic death domain. Functions ascribed to the p75NTR receptor are diverse, complex, and sometimes contradictory. p75NTR has been implicated in both promoting survival and inducing apoptosis, enhancing neurite outgrowth and facilitating growth-cone collapse, and mediating differentiation and enhancing proliferation. Moreover, p75NTR may also play a role in myelination (Cosgaya et al., 2002; Yamauchi et al., 2004; Du et al., 2006). The lack of catalytic activity in the

cytoplasmic domain of p75NTR suggests that the signaling of this receptor is carried out by interacting proteins that are either constitutively associated with or are recruited to the receptor in response to neurotrophins.

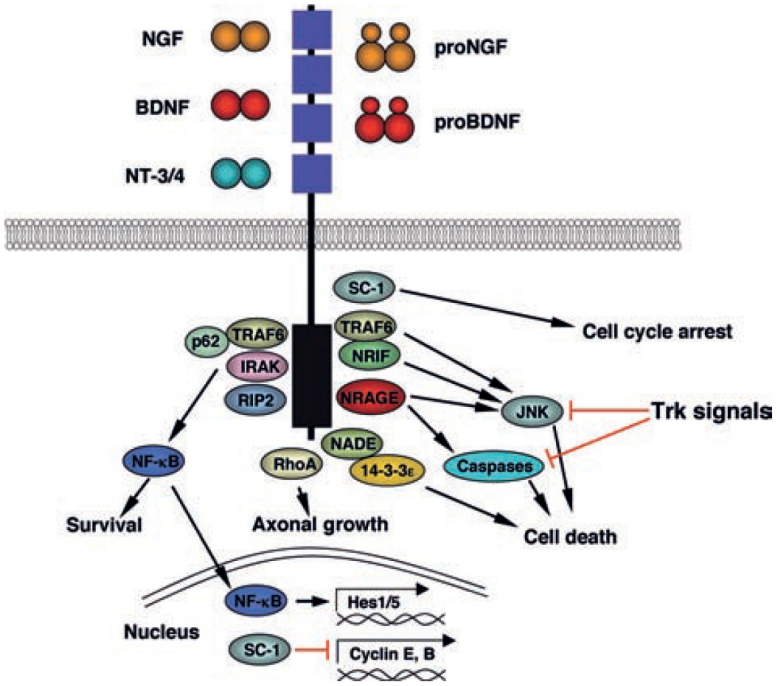


Figure 1.7. p75NTR receptor-mediated signaling pathways. (Arevalo and Wu, Cellular and Molecular Life Sciences, 2006)

An important pro-survival signaling pathway activated by NGF, but not BDNF or NT-3, through p75NTR is the NF-κB pathway. The activation of NF-κB requires several proteins, including TNF receptor-associated factor-6 (TRAF6), IL-1 receptor-associated kinase (IRAK), and receptor-interacting protein-2 (RIP2). Upon activation in response to neurotrophins, NF-κB translocates to the nucleus and modulates dendritic growth (Salama-Cohen et al., 2005). p75NTR regulates the activity of the small GTPase RhoA, a member of the Rho family of proteins that have been shown to control the organization of the actin cytoskeleton in many cell types (Jaffe and Hall, 2005). In the absence of neurotrophins, a constitutive interaction between p75NTR and RhoA maintains RhoA activation and inhibition of axonal growth. Neurotrophin binding to p75NTR causes dissociation of RhoA from the receptor, blocking RhoA activity and leading to axonal growth (Yamashita et al., 1999). Otherwise cell death signaling involves caspase activation, as well as Bax/Bad, Bcl-2, and Bcl-xL (Coulson et al., 1999). The apoptotic effects mediated by p75NTR may depend on the orchestration of several downstream

molecules of neurotrophins. In mature oligodendrocytes, NGF binding of p75NTR leads to the activation of Jun kinase (JNK) and to apoptosis. JNK activation may occur through interaction of TRAF6 with p75NTR (Arevalo and Wu, 2006). Neurotrophin receptor interacting factor (NRIF) is a ubiquitously expressed zinc finger protein that may transduce cell death signals during development and functions in association with TRAF6 to induce activation of JNK. Neurotrophin receptor-interacting MAGE homolog (NRAGE) also interacts with p75NTR to mediate neurotrophin-induced cell death through a mechanism that involves cell cycle arrest, JNK activation, cytosolic cytochrome c accumulation, and activation of caspases-3, -7, and -9. p75NTR-associated cell death executor (NADE) protein may induce cell death upon NGF binding to p75NTR. SC-1 is a transcriptional repressor which may function by forming a complex with histone deacyetylases (HDACs) promoting cell cycle arrest (Arevalo and Wu, 2006).

1.3.3 Neurotrophin expression within the immune system

The actions of NT in the nervous system have been well studied and extensively reviewed (Farinas, 1999; Huang and Reichardt, 2001), although the concept that the role of NT is confined to cells of the nervous system is being reconsidered. Some studies revealed significant actions of neurotrophins in a wide variety of tissues outside the nervous system, especially in the immune system (Otten et al., 1994; Sariola, 2001; Aloe et al., 1999; Tessarollo, 1998).

Neurotrophins are thought to have a role in the physiology of human lymphoid organs, such as bone marrow (Labouyrie et al., 1999), spleen (Labouyrie et al., 1997), lymph nodes (Garcia-Suarez et al., 1997) and thymus (Ciriaco et al., 1996; Berzi et al., 2008), since they or their receptors have been found in those tissues. In particular immunocompetent cells, as T-lymphocytes, macrophages and B cells express neurotrophin receptors and also synthesize and release neurotrophins, suggesting that there might be autocrine and paracrine actions of neurotrophins on these cells (Berzi et al., 2008; Moalem et al., 2000; Kerschensteiner et al., 1999). NGF was the first neurotrophin shown to be expressed by immune cells (T and B lymphocytes, macrophages, and mast cells) (Ehrhard et al., 1993; Leon et al., 1994; Santambrogio et al., 1994). TrkA and TrkB and TrkC are detected in T cells (Berzi et al., 2008; Lambiase et

al., 1997; Besser and Wank, 1999). p75NTR expression in T lymphocytes is still controversial (Ehrhard et al., 1993; Kittur et al., 1992). B lymphocytes express the two NGF receptors, p75NTR and TrkA (Berzi et al., 2008) and therefore can react to NGF stimulation (Torcia et al., 1996). More recently, BDNF expression in immune cells was strongly debated. BDNF can be produced by essentially all major cell types of the human peripheral immune system, includingCD4+ and CD8+ T lymphocytes, B lymphocytes, and monocytes in vitro (Kerschensteiner et al., 1999).

It is well known that the nervous and immune systems interact in both health and disease, and NT seems to participate in the communication network made up of cytokines, growth factors, neuropeptides and hormones which regulate immune and nervous system interactions (Vega et al., 2003). For example in inflammatory brain lesions of MS patients, BDNF is found in infiltrating T cells and macrophages, as well as in neurons and reactive astrocytosis (Kerschensteiner et al., 1999; Stadelmann et al., 2002). Studies in experimental models of ischemic, traumatic, or degenerative CNS disorders and airway inflammation describe the production of BDNF and other members of the NGF neurotrophin family by infiltrating immune cells (Kerschensteiner et al., 1999; Barouch and Schwartz, 2002). In some circumstances, the immune infiltrates commonly found in inflammatory lesions of the nervous system may have a protective rather than destructive role, suggesting that BDNF production by activated T cells, B cells, and monocytes might have a neuroprotective effect (Kerschensteiner et al., 1999).

Regarding the BDNF receptor p75NTR, it is known that p75NTR signalling in macrophages lowers major histocompatibility complex II inducibility and blocks migration and survival in response to inflammatory stimuli (Flugel et al., 2001; Caroleo et al., 2001; Neumann et al., 1998), indicating that neurotrophins may downregulate the aggressive macrophagic phenotype. Moreover germinal center B cells strongly express p75NTR and BDNF, suggesting a role for neurotrophins in B cell maturation (Berzi et al., 2008).

4. NEUROTROPHIN AND NEUROTROPHIN RECEPTORS IN SKELETAL MUSCLE

Muscle fiber contraction is regulated by the activation of motorneurons both under physiological and pathological conditions. Regulation of skeletal muscle innervations, differentiation and function is controlled by a variety of signals from different sources, such as growth factors, hormones, and signaling molecules from innervating motorneurons and contacting Schwann cells. Among these factors the neurotrophins were described as modulators for the development and the maintenance of motorneuron survival (Henderson et al., 1998; Hamburger, 1977). Postsynaptic muscle fibers may release NT that are taken up by presynaptic motor nerve terminals, which express the appropriate receptors. NT are then transported retrogradely to the neuronal cell body, where they may support motor neuron survival (Yano and Chao, 2004). Furthermore, NT potentiate presynaptic release of neurotransmitter (Wang and Poo, 1997) and are essential for maintenance of postsynaptic regions in muscle (Wang and Poo, 1997; Xie et al., 1997; Gonzalez et al., 1999). The observation that the receptors for neurotrophic factors are also expressed in skeletal muscle fibers was surprising and led to the hypothesis that NT might modulate differentiation and functional properties of this tissue (Hamburger, 1977).

1.4.1 Neurotrophins and Neurotrophin Receptors expression in developing and adult muscle

NT and neurotrophin receptors are highly expressed during the earliest stages of muscle development (Henderson et al., 1998). Studies reporting this expression were almost performed on animal models, in vivo and in vitro, but available data are controversial. For example p75NTR is expressed in developing rat myoblasts and in adult rat and chicken muscle (Lomen-Hoerth and Shooter, 1995; Raivich et al., 1985). In developing chick, all somitic cells strongly express p75NTR mRNA during early development. Subsequently, as the somite becomes subdivided into dermatome, myotome, and sclerotome, p75NTR mRNA level is high in dermatome and sclerotome, and decreases to low levels in the myotome (Heuer et al., 1990). When the cells of the myotome differentiate into muscle cells, p75NTR mRNA is re-expressed. Similar observations were made in developing rat

(Wheeler and Bothwell, 1992). In human cultured fetal muscle cells, p75NTR is detected during the first 2 weeks in vitro, whereas in satellite cells from adult muscle, its expression is maintained for up to 7 weeks. Otherwise adult muscle fibers do not show immunostaining for p75NTR, indicating a prominent role for this receptor in the earliest phases of muscle development (Baron et al., 1994). Since the activation of p75NTR may lead to different biological response, its expression in skeletal muscle raises the question whether NTs exert such effects on mitosis, migration, and survival of skeletal muscle cells. Regarding Trk receptors, while TrkA is expressed only in the muscle of adult rat and TrkB is detected both in rat and adult mouse (Lomen-Hoerth and Shooter, 1995; Sakuma et al., 2001; Klein, 1994). Depending on the type of skeletal muscle, expression of the different isoforms of TrkB varies. TrkC is also expressed both as a full-length and truncated isoform, but its distribution is not well studied (Escandon et al., 1994). In general, expression of Trk receptors at the time when myoblasts divide, differentiate, and form myotubes seems very low. In contrast, p75NTR appears as the predominant NT receptor during early development.

Expression of NT in skeletal muscle has been investigated mostly with regard to how these factors modulate survival and differentiation of innervating motorneurons. NGF, BDNF, NT-3, and NT-4 have been mostly studied in developing rat and chicken skeletal muscle (Ip et al., 2001). Low levels of BDNF are detected in developing and postnatal avian and rodent skeletal muscle (Ip et al., 2001; Maisonpierre et al., 1990; Timmusk et al., 1993; Griesbeck et al., 1995; Koliatsos et al., 1993). However, other studies can not detect any BDNF expression in developing muscle of mouse and rat (Sakuma et al., 2001; Schecterson and Bothwell, 1992). NT-3 and NT-4 have been more widely studied during embryonic development and the postnatal phase in rat and chicken (Timmusk et al., 1993; Griesbeck et al., 1995), suggesting an important role of these neurotrophins for the development of muscle spindles.

Regarding NT and NT receptor presence in adult human muscle, available data are few and controversial. For example some studies report p75NTR expression in vivo in human adult muscle (Yamamoto et al., 1996), while in vitro is found only in myoblasts and not in differentiated myotubes (Rende et al., 2000). TrkA is described only in human myoblasts and mytotubes in vitro (Rende et al., 2000), while all TrkB isoforms are detected by PCR in adult muscle (Yamamoto et al., 1996).

Regarding the neurotrophins, some studies suggest the expression of all three neurotrophins in skeletal muscle (Yamamoto et al., 1996; Funakoshi et al., 1993; Sheard

et al., 2002), albeit the sources of neurotrophins in human tissues have not been clearly characterized.

1.4.2 p75NTR and BDNF involvement in skeletal muscle differentiation

Myofibers are formed by fusion of myoblasts, but little is known about exogenous regulators that control the survival, differentiation and fusion of myoblasts. Some evidence suggest a role for the neurotrophin in the regulation of these processes.

Many studies report NT and NT receptor expression in muscle precursor cells. NGF and p75NTR are expressed in C2C12 (Erck et al., 1998) and in primary murine myoblasts (Deponti et al., 2009). Cultures of L6 rat myoblasts display both p75NTR and BDNF (Mousavi and Jasmin, 2006; Rende et al., 1999). BDNF results also expressed in primary murine cells (Clow and Jasmin, 2010; Seidl et al., 1998).

Early functional investigations devoted to the elucidation of the role of neurotrophins in myogenesis were performed in vitro on murine or rat myoblasts cultures. Some studies focus their attention on NGF and its receptor p75NTR. NGF is able to replace serum as an essential supplement for development of cultured primary rat myotubes (Brodie and Sampson, 1987). Functional studies also show that NGF influences the expression of sodium channels in differentiating muscle (Brodie and Sampson, 1990; Vigdor-Alboim et al., 1999). In addition primary mouse myoblasts treated with a blocking antibody or with a NGF antagonist show lower fusion capability (Deponti et al., 2009). Role of p75NTR in myoblast differentiation is controversial. Some studies report that this receptor is downregulated during muscle cell differentiation in murine (Erck et al., 1998; Seidl et al., 1998), in rat (Mousavi and Jasmin, 2006) and in human primary myoblasts (Rende et al., 2000). Functional experiments in the mouse C2C12 cell line demonstrate that p75NTR overexpression improvs myogenesis (Seidl et al., 1998), while inhibits fusion in rat myoblasts (Rende et al., 2000). Finally, a recent study in the mouse enhances the role of p75NTR in muscle regeneration, since the injection of a p75NTR blocking peptide *in vivo* reduces regeneration following cardiotoxin-induced injury (Deponti et al., 2009).

Even BDNF seems to have an important role in skeletal muscle regeneration, but also in this case available data report opposite results. BDNF is expressed in cultured rat myoblasts, but this expression decreased during differentiation. BDNF RNAinterfering in

these cultures enhancs myoblasts fusion, indicating a role for this molecule as inhibitor of the differentiation process (Mousavi and Jasmin, 2006). On the contrary, recent report in mouse model carrying selective depletion of BDNF in skeletal muscle cells demonstrate that lack of the neurotrophin causes abnormability in proliferation and differentiation of primary myoblasts. Moreover, after injury generated by cardiotoxin injection in vivo, BDNF-depleted muscle shows delayed expression of several molecular markers of regeneration, as well as reduced numbers of regenerating fibers. These evidence suggest that BDNF plays an important role in regulating satellite cell function and regeneration in vivo (Clow and Jasmin, 2010).

1.4.3 Role of Neurotrophins in muscle pathology

Mechanical and metabolic disturbances, as they occur during physiological exercise, denervation and in muscle diseases, may alter the expression of different neurotrophins and neurotrophin receptors in muscle (Chevrel et al., 2006).

For example NGF expression decreases in muscular dystrophic mice (Furukawa et al., 1979). Moreover, in a model of laminin alpha2 (merosin) deficient congenital muscular dystrophy, the dy mice, the amount of NT-4 is markedly lower in transgenic muscles compared to control mice (Sakuma et al., 2002). Regarding NGF expression in human pathology, its concentration, as measured by enzyme immunoassay (EIA), is significantly (140%) higher in patients with amyotrophic lateral sclerosis (ALS) than in the control or muscular dystrophies. On the contrary in inflammatory myopathies, the increase is not significant (Stuerenburg and Kunze, 1998).

Another report confirmes that mRNA and protein levels of NGF, BDNF, NT-3, and NT-4 are increased in postmortem biceps tissue of ALS patients in comparison to controls (Kust et al., 2002). In muscle from patients with Duchenne muscular dystrophy (DMD), NGF is expressed in regenerating fibers and connective tissue myofibroblasts (Toti et al., 2003). Rest fibers from dystrophic patients, as well as muscle fibers from healthy patients and regenerative muscle fibers in PM do not show NGF immunoreactivity. The outer surface of some regenerating muscle fibers, in muscle biopsy from patients with muscular dystrophies, are positively stained for p75NTR (Baron et al., 1994; Zhao et al., 1991). The available data on the expression of NT and their receptors in human muscle diseases

are scarce and thus difficult to interpret. However, many studies available suggest a potential involvement of p75NTR in human muscle development and regeneration.

2. AIM OF THE THESIS

Neurotrophins (NT) are growth factors fundamental for the development and the maintenance of the central nervous system, where they regulate neuronal cell numbers, axonal growth and synaptic transmission. Their function may be mediated by two kinds of receptor: the tyrosine kinase Trk receptors and the p75NTR. While Trk receptors mainly promote survival and differentiation processes, p75NTR activation may lead to different responses, such as survival, axonal growth, cell cycle arrest or death (Bibel and Barde, 2000). Several studies report expression of NT or their receptors out of the nervous system, as the immune system, where neurotrophins may have a role in survival and activation of immune cells (Vega et al., 2003). NT and their receptors may be expressed also in skeletal muscle and previous few studies suggest that they are critical for development, function and regeneration of skeletal muscle fibers (Deponti et al., 2009; Clow and Jasmin., 2010; Chevrel et al., 2006).

The aim of this thesis was to study the expression of the neurotrophin receptor p75NTR in human skeletal muscle, and to investigate its involvement in myogenesis and in muscle regeneration. Then this work focused on the ligands that trigger p75NTR signalling.

This study is divided in four distinct parts:

- PART 1: ***Human neurotrophin receptor p75NTR defines differentiation-oriented skeletal muscle precursor cells: implications for muscle regeneration.***

  Here we analysed the expression and the function of the neurotrophin receptor p75NTR in human skeletal muscle precursor cells. Then we investigated the role of this molecule in muscle differentiation and regeneration.

- PART 2: ***The neurotrophin receptor p75NTR is induced on mature myofibers in inflammatory myopathies and promotes myotube survival to inflammatory stress.***

  In this part we investigated the effects of inflammation on p75NTR expression and the contribution of cellular compartments other than satellite cells and regenerating myofibers to p75NTR signal in diseased skeletal muscle. Then we aimed to understand the effect of muscle p75NTR activation in response to inflammation.

- PART 3: ***A role for inflammatory mediators in the modulation of the neurotrophin receptor p75NTR on human muscle precursor cells.***

  Here we wondered whether inflammation could modify p75NTR expression on muscle precursor cells.

- PART 4: ***p75NTR ligands in human skeletal muscle: a role for autocrine and immune cell derived BDNF in human myogenesis and muscle regeneration.***

  We analyzed the expression of neurotrophins in human skeletal muscle to define a candidate ligand for p75NTR. In particular we studied the production of BDNF by muscle cells and its involvement in myogenesis. Finally we characterized how immune cells may support muscle regeneration by production of neurotrophic factors.

We analyzed ex vivo non myopathic muscle from young and adult subjects, and specimens from myopathic muscle such as inflammatory myopathies (polymyositis, dermatomyositis and inclusion body myositis), where muscle degeneration is mediated by massive immune cell infiltration, and biopsies from patients affected by Becker Muscular Dystrophy, where muscle degeneration is due to genetic causes.

Through mRNA and protein localization analysis we evaluated the expression of our targets on muscle satellite cells, on the myofibers and infiltrating immune cells.

This ex vivo investigation was paralleled by in vitro analyses of human primary myoblasts, in order to define the involvement of these molecules in myogenesis and differentiation, through mRNA analysis, protein localization, RNAinterference and gene profiling approaches.

3. MATERIALS AND METHODS

3.1 PATIENTS AND TISSUES

Muscle biopsies were performed for diagnostic reasons and stored in the institutional tissue bank. Informed consent for biopsy and its storage for research purposes were obtained in all cases. Tissue samples were frozen within 1 hour and stored in liquid nitrogen pending molecular or histological analyses. Biopsies were taken from quadriceps femoris muscles in most cases, and specimens with a clear diagnosis, based on clinical, electromyographic, and histological findings were selected (Dalakas, 1991; Engel and Franzini-Armstrong, 1994).

A total of 45 patients suffered from idiopathic inflammatory myopathies: polymyositis (PM, n=16, age range 29-76 years, mean age 64±12), dermatomyositis (DM, n=11, age range 7-71 years, including 3 juvenile cases, mean age 39±22) or inclusion body myositis (IBM, n=18, age range 50-80 years, mean age 69±8). Furthermore, 7 muscle biopsies from Becker Muscular Dystrophy (BMD) patients (age range 11-51 years, mean age 33±16) showing typical histological patterns of necrosis and regeneration but deprived of immune cell infiltration were selected and analyzed.

As controls, muscle specimens from infant (n=9, age range 1-9 years, mean age 5±3) and adult (n=10, age range 19-44 years, mean age 36±12) individuals initially suspected of neuromuscular disease but then ultimately deemed to be free of muscle disease were analyzed. These subjects were visited because of myalgia, slight but asymptomatic increase of creatine kinase or presence of dysmorphic features. Clinical examination was normal in all cases. All muscle biopsies were evaluated by histological and histoenzymatic reactions, and were normal. In some subjects motor performance by exercise on a cycloergometer and metabolic parameters were also assessed to exclude a metabolic myopathy.

3.2 MYOBLAST CELL CULTURES

Three human primary cell lines of myoblasts isolated from non-myopathic tissue specimens were kindly provided by the Telethon sponsored institutional BioBank. Growth medium consisted of Dulbecco modified Eagle medium (Euroclone) containing 20% fetal bovine serum (PAA), 100 U/mL penicillin, 100 mg/L streptomycin, 292 ng/mL

L-glutamine (Euroclone), 10 µg/mL insulin (Sigma), 25 ng/mL fibroblast growth factor (Peprotech), and 10 ng/ml epidermal growth factor (Invitrogen).

CD56/NCAM-positive myoblasts were immunoselected by mouse monoclonal anti-human CD56 antibody (clone MY31; BD Biosciences) and antimouse IgG microbeads (Miltenyi Biotec). The purity of myoblast preparations was assessed by cytofluorimetric analyses for CD56/NCAM and was greater than 95%.

Myoblasts were induced to differentiate in medium containing 2% horse serum (PAA). Fusion index was calculated as the percentage of nuclei within myotubes (with more than 2 nuclei) on total nuclei number.

For stimulation with cytokines, 100 ng/ml IL-1α (R&D Systems) or 150 U/ml IFN-γ (Roche diagnostics) were added to the culture medium.

3.3 IMMUNOHISTOCHEMISTRY AND DOUBLE IMMUNOFLUORESCENCE

Six-µm thick tissue sections were fixed with methanol, blocked with 5% animal serum and stained with the following primary antibodies:

- monoclonal anti-human BDNF antibody (clone 35928, dilution 1:150, R&D Systems),
- monoclonal anti-human BDNF antibody (clone 35909, dilution 1:150, R&D Systems),
- monoclonal anti-human NGFR antibody (clone 74902, dilution 1:20, R&D Systems),
- monoclonal anti-human NGFR antibody (clone NGFR5, dilution 1:20, Invitrogen),
- monoclonal anti-human NGFR antibody (clone C40-1457, dilution 1:50, BD Biosciences),
- monoclonal anti-human TrkB antibody (clone 72509, dilution 1:100, R&D Systems),
- monoclonal mouse anti-human CD56 (clone 555514, dilution 1:50, BD Biosciences),
- monoclonal mouse anti-human CD56 (clone MY31, dilution 1:20, BD Biosciences),
- rabbit polyclonal anti-human dystrophin (dilution 1:800, provided by Dr. Mora from Neurological Institute Carlo Besta of Milan),
- polyclonal rat anti-human laminin (dilution 1:50, provided by Dr. Mora from Neurological Institute Carlo Besta of Milan)
- mouse monoclonal anti-human desmin (clone D33, dilution 1:250, Dako),

- mouse monoclonal anti-human MyoG (clone F5D, dilution 1:50, Dako),
- mouse monoclonal anti-human dysferlin (clone HAM1, dilution 1:100, Novocastra),
- mouse monoclonal anti-human β-dystroglycan (clone 43DAG1/8D5, dilution 1:100, Novocastra),
- mouse monoclonal anti-human CD68 (clone KP1, dilution 1:100, Dako),
- monoclonal mouse anti-human CD4 (clone 4B12, dilution 1:50, Dako),
- monoclonal mouse anti-human CD8 (clone C8/144B, dilution 1:50, Dako),
- rabbit monoclonal antibody anti-human CD3 (clone SP7, dilution 1:300, Lab Vision),
- rabbit monoclonal antibody anti-human CD20 (clone EP459Y, dilution 1:250, Epitomics).
- mouse monoclonal antibody anti-human Ki67 (clone MIB-1, dilution 1:200, Dako)
- mouse monoclonal antibody anti-human perforin (clone δG9, dilution 1:500, BD Biosciences)

Negative stainings consisted of appropriate isotype controls.

For immunohistochemistry, EnVision Detection System (Dako) and counterstaining with hematoxylin were used. For double immunofluorescence, Alexa 488/594 donkey anti-mouse IgG, Alexa 488/594 donkey anti-rabbit IgG, and Alexa 488/594 donkey anti-rat IgG (dilution 1:1000, Molecular Probes, Invitrogen) were used. The slides were counterstained with 4',6-diamidino-2-phenylindole (DAPI, Sigma) and were mounted with FluorSave (Calbiochem).

The Zenon technique (Invitrogen) was used to perform staining for confocal imaging with multiple mouse monoclonal antibodies.

For immunofluorescence on adherent cells, cells were grown on Permanox slides. Staining protocol was as described above.

Apoptotic cells were detected with DeadEndTM Fluorometric TUNEL System according to the manufacturer's protocol (Promega).

Fluorescence images were captured with a confocal laser-scanning microscope equipped with EZ-C1 Software (Nikon, Sesto Fiorentino, Italy) or with Leica TCS SP5 (Milan, Italy). The ImageProPlus software (Media Cybernetics) and ImageJ software (download at: http://rsbweb.nih.gov/ij/) was used for image analysis.

3.4 ELECTRON MICROSCOPY

Twelve µm thick frozen sections were fixed in 4% paraformaldehyde-0.05% glutaraldehyde solution and stained for p75NTR as for conventional immunohistochemistry. Sections were post-fixed in OsO4, dehydrated, and embedded in Spurr resin. After polymerization, the resin blocks were trimmed and sectioned. Ultrathin sections stained with uranyl acetate and lead citrate were examined under a Philips EM410 electron microscope.

3.5 FLOW CITOMETRY

Labeling with mouse monoclonal anti-human p75NTR (clone C40-1457), mouse monoclonal anti-human CD56 (clone MY31), or the corresponding isotype controls (all from BD Biosciences) was followed by detection with phenylephrine-labeled F(ab')2 fragments goat anti-mouse Ig (Dako).

For cell cycle analyses, cells were fixed in 70% ethanol, incubated overnight at 4-C, then stained with propidium iodide solution (50 Kg/mL propidium iodide (Sigma), 0.1 mg/mL RNase A (Ambion) in PBS+ 0.05% Triton X-100).

Cytofluorimetry analyses were performed on FACSCalibur (BD Biosciences).

CellQuest software (BD Biosciences) was used for acquisition and FlowJo (Tree Star Inc, Ashland, OR) for data analysis. Thresholds for positivity were set on isotype controls.

3.6 TREATMENT WITH ANTI-p75NTR BLOCKING ANTIBODY

Myoblasts were seeded in 4-well chamber slides and induced to differentiate in medium supplemented with 10 µg/mL mouse anti-human p75NTR blocking monoclonal antibody (clone NGFR5; Invitrogen) or purified mouse Ig isotype (BD Biosciences). A second antibody treatment was given the next day.

3.7 TREATMENT WITH ANTI-BDNF BLOCKING ANTIBODY

Myoblasts were seeded in 4-well chamber slides and induced to differentiate in medium supplemented with 10μg/ml rabbit polyclonal anti-human BDNF blocking antibody (Millipore) or purified rabbit Ig (Dako). Antibody treatment was repeated for three consecutive days.

3.8 SELECTION ACCORDING TO p75NTR EXPRESSION

Myoblasts were separated by magnetic cell sorting (Miltenyi Biotech) after incubation with mouse monoclonal anti-human p75NTR antibody (clone C40-1457, BD Biosciences). Both negative and positive fractions were collected and induced to differentiate the following day. p75NTR expression was monitored by real-time polymerase chain reaction (PCR) immediately after selection and reached a 10-fold difference in the 2 preparations. Selection was repeated 4 times on the same cell line and reached similar purity.

3.9 p75NTR AND BDNF RNA INTERFERENCE

Small-interfering RNA fragments (siRNA) were purchased at Eurofins MWG. The following siRNA were used:

- p75NTR siRNA1 (5'-UGCGGCAAGAAGGAAUUGATT-3');
- p75NTR siRNA2 (5'-GGUGGAGAAGCUUCUCAACTT-3');
- BDNF siRNA1 (5'-GGACCAUGUUGCUAACUUGTT-3');
- BDNF siRNA2 (5'-UUAAUUACACUUGCAGUUGTT-3');
- non-specific control 47% GC content (5'-AGGUAGUGUAAUCGCCUUGTT-3').

Preliminary experiments were performed in order to determine the optimal concentration for silencing. siRNA were diluted at 20nM in Optimem (Invitrogen). Transfection was obtained by Interferin (Polyplus; Celbio). Differentiation medium was given 2 or 3 days after gene silencing. Silencing efficiency in differentiated cultures was monitored

between days 7 and 10 by quantitative PCR. For the proliferation assay, myoblasts were cultured in chamber slides with growth medium containing p75NTR or control siRNA for some days and then labeled with DAPI. The number of nuclei/area was counted. For cell cycle analysis, myoblasts were analyzed 3 days after siRNA transfection.

3.10 RNA EXTRACTION, cDNA SYNTHESIS, AND REAL-TIME PCR

Total RNA was extracted by TriReagent (Ambion) and reverse transcribed using random hexamer primers and Superscript III reverse transcriptase (all from Invitrogen) following the manufacturers' instructions. Real-time PCR was performed using TAQMan Universal Master Mix (Applied BioSystems, Monza, Italy) on 7500 Fast Real-Time PCR System. The following amplification sets were synthesized: p75NTR (Fw 5'-TGTGCGAGGACACCGAGC-3', Rev 5'-GGGTGTGGACCGTGTAATCC-3', probe 5'FAM-TGCGAGGAGATCCCTGGCCGT-3'BHQ1); BDNF (Fw 5'-AAAAGGCATTGGAACTCCCA-3', Rev 5'-TGCTATCCATGGTAAGGGCC-3', probe 5'FAM-TGCCGAACTACCCAGTCGTACGTGC-3'BHQ1); TrkB extracellular domain (ECD) (Fw 5'-AGGAGAAGATCAAGATTCTGTCAA-3', Rev 5'-GGTCTGAGGTTGG AGATTCG-3', probe 5'FAM-ACTGTGCATTTTGCACCAACTATCACATTTCT-3' TAMRA).

Amplification sets for NGF, NT-3, dysferlin (DYSF), myogenin (MYOG), actin alpha 1 (ACTA1), troponin C type 1 (TNNC1), myocyte enhancer factor 2C (MEF2C), nicotinic cholinergic receptor beta 1 (CHRNB1) and myosin light chain 4 (MYL4) were purchased at Applied BioSystems. Messenger RNA levels of target genes were graphically reported as the percentage of the housekeeping gene Cyclophylin A (PPIA).

3.11 MICROARRAY ANALYSIS

Total RNA extracted from $p75NTR^{high}$ and $p75NTR^{low}$ cells (4 independent samples for each group, selected as previously described) was used for microarray experiments on Illumina Human_Ref-8_V3 arrays. Quantification and quality analysis of RNA were

performed on a Bioanalyzer 2100 (Agilent, Cernusco sul Naviglio, Milan, Italy). Reverse transcription and biotinylated cRNA synthesis were performed using the Illumina TotalPrep RNA Amplification Kit (Ambion) according to the manufacturer's protocol. Hybridization of the cRNAs was carried out on Illumina Human_Ref-8_V3 arrays (Illumina, Son, Netherlands). These arrays contain about 22,000 probes exploring the transcripts contained in the Refseq database. Array hybridization, washing, staining, and scanning in the Beadstation 500 were performed according to standard Illumina protocols. The BeadStudio software (Illumina) was used to analyze raw data grouped by experimental condition. After cubic spline normalization, genes were filtered for detection (detection = 1 in at least 1 experimental group) and selected for statistically significant differential expressions using the Illumina custom test (iterative robust least squares fit, differential score). Stringent criteria were applied: minimal fold change of 1.7 and $p \leq 0.01$ (differential score, ≥ 20). Only 89 probes passed selection. Gene Ontology analysis was then conducted using DAVID (Dennis et al., 2003). Hierarchical clustering algorithm (complete linkage) with the default parameters was used to the perform cluster analysis, as implemented in the Mev software. The bioinformatician was not aware of the cell type under analysis. The transcriptome diagram was built using Pathvisio1.1 with plug-ins (van Iersel et al., 2008), an open source graphical editor for constructing biological pathways.

3.12 STATISTICAL ANALYSIS

The normality of the distribution was assessed by Kolmogorov-Smirnov statistics, and logarithmic transformation of data was applied if necessary. Analysis of variance (in case of normal distribution) or nonparametric Mann-Whitney U test (in case of non-normal distribution) was performed to compare means. Paired sample t-test was used to compare means at different time points. All p values were 2-sided and subjected to a significance level of 0.05.

4.
RESULTS AND DISCUSSION

PART 1:

HUMAN NEUROTROPHIN RECEPTOR p75NTR DEFINES DIFFERENTIATION-ORIENTED SKELETAL MUSCLE PRECURSOR CELLS: IMPLICATIONS FOR MUSCLE REGENERATION

RESULTS

p75NTR is a marker for satellite cells and regenerating fibers in vivo

By immunohistochemistry and immunofluorescence, p75NTR was localized in human skeletal muscle on cells in tight contact with mature myofibers but was absent on the myofibers themselves (Figure 4.1A). We made use of the CD56/NCAM marker to identify satellite cells in muscle tissue. Confocal imaging confirmed p75NTR expression on CD56/NCAM-positive cells (Figure 4.1B). Quantitative analyses revealed that 88.6% ± 9.5% of CD56/NCAM-positive muscle precursor cells expressed p75NTR in human adult skeletal muscle (Figure 4.1C).

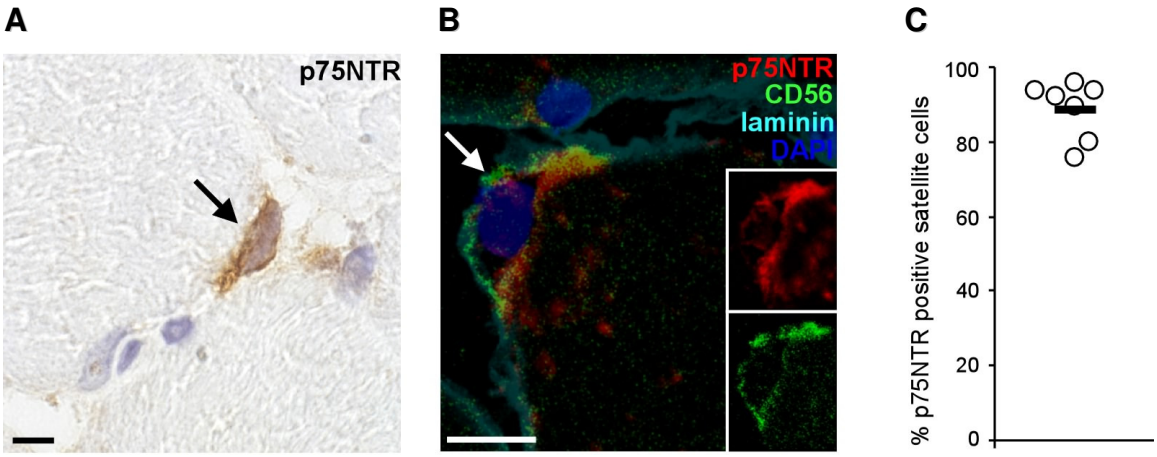


Figure 4.1. p75NTR is expressed in human muscle satellite cells. A: Immunohistochemistry for p75NTR in adult muscle biopsy. B: Double immunofluorescence for p75NTR, CD56, laminin and DAPI. Arrows indicate positively stained satellite cells. C: Percentage of CD56/NCAM positive satellite cells expressing p75NTR in 7 muscle biopsies from healthy adult individuals. Circles represent distinct tissue samples. Black bar indicate average. Scale bar 5 µm.

Regeneration (as assessed by the number of CD56/NCAM-positive fibers) was only rarely detected in healthy skeletal muscle (Figure 4.2A, first column). Therefore, we analyzed regeneration levels in biopsies from inflammatory myopathies in which the pathogenetic process is presumably of autoimmune origin (polymyositis PM, dermatomyositis DM, and inclusion body myositis IBM). We included in our study the analysis of Becker Muscular Dystrophy (BMD) biopsies, a genetic muscle pathology in which degeneration is caused by defects in the dystrophin gene, and characterized by the

absence of inflammatory infiltration. Compared with healthy muscle, the inflammatory myopathies exhibited the highest percentages of regenerating fibers, whereas BMD had quite low regenerative levels (Figure 4.2A). Importantly, numerous newly forming fibers showed strong p75NTR expression (Figure 4.2B, C).

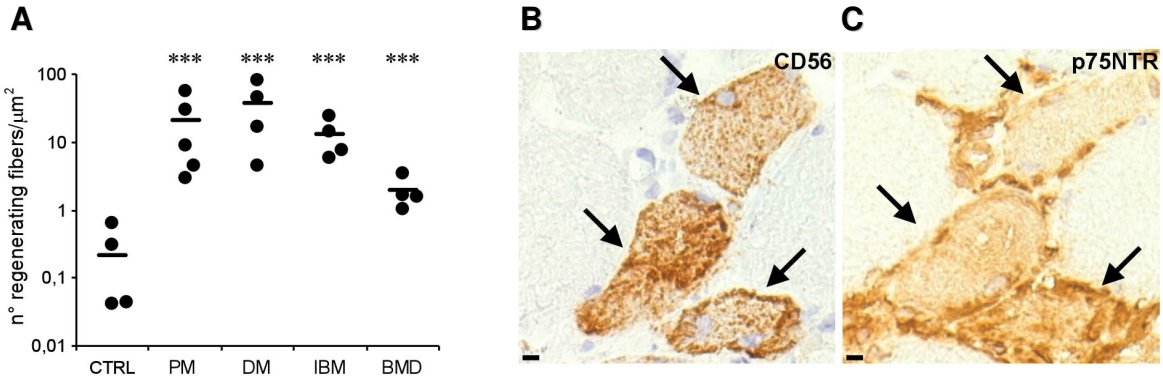


Figure 4.2. p75NTR is a marker for regenerating fibers. A: Quantification of CD56/NCAM positive regenerating myofibers. Black bars indicate average values for each group, dots represent distinct tissue samples. *** p <0.001 when compared to control. B-C: p75NTR expression (B) on CD56/NCAM positive (C) regenerating fibers in a DM muscle. Arrows indicate positively stained fibers. Scale bar 10 µm.

No significant differences in the percentage of regenerating fibers displaying p75NTR were found among the different muscle diseases (average ± SD in each group were 81% ± 3% in PM, 49% ± 29% in DM, 83% ± 8% in IBM, 66% ± 18% in BMD). Approximately 70% ± 7.98% (average ± SEM among groups) of CD56/NCAM-positive fibers coexpressed p75NTR, indicating an in vivo role for this molecule in the first phases of cell fusion and differentiation. Overall, these observations support the hypothesis that p75NTR-positive satellite cells constitute a pool of precursor cells critical for tissue repair and that p75NTR may sustain regenerative processes in vivo.

p75NTR is expressed in vitro by human muscle precursor cells and is transiently enhanced during myogenesis

The analysis was next extended to human muscle cells in vitro. First, primary myoblasts were purified from dissociated muscle cultures based on the expression of the CD56/NCAM marker and were cultured in vitro. Selection efficiency was monitored by cytofluorimetric analysis and was >95%.

First, immunofluorescence experiments with antibodies directed to p75NTR were performed. Figure 4.3 shows that part of human muscle precursor cells displayed the NT receptor under basal conditions.

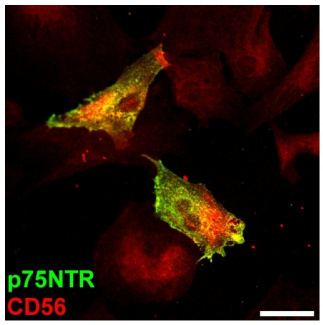


Figure 4.3. Double immunofluorescence for p75NTR and CD56 in cultured myoblasts. Scale bar 30 µm.

An in vitro model of myoblast differentiation into myotubes was then adopted to verify regulation of the receptor p75NTR during myogenesis. Myoblasts were induced to differentiate at day 0 in medium with low serum concentration. After few days mononucleated cells changed their morphology and became elongated (Figure 4.4, second panel, day 4), then they started to fuse to generate plurinucleated elements, the myotubes (Figure 4.4, third panel, day 7).

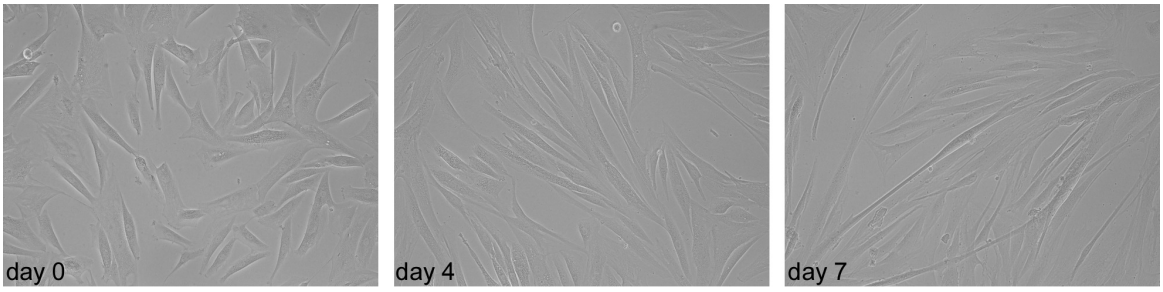


Figure 4.4. Myoblast differentiation in vitro was monitored for 7 days. Bright field images report representative images at different time points.

Messenger RNA was isolated at different time points from cells exposed to differentiation medium, and quantitative PCR for p75NTR was performed on relative cDNA. Compared with basal stage (day 0), p75NTR transcription augmented and reached a peak during the first days of the myogenic process, but returned to initial levels at later time points (Figure 4.5A). Cytofluorimetric analyses on distinct cell preparations showed that 20% to 40% of primary myoblasts expressed the receptor under normal growth conditions. Furthermore, myoblasts promptly upregulated p75NTR when exposed to differentiation stimuli (Figure 4.5B).

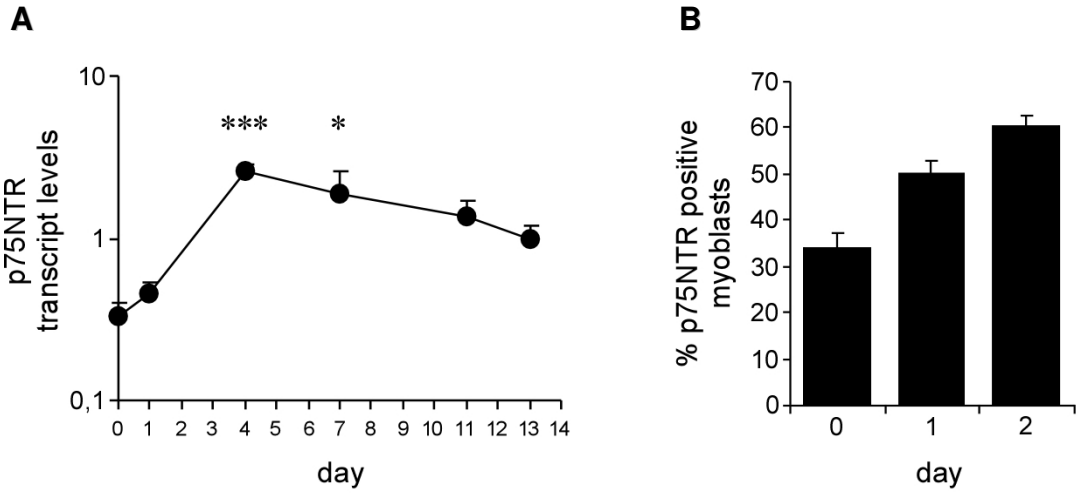


Figure 4.5. Upregulation of p75NTR mRNA levels upon differentiation. A: Quantitative real-time PCR for p75NTR at different timepoints. * $p < 0.05$, *** $p < 0.001$ compared to baseline (day0). B: Flow cytometry experiments showing percentages of p75NTR positive mononucleated myoblasts after exposure to differentiation medium.

From day 3 on, p75NTR expressing multinucleated myotubes were detected, as demonstrated by immunofluorescence on differentiated cultures (Figure 4.6A). Consistent with transcript levels given in Figure 4.5A, approximately 90% of the myotubes were p75NTR positive at day 5, whereas only 25% were at day 14 (Figure 4.6B, squares).

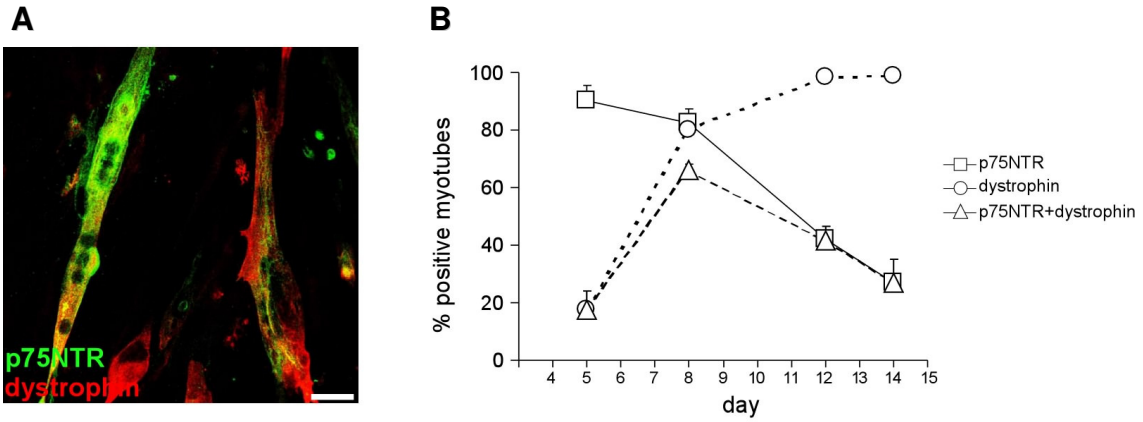
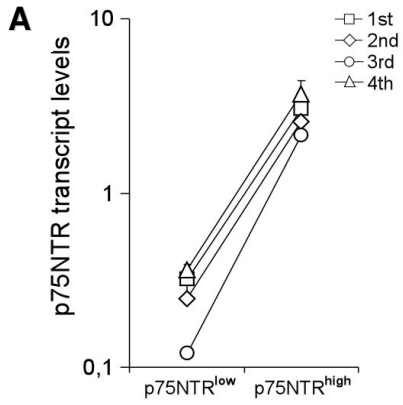


Figure 4.6. p75NTR is expressed in cultured myotubes. A: Immunofluorescence for p75NTR and dystrophin in myotubes at day 11. Scale bar 30 µm. B: Kinetics of p75NTR expression on myotubes and correlation with dystrophin induction. Costainings for p75NTR and dystrophin were performed on differentiating cultures. The percentages of p75NTR or dystrophin or p75NTR+dystrophin positive myotubes were measured at different timepoints.

p75NTR defines differentiation-oriented muscle precursor cells

Further characterization of p75NTR expressing cells was performed by gene profiling experiments. p75NTR-positive myoblasts were separated from p75NTR-negative myoblasts by magnetic cell sorting, obtaining 2 populations with an approximate 10-fold difference in p75NTR mRNA levels immediately after selection (Figure 4.7A). These RNA were used for hybridization on standard Illumina microarrays containing approximately 22,000 probes for human genes and the transcriptome of the $p75NTR^{high}$ population was compared with the one of $p75NTR^{low}$ cells. Eighty-nine gene probes passed the significance threshold of 0.01 and displayed a minimal fold change of 1.7; most were genes more expressed in $p75NTR^{high}$ than in $p75NTR^{low}$ cells (79 upregulated vs 10 downregulated probes) and related to muscle. In fact, significantly enriched gene ontology categories regarded muscle development ($p < 3.9 \times 10^{-9}$) and contraction ($p < 5.4 \times 10^{-12}$) (Figure 4.7B).



B

Gene Ontology	n° genes	Representative genes	p-value
Muscle Contraction	13	DYSF TTN TNNC1	5.4×10^{-12}
Muscle Development	11	MYOG MEF2C CD34	3.9×10^{-9}

Figure 4.7. A: p75NTR mRNA analysis in myoblasts after immunoselection. Receptor expression was tenfold higher in p75NTRhigh than in p75NTRlow populations. Selection was repeated four times with comparable results. B: Muscle-related Gene Ontology categories were significantly enriched in p75NTRhigh myoblasts.

Diagrammatic representation of the main transcriptome data is given in Figure 4.8. Each muscle-related gene product is positioned in the cellular context and is shown as gene symbol contained in an ellipse whose color reflects the extent of fold change.

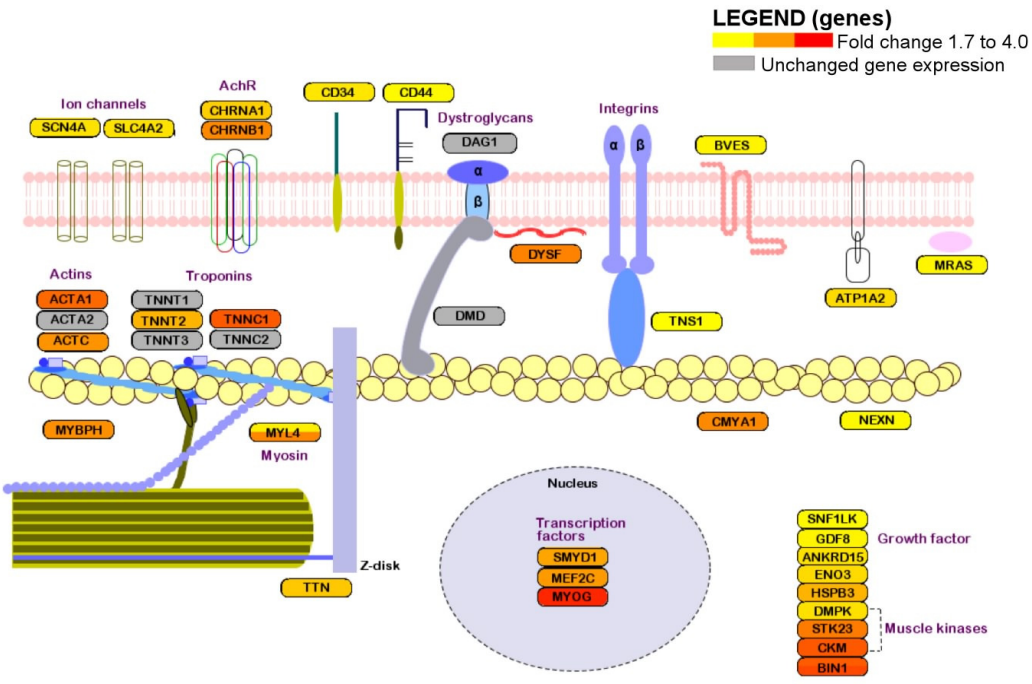


Figure 4.8. A: Graphical representation of the p75NTR-associated transcriptome. Each muscle related gene product is positioned in the cellular context and shown as gene symbol contained in an ellipse whose color reflects fold change extent.

Titin, dysferlin, α and β subunits of the nicotinic receptor, α1 actin, troponin T type 2, and troponin C type 1 were among the upregulated structural genes. Muscle development-related gene products included MyoG, MEF2C, α-enolase, dystrophia myotonica protein kinase, and CD34. Moreover, cluster analysis of the upregulated genes showed that several muscle-related genes clustered together, suggesting coregulation among them (Figure 4.9). In addition, they were located mainly in the clusters at high fold change (Figure 4.9, upper clusters).

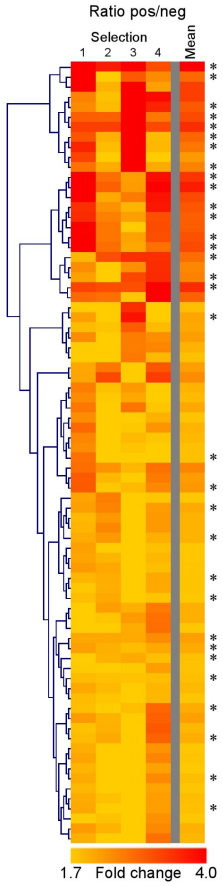


Figure 4.9. Fold change-based cluster analysis of upregulated genes. Each column represents expression ratio between $p75NTR^{high}$ and $p75NTR^{low}$ populations in each of the four performed experiments. The last column reports the average ratio of all groups. Muscle-related genes (labelled with "*") clustered together and were located mainly in the clusters at high fold change. Gene expression is represented in a color gradient ranging from yellow to red for fold change values from 1.7 to 4.0 respectively.

For validation, we analyzed transcript levels of 5 upregulated genes (ACTA1, CHRNB1, MEF2C, TNNC1, and MYL4) in $p75NTR^{high}$ and $p75NTR^{low}$ cells by real-time PCR. All of these genes were more expressed in the $p75NTR^{high}$ population ($p < 0.05$ for ACTA1 and CHRNB1; $p < 0.01$ for MEF2C and TNNC1; $p < 0.001$ for MYL4) (Figure 4.10).

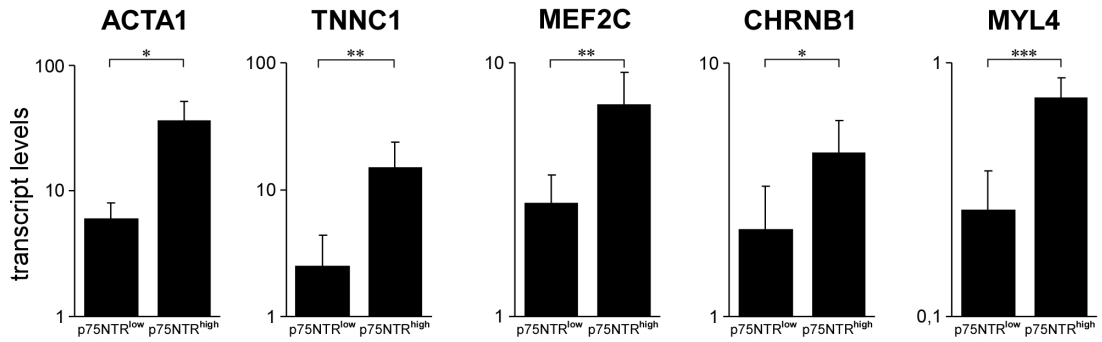


Figure 4.10. Real-Time PCR for ACTA1, TNNC1, MEF2C, CHRNB1 and MYL4 in p75NTR^{high} and p75NTR^{low} cells. Graphs report the average gene expression among four distinct cell preparations. Error bars represent SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Finally, we validated at the protein level the differential expression for 2 more upregulated genes, MyoG and dysferlin. MyoG is an important nuclear transcription factor regulating terminal differentiation of muscle cells, as it induces the coordinated expression of several structural genes, including troponins, myosin chains and titin (Davie et al., 2007). Dysferlin is a membrane protein essential for correct myofiber structure and function, whose absence causes muscular dystrophy characterised by impaired muscle regeneration (Chiu et al., 2009). Protein detection by immunofluorescence (Figure 4.11A, B) and relative quantification in p75NTR-positive or negative myoblasts confirmed that both MyoG and dysferlin were indeed preferentially expressed in p75NTR-positive cells ($p = 0.002$ for both, Figure 4.11C).

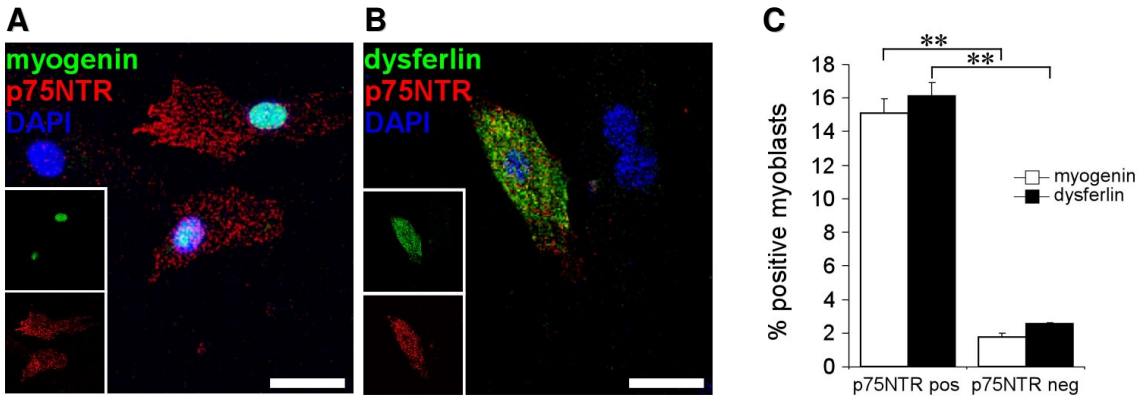


Figure 4.11. A-B: Double immunofluorescence for p75NTR and MyoG (A) or dysferlin (B) in cultured myoblasts. C: Percentage of MyoG or dysferlin expressing cells among p75NTR positive and negative myoblasts. Scale bar 30 µm in E and F. ** $p < 0.01$.

Overall, the transcriptional repertoire displayed by p75NTR-expressing cells suggests that these cells have a strong differentiation potential.

p75NTR regulates precursor cell differentiation

The functional role of p75NTR in muscle cell differentiation was investigated in vitro. Initially, we hindered p75NTR activity by the addition of an anti-p75NTR blocking antibody to myoblasts cultures and assessed the extent of myogenic fusion. Muscle cells treated with the blocking antibody displayed lower fusion index ($p = 0.001$) than control cells, indicating that p75NTR may influence precursor cell differentiation (Figure 4.12A). Figure 4.12C report a representative staining for desmin in myotubes cultures exposed to α-p75NTR antibody or isotype control. White arrows highlight the higher number of multinucleated myotubes in control cultures than in antibody-treated cultures. Next, we separated p75NTR-positive myoblasts from the p75NTR negative cells by magnetic beads and induced in vitro differentiation. Under these conditions, $p75NTR^{high}$ muscle cells formed significantly more myotubes than the $p75NTR^{low}$ population ($p < 0.001$; Figure 4.12B). As expected, the addition of the blocking antibody to $p75NTR^{high}$ precursor cells inhibited cell fusion ($p < 0.001$; Figure 4.12B, third column).

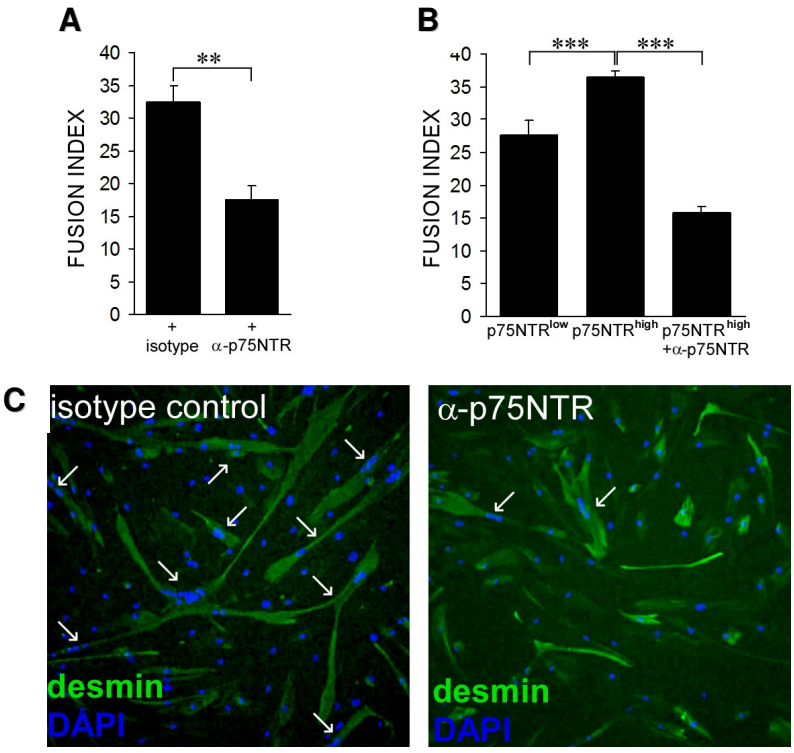


Figure 4.12. A: Fusion index in a blocking experiment with anti-p75NTR antibody or isotype control administered during cell differentiation. B: Extent of myogenesis in $p75NTR^{low}$, $p75NTR^{high}$, and $p75NTR^{high}$ myoblasts treated with anti-p75NTR blocking antibody. Fusion was evaluated at day 6 after differentiation induction. ** $p < 0.01$, *** $p < 0.001$. C: Representative staining for desmin and DAPI in a blocking experiment. Arrows indicate myotubes (cells with more than 2 nuclei).

Because fusion was not strongly impaired in p75NTR^{low} cultures, we checked p75NTR levels during differentiation. Interestingly, p75NTR was readily induced in p75NTR^{low} cells and reached values comparable to those in unseparated cells by a few days (Figure 4.13), indicating that differentiation stimuli force p75NTR expression also in p75NTR-negative cells.

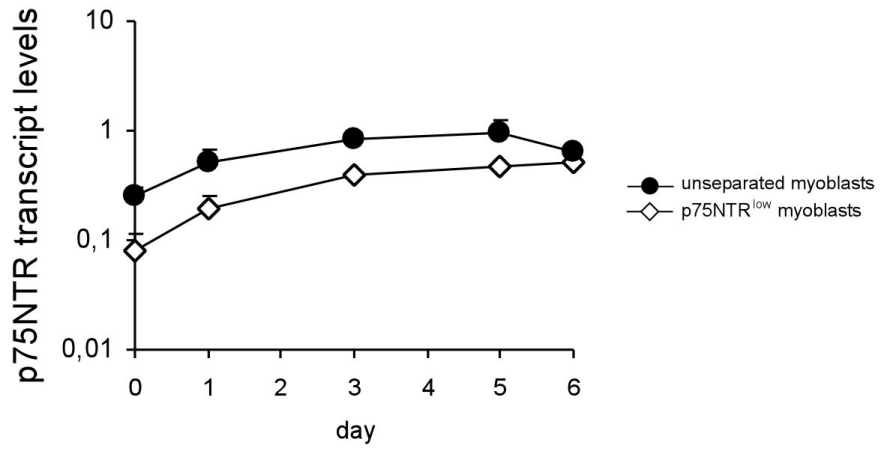


Figure 4.13. Quantitative real Time PCR for p75NTR in unseparated myoblasts and p75NTR^{low} myoblasts induced to differentiate.

Finally, RNA interference experiments for p75NTR were performed. Myoblasts were transfected with p75NTR or scrambled siRNA and were induced to differentiate after 48 hours. The efficiency of silencing in different experiments ranged between 70% and 96%, as assessed by real-time PCR on cultures at days 7 to 10. Figure 4.14A shows the reduced p75NTR mRNA levels after silencing in a representative experiment. Immunofluorescence for p75NTR showed at least 50% reduction in protein expression on myotubes after gene silencing (Figure 4.14B). Most importantly, myoblasts treated with p75NTR siRNA showed a consistent decrease of the fusion index compared with cells treated with control siRNA ($p < 0.001$; Figure 4.14C). Similar results were obtained with a second p75NTR siRNA (not shown).

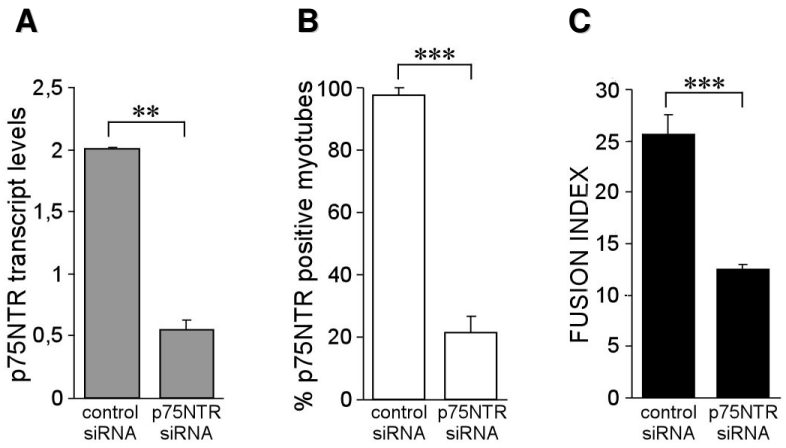


Figure 4.14. p75NTR mRNA (A), protein levels (B) and fusion index (C) in p75NTR silenced and control myotubes. ** $p < 0.01$, *** $p < 0.001$. p75NTR silencing efficiency and relative fusion index were evaluated in myotubes at day 8.

Because p75NTR signaling might affect several cellular programs, including differentiation, or exert a specific action on myogenesis, we addressed this key point by determining whether p75NTR controlled other cell functions. Proliferation of myoblasts was found to be unaltered in p75NTRsilenced cells versus control cells (Figure 4.15A). Furthermore, cell cycle progression did not change upon p75NTR silencing (Figure 4.15B). Taken together, consistent with the transcriptome data showing p75NTR as a prototypic marker for differentiation prone muscle precursor cells, these functional data demonstrate that p75NTR specifically regulates myogenesis.

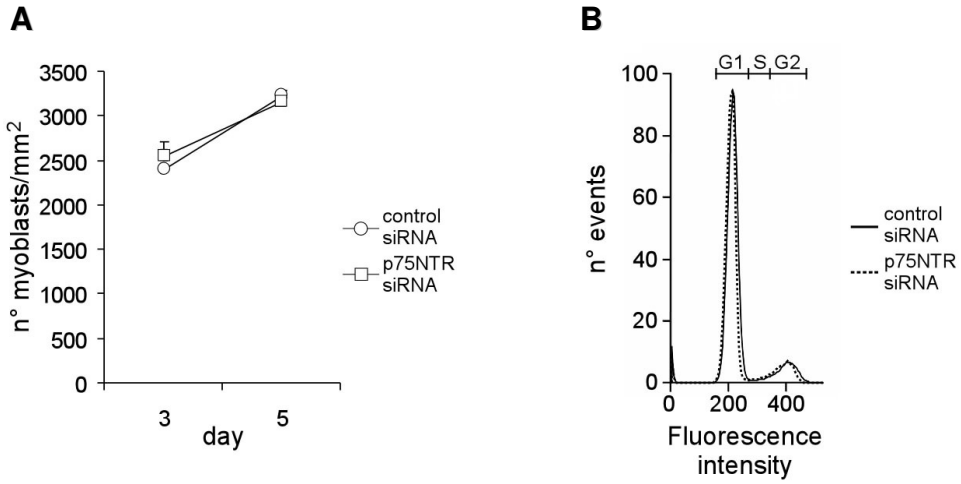


Figure 4.15. A: Myoblast proliferation in p75NTR silenced or control myoblasts. siRNA administration was performed at day 0, cells were maintained in growth medium for a few days and counted. B: Cell cycle analysis as detected by propidium iodide incorporation in silenced myoblasts. Evaluation was performed at day 3 after transfection with p75NTR or control siRNA.

p75NTR specifically controls dystrophin induction in myotubes

The kinetics of p75NTR in newly forming myotubes in vitro was compared with that of dystrophin, a fundamental muscle protein induced upon maturation. In contrast to p75NTR, dystrophin was present on few myotubes at day 5, reached a peak at day 8, and remained high thereafter (Figure 4.6B, circles). Notably, when coexpression of p75NTR and dystrophin was checked (Figure 4.6B, triangles) at day 5, dystrophin started to emerge exclusively on p75NTR-positive myotubes, indicating that sustained expression of p75NTR on multinucleated elements precedes dystrophin induction and might, therefore, regulate it. We wondered whether p75NTR was needed for dystrophin induction. The dystrophin gene was expressed in myoblasts, but the microarray analysis did not reveal any difference for this molecule between p75NTRhigh and p75NTRlow cells (not shown). Quantitative PCR for dystrophin on cDNA from p75NTR-silenced and control myoblasts confirmed that there was no association between p75NTR and dystrophin levels under basal conditions (not shown). We then induced differentiation of p75NTR-silenced myoblasts and measured dystrophin expression on myotubes (some degree of myogenesis was still present in such cultures). This analysis showed that p75NTR silencing significantly reduced the percentage of dystrophin-expressing myotubes ($p = 0.002$; Figure 4.16), demonstrating that p75NTR may regulate the correct induction of a structural protein, such as dystrophin in differentiated muscle cells.

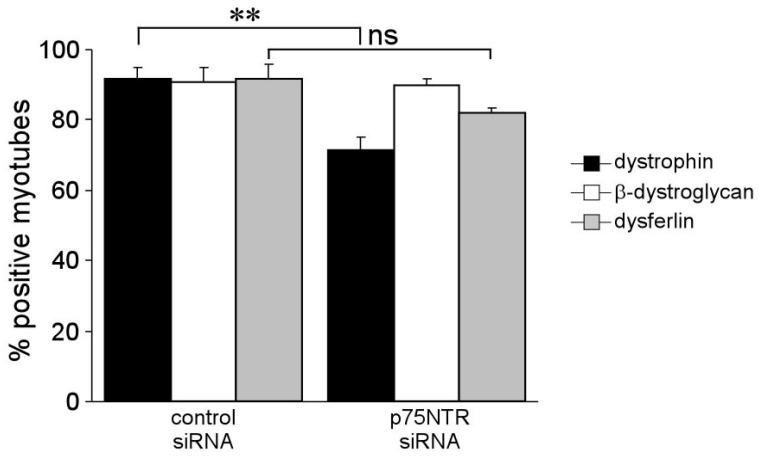


Figure 4.16. Quantification of dystrophin, β-dystroglycan and dysferlin positive myotubes in differentiated cultures at day 8 after differentiation induction. ** $p < 0.01$, ns not significant.

Similarly, the addition of the anti p75NTR blocking antibody during differentiation reduced the percentage of dystrophin-positive myotubes (not shown). Regulation of dystrophin by p75NTR was specific because the expression on myotubes of other muscle proteins (e.g. β-dystroglycan and dysferlin) was not altered upon p75NTR silencing (Figure 4.16).

DISCUSSION

Here we identified p75NTR as a novel marker for human differentiation-prone muscle precursor cells and demonstrated its involvement in myogenic processes *in vitro* and *in vivo*.

Our immunohistochemical experiments demonstrated that this receptor is displayed by satellite cells in human adult skeletal muscle. p75NTR positive satellite cells have been detected also in rodent muscle (Mousavi and Jasmin, 2006) but the size of this pool of precursor cells has never been determined. We found that in human muscle the great majority of satellite cells express p75NTR on the surface, indicating this molecule as a novel marker for that cell type.

Next, we wondered whether extensive muscle repair as it occurs under pathological settings is associated to p75NTR expression. Two classes of human muscle diseases were analysed: inflammatory myopathies (PM, DM and IBM) and Becker muscular dystrophy. The first is a group of acquired disorders of skeletal muscle characterised by inflammation-mediated muscle injury (Dalakas and Hohlfeld, 2003), while BMD is a genetic disease and represents a relatively mild form of muscular dystrophy (Lim and Rando, 2008). A previous report described p75NTR on regenerating fibers in Duchenne muscular dystrophy (Baron et al., 1994). Consistently, we found that newly forming fibers in inflammatory myopathies and in BMD express p75NTR, further supporting the notion that this receptor regulates regenerative processes in human skeletal muscle. Although expression of p75NTR on regenerating fibers in the animal has not been reported so far, functional studies in the mouse showed that injection of a p75NTR blocking peptide *in vivo* reduces regeneration following cardiotoxin-induced injury (Deponti et al., 2009).

A couple of *in vitro* studies on rodent cell lines reported about the downregulation of p75NTR during muscle cell differentiation (Rende et al., 2000; Mousavi and Jasmin, 2006; Seidl et al., 1998). Our experiments on human primary muscle cells demonstrated prompt upregulation of p75NTR on myoblasts when exposed to differentiating stimuli and robust but transient expression on mature myotubes. This is consistent with the presence of p75NTR in regenerating fibers and its lack of expression on mature myofibers *in vivo*. Fusion experiments with $p75NTR^{high}$ myoblasts revealed that they differentiate more efficiently than $p75NTR^{low}$ cells. Furthermore, blockade of p75NTR at the protein

or at the mRNA level impairs fusion, clearly demonstrating a direct role for this receptor in muscle cell differentiation. Similar results were obtained recently with primary mouse myoblasts, where treatment with a blocking antibody or with a NGF antagonist inhibited fusion (Deponti et al., 2009). Viceversa, p75NTR overexpression in the mouse C2C12 cell line improved myogenesis (Seidl et al., 1998). Of course, the possibility that, in vivo, also p75NTR-negative satellite cells contribute to the formation of new myofibers cannot be excluded. Our results indicate that p75NTR-negative myoblasts upregulate the NT receptor in response to differentiation stimuli, and that p75NTR-positive cells are more efficient in myogenesis than p75NTR-negative cells, suggesting that p75NTR-bearing cells might have a functional advantage in vivo. Further insights on the mechanisms came from transcriptome analysis, demonstrating that p75NTR expressing myoblasts display a repertoire of gene products that justifies their proficiency to differentiation and maturation. Among them, both MyoG and MEF2, two important positive regulators of differentiation (Le Grand and Rudnicki, 2007), are more expressed in p75NTR positive myoblasts, which bear also increased transcripts for a number of muscle structural proteins.

All together, we propose p75NTR as a novel marker for human differentiation-oriented muscle precursor cells. Our *in vitro* experiments show that p75NTR expressing myoblasts have enhanced myogenic properties though maintaining unchanged proliferation extent. Of note, while most of satellite cells display the neurotrophin receptor *in vivo*, only a minor percentage of muscle precursor cells expresses it *in vitro*. As demonstrated by our *in vitro* experiments, p75NTR is not necessary for proliferation and survival of undifferentiated cells, while its expression is finely regulated to allow correct myotubes maturation. It is possible that culture conditions may favour growth of p75NTR negative myoblasts since its induction is required only during differentiation. In future, muscle stem cell expansion protocols could be developed to maintain or select the pool of precursor cells expressing the neurotrophin receptor.

Another important aspect emerging from our studies is that p75NTR signalling may regulate distrophin induction. In fact, dystrophin protein is induced exclusively on p75NTR positive myotubes during the first phases of differentiation, suggesting that the neurotrophin receptor is not dispensable for muscle cell maturation. Moreover, p75NTR silencing in differentiating myoblasts leads not only to decreased cell fusion but also to reduced dystrophin expression on myotubes. The effect is specific for dystrophin as two other muscle proteins as dysferlin and β-dystroglycan are not affected by p75NTR

blockade. Dystrophin is fundamental not only for muscle structure but also for myofiber survival (Ervasti, 2007). In fact, reduction of dystrophin levels leads to muscle loss, as dystrophin itself triggers signalling molecules that inhibit atrophy pathways (Acharyya et al., 2005). Intriguingly, phenotypic elimination of the neurotrophin NGF in adult transgenic mice leads to muscle atrophy, underlying the importance of this trophic stimulus for skeletal muscle homeostasis (Ruberti et al., 2000).

We can conclude that neurotrophins profoundly tailor muscle regeneration as they may act on precursor and differentiating cells.

PART 2:

THE NEUROTROPHIN RECEPTOR p75NTR IS INDUCED ON MATURE MYOFIBERS IN INFLAMMATORY MYOPATHIES AND PROMOTES MYOTUBE SURVIVAL TO INFLAMMATORY STRESS

RESULTS

p75NTR is upregulated in inflamed muscle on distinct cell types including mature skeletal myofibers

We investigated the expression of the neurotrophin receptor p75NTR in human skeletal muscle. First of all, we checked transcript levels in infant and adult healthy specimens. Total RNA was extracted by muscle biopsies and retro-transcribed. Quantitative PCR experiments showed detectable and comparable levels of p75NTR in the two groups (Figure 4.17A) and no significant correlation between ageing and p75NTR expression (P>0.05, Figure 4.17B, white dots). Next, we wondered whether muscle pathology was characterized by altered levels of this receptor. Interestingly, PCR analyses showed increased levels for p75NTR mRNA in PM, DM and IBM specimens and, on the contrary, a clear reduction in BMD tissues (P = 0.003 for PM, DM, IBM, P = 0.001 for BMD when compared to adult control group, P < 0.05 for DM and BMD, P < 0.01 for IBM when compared to infant control group, Figure 4.17A). As inflammatory myopathy patients had a large age range, we verified whether ageing was affecting p75NTR expression. As for control tissues, no correlation was evident between ageing and p75NTR levels (P > 0.05, Figure 4.17B, black dots).

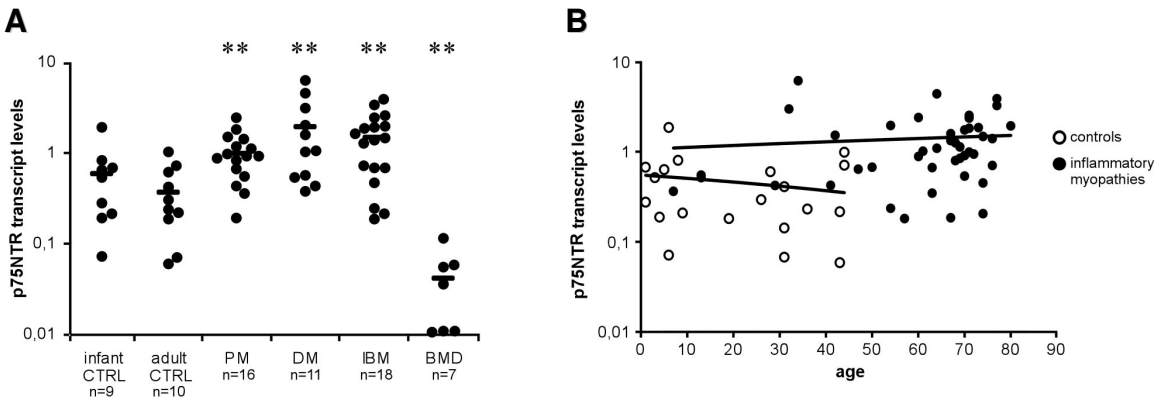


Figure 4.17. A: p75NTR mRNA tissue levels in infant and adult non myopathic biopsies and in PM, DM, IBM and BMD specimens. Each dot represents a tissue sample, black bars indicate average values for each group. B: Correlation between p75NTR transcript levels and age in non-myopathic tissues and in inflammatory myopathy biopsies. ** P<0.01 vs. adult control.

Immunohistochemistry experiments with specific p75NTR reactive antibodies were then performed aiming to allocate p75NTR protein to specific cell types and structures. Among pathological biopsies, we selected representative tissues with p75NTR RNA levels around the mean of the group. Consistent with molecular analyses, we noticed enhanced immunoreactivity for p75NTR in all analyzed inflammatory myopathies but not in BMD (Figure 4.18C-F) when compared to control muscle (Figure 4.18A,B).

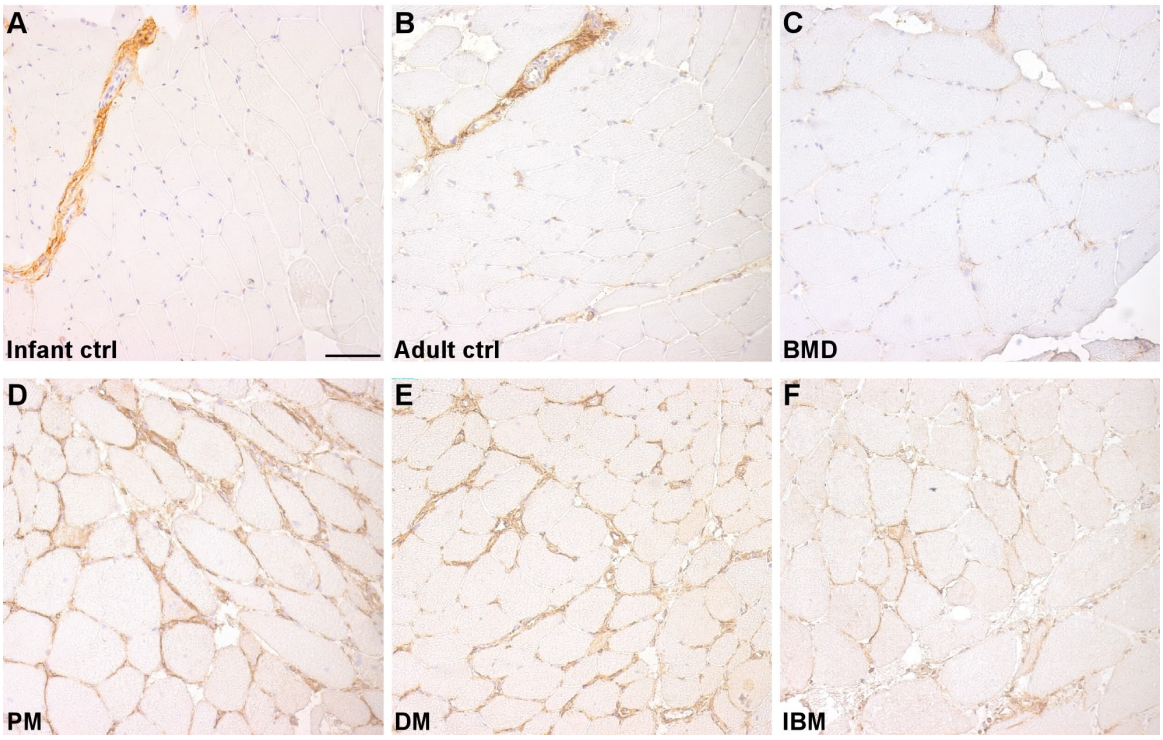


Figure 4.18. Immunohistochemistry for p75NTR in infant and adult healthy muscle and in pathological tissues from BMD, PM, DM and IBM patients. Scale bar 100 µm.

Part of the signal was localized in fibrotic areas at perimysium and endomysium (Figure 4.19A). Furthermore, some immune cells infiltrating muscle tissue stained positive. Immunofluorescence together with confocal imaging experiments on sections from inflamed muscle showed that several macrophages, T lymphocytes and, in DM, B cells displayed the neurotrophin receptor (Figure 4.19B-D).

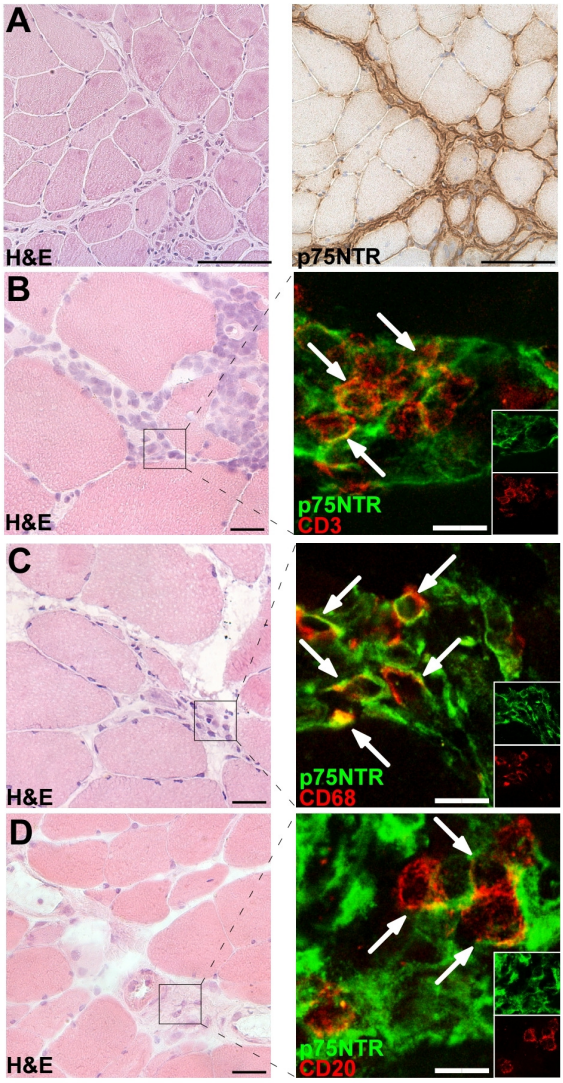


Figure 4.19. A: Haematoxylin-eosin and immunohistochemistry for p75NTR showing receptor localization at the endomysium. Scale bars 30 µm. B-D: Haematoxylin-eosin and double immunofluorescence staining for p75NTR and T cells (B), macrophages (C) and B cells (D) on serial sections from PM; IBM or DM biopsies. Scale bar 30 µm in left panels, 10 µm in right panels.

As previously discussed, regenerating CD56 positive muscle fibers strongly expressed p75NTR (Figure 4.20A). Most interestingly, in inflammatory myopathies and not in BMD or healthy muscle, p75NTR immunoreactivity was observed in all myofibers (Figure 4.18C-F). In fact, not only regenerating (CD56 positive) but also mature (CD56 negative) skeletal muscle fibers displayed immunoreactivity for p75NTR at the cytoplasmic and membrane level, as shown by immunohistochemistry and electron microscopy examinations (Figure 4.20A,B), suggesting a link between the upregulation of this protein in differentiated muscle cells and inflammation.

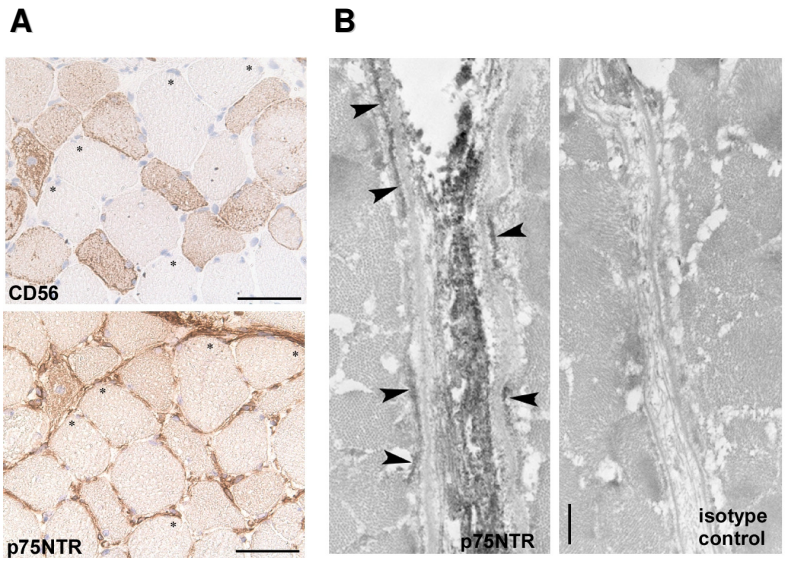


Figure 4.20. A: Representative stainings for CD56 and p75NTR on serial sections from an IBM biopsy. Asterisks indicate p75NTR expressing mature fibers. Scale bar = 30 μm. B: Electron microscopy for p75NTR in mature myofibers. Arrowheads indicate immunoreactivity on the cell membrane. Scale bar 1μm.

Overall, distinct cell types, including mature myofibers, contribute to the increased p75NTR transcript levels observed in inflammatory myopathies.

The inflammatory cytokine IL-1 increases p75NTR expression on myotubes

We then focused our attention on the observation that mature skeletal myofibers displayed enhanced p75NTR immunoreactivity in inflamed muscle. To verify whether inflammation could regulate p75NTR levels on differentiated muscle cells, we set up appropriate tests on in vitro generated multinucleated myotubes. Primary cultures of purified human myoblasts were induced to fuse into myotubes and these were exposed to inflammatory mediators. As assessed by quantitative PCR, p75NTR transcript levels resulted significantly upregulated in IL-1 treated myotubes but not in IFN-γ treated myotubes ($P = 0.009$, Figure 4.21A). Consistently, a higher percentage of myotubes expressed p75NTR protein at day 3 after IL-1 but not IFN-γ administration, as detected by immunofluorescence ($P < 0.001$, Figure 4.21B,C). Therefore, differentiated muscle cells may upregulate p75NTR in vitro in response to the inflammatory factor IL-1.

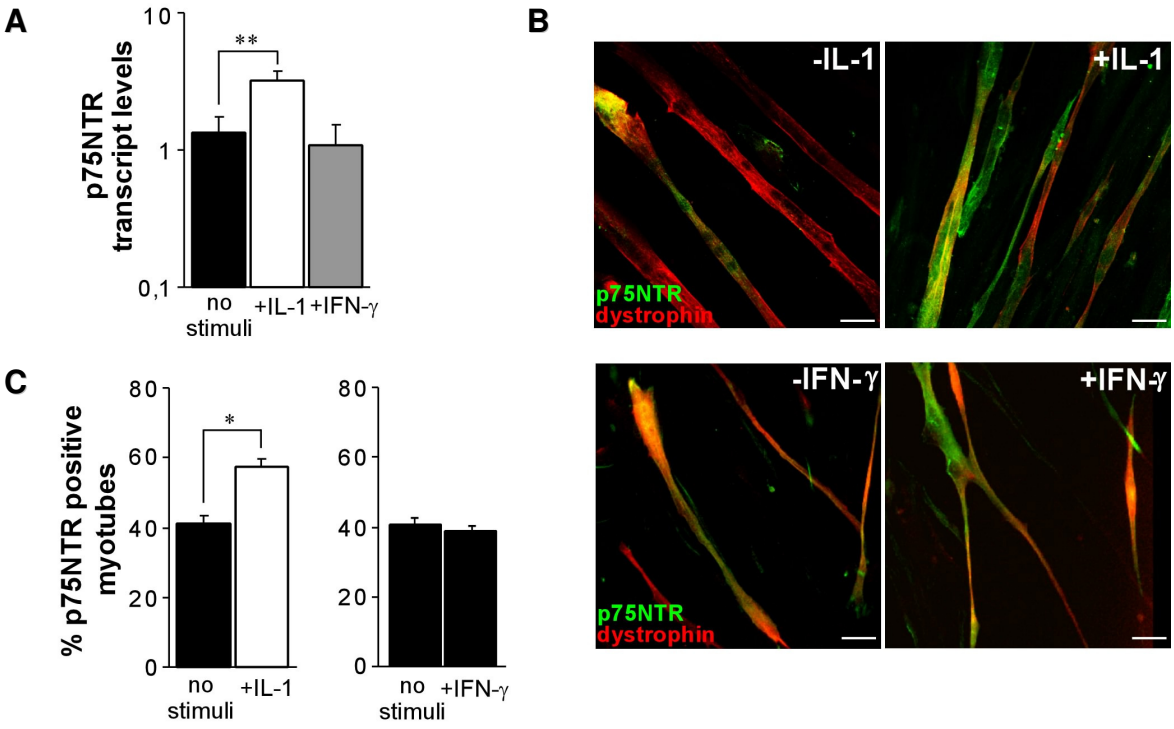


Figure 4.21. A: mRNA levels for p75NTR in myotubes after exposure to IL-1α and IFN-γ. Transcript levels were measured after 24 hours stimulation. B-C: Immunofluorescence for p75NTR and dystrophin on IL-1 or IFN- γ treated or control myotubes (B) and relative quantification (C). Myotubes were exposed to stimuli or control medium for 72 hours. Scale bar 30 μm in B. ** P<0.01, *** P<0.001.

p75NTR controls myofiber resistance to inflammatory stress

In order to clarify the role of the neurotrophin receptor in skeletal myofibers, p75NTR silencing experiments in differentiated myotubes were performed and the effects of p75NTR depletion on myotube integrity were evaluated. Myotubes cultures at day 8-11 were trasfected with p75NTR or control siRNA and at the same time, they were exposed to IL-1. The efficiency of silencing in different experiments ranged between 73% and 99%, as assessed by Real-Time PCR on cultures at day 2-4 after transfection (Figure 4.22A). As already described p75NTR was significantly upregulated control mytubes after exposure to IL-1 (Figure 4.22A). Upon exposure to IL-1 the p75NTR-silenced multinucleated cells showed morphological alterations associated with nuclei condensation (Figure 4.22B). TUNEL assay was then performed to find out presence of apoptotic myotubes. After 96 hours from siRNA transfection and exposure to IL-1 we observed that p75NTR silencing per se did not alter myotube survival, unless cultures

were exposed to inflammatory stimuli. In fact, after treatment with IL-1, increased numbers of apoptotic myotubes were detected (P = 0.02, Figure 4.22B,C), demonstrating a direct role for p75NTR in myofiber survival to inflammatory stress.

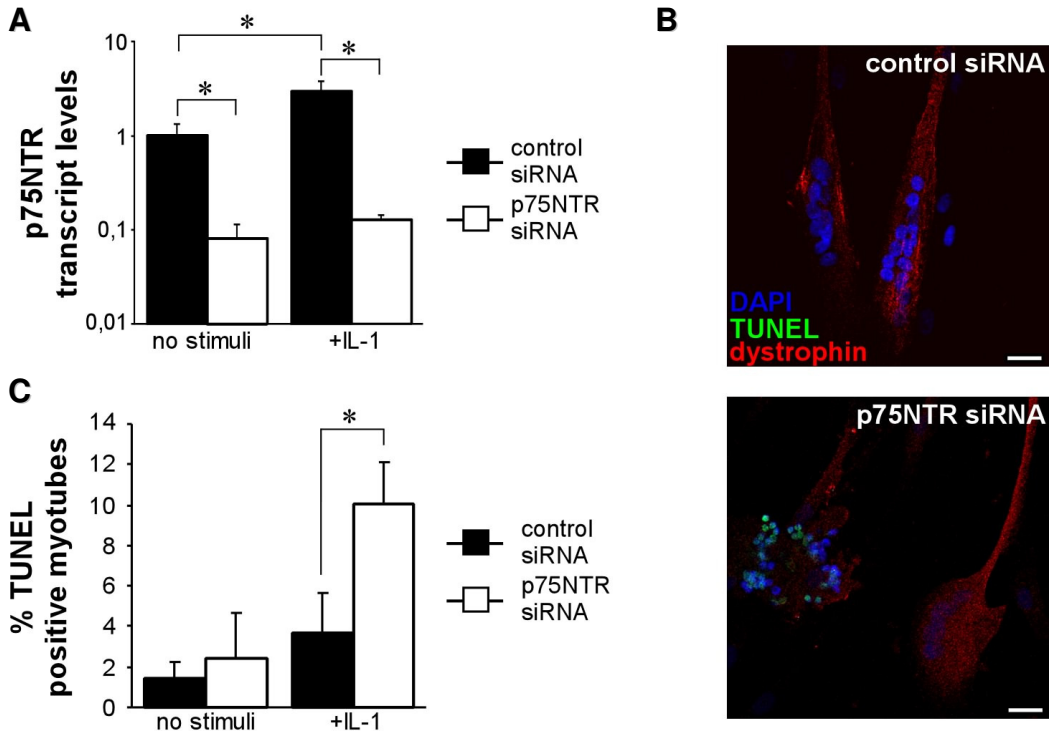


Figure 4.22. A: p75NTR mRNA levels in myotubes transfected with p75NTR or non specific siRNA and exposed to IL-1. B-C: Immunofluorescence (B) for dystrophin, TUNEL and DAPI in p75NTR silenced or control myotubes and relative quantification (C). Analysis was performed after 48-96 hours. Scale bar 30 μm. * P<0.05.

DISCUSSION

The analyses of idiopathic and genetic skeletal muscle disorders highlighted alterations in p75NTR expression under pathological settings.

The inflammatory myopathies (PM, DM and IBM) are a heterogeneous group of acquired diseases of skeletal muscle characterized by inflammation-mediated tissue injury. Many studies reported elevated expression of pro-inflammatory cytokines, such as IL-1, IL-2, IL-18, IFN-γ and TNF-α in inflammatory myopathies (Lepidi et al., 1998; Tucci et al., 2007). Interestingly, several cell types, including immune and muscle cells, may release locally such inflammatory mediators (Lundberg et al., 1997, De Bleecker et al., 1999, Schmidt et al., 2008). For example, IL-1α was detected in endothelial, immune and smooth muscle cells (Lundberg et al., 1997), and localized mainly at the connective tissue and capillaries in PM and DM, while in myofibers in IBM (Schmidt et al., 2008). Moreover, myofibers may respond to IL-1 as they express IL-1 receptors at the sarcolemma in inflamed muscle (Grundtman et al., 2007). In contrast to inflammatory myopathies, Becker Muscular Dystrophy (BMD) is a genetic pathology characterized by muscle atrophy due to a reduction in amount or alteration in the size of the dystrophin protein (Nowak and Davies, 2004). Our quantitative analysis of p75NTR transcripts showed that the neurotrophin receptor was easily detected in infant and adult muscle, and that its levels were altered in diseased tissues. In fact, while BMD showed lower p75NTR expression than control muscle, all inflammatory myopathies displayed augmented transcript levels.

As already described p75NTR is displayed by human muscle satellite cells, and takes part to myogenesis in vitro and to human muscle regeneration in vivo. Therefore, both muscle satellite cells and regenerating fibers contribute to p75NTR transcript levels. Now, as demonstrated in the first aim of this study, BMD tissues display almost 10-fold lower regeneration levels compared to inflammatory myopathies and only two thirds of the regenerating fibers express p75NTR. Thus, the contribution of the regenerating fibers to p75NTR transcript levels in BMD is extremely limited. We showed previously that almost all satellite cells express p75NTR in control tissue, but it is not known whether this pool of satellite cells is maintained in injured tissue. The decreased expression of p75NTR in BMD might indicate that less p75NTR positive precursor cells are present in this pathological condition. The maintenance of the p75NTR positive satellite cells in

genetic or idiopathic myopathies is an issue that deserves further investigation, as we have shown that p75NTR positive precursor cells display high differentiation potential. Interestingly, immunohistochemistry experiments revealed enhanced p75NTR immunoreactivity on several other cell types in inflammatory myopathies. Strong upregulation was in fact evident at endomysium and perimysium, both around vessels and on fibrotic tissue. Fibrosis is a process triggered by chronic damage and inflammation, counteracting atrophy and assuring preservation of tissue continuity. It is characterized by the continuous deposition and remodelling of extracellular matrix components produced by specific cells, the myofibroblasts (Tomasek et al., 2002; Pinzani and Vizzutti, 2008). Fibrosis is a typical feature of chronic myopathies and is associated to decreased strength and elasticity of muscle (Dalakas, 2001). Myofibroblasts mainly derive from differentiation of fibroblasts recruited at the lesion site, and share the phenotypic features of both fibroblasts and smooth muscle cells (for review (Tomasek et al., 2002)). However, myofibroblasts may originate from other specialized cells. For instance, hepatic stellate cells in injured liver may transdifferentiate into myofibroblasts which secrete growth factors necessary for the restoration of normal liver structure (Balabaud et al., 2004). Similarly, muscle-derived stem cells can differentiate into myofibroblasts after muscle injury (Li et al., 2004). A series of observations indicate a role for p75NTR in tissue fibrosis. In fact, this receptor regulates hepatocellular transdifferentiation into myofibroblasts (Passino et al., 2007) and sustains fibrin deposition (Sachs et al., 2007), indicating that p75NTR is involved in generation of fibrotic tissue. However, loss of p75NTR signaling causes delayed fibrosis resolution and reduced apoptosis of hepatic myofibroblasts, demonstrating that the neurotrophin receptor promotes recovery from fibrosis at later stages (Kendall et al., 2009). We can therefore hypothesize that p75NTR on fibrotic tissue in skeletal muscle may exert similar tasks. Studies in appropriate animal models could shed new light on this issue.

Inflammatory myopathies are typically characterised by massive infiltration of T cells and macrophages into the tissue. In this study, we observed that many of these immune cells displayed immunoreactivity for p75NTR. While no information is available on the role of p75NTR in T cells, it is known that p75NTR signaling in macrophages lowers major histocompatibility complex II inducibility and blocks migration and survival in response to inflammatory stimuli (Flugel et al., 2001; Caroleo et al., 2001; Neumann et al., 1998), indicating that neurotrophins may downregulate the aggressive macrophagic phenotype. Furthermore, p75NTR expressing B cells were found in DM tissues. A recent report

demonstrated that germinal center B cells strongly express p75NTR and the ligand BDNF, suggesting a role for neurotrophins in B cell maturation (Berzi et al., 2008). We showed that mature myofibers upregulated p75NTR in PM, DM and IBM but not in BMD, indicating a relationship between tissue inflammation and the induction of p75NTR expression in mature myofibers in vivo. Indeed, in vitro experiments showed that IL-1, but not IFN-γ, induced higher levels of p75NTR transcript and protein in myotubes, demonstrating that some inflammatory stimuli can enhance p75NTR expression in differentiated myocytes. IL-1 is a pro-inflammatory cytokine rapidly secreted by innate immune cells in response to various stimuli such as microbial products and danger signals (for review (Apte and Voronov, 2002)). This cytokine is released at sites of inflammation, where it activates endothelial cells to express adhesion molecules that allow leukocyte migration into the tissue. Furthermore, IL-1 triggers a cascade of inflammatory mediators resulting in rapid activation of stroma and immune cells (Apte and Voronov, 2002; Dinarello, 2000). IL-1 may directly target myoblasts and inhibit their differentiation by transcriptional suppression of myogenic transcription factors through the nuclear factor NF-kB (Guttridge et al., 1999; Guttridge et al., 2000; Langen et al., 2001) and by impairing insulin growth factor-1 (IGF-1) dependent protein synthesis (Broussard et al., 2004). In our study we could demonstrate that p75NTR protected differentiated muscle cells from IL-1 induced apoptosis. Clearly, further studies are needed to define the mechanistic events controlled by p75NTR and linked to the response to inflammatory mediators in differentiated muscle cells. IL-1 is considered a muscle wasting mediator since it causes myofibrillar protein loss by activation of the ubiquitin-proteosome pathway (Llovera et al., 1998; Li et al., 2009). Till now no rescue mechanisms from muscle degeneration have been elucidated. Overall, we identified a p75NTR-dependent survival pathway to inflammatory stress in differentiated muscle cells and speculate that the observed p75NTR upregulation on mature myofibers in inflamed muscle may deliver rescue signals from atrophy.

PART 3:

A ROLE FOR INFLAMMATORY MEDIATORS IN THE MODULATION OF THE NEUROTROPHIN RECEPTOR p75NTR ON HUMAN MUSCLE PRECURSOR CELLS

RESULTS

Inflammatory mediators modulate the neurotrophin receptor p75NTR on human muscle precursor cells

Primary cultures of human myoblasts were exposed to the inflammatory mediators IL-1α or IFN-γ. p75NTR expression was monitored both at transcript and protein levels. As assessed by quantitative PCR, after 18 hours from stimuli administration, the amount of p75NTR mRNA was significantly lower in myoblasts treated with the inflammatory mediators ($p < 0.001$, Figure 4.23A). Consistently, the cytofluorimetric analysis confirmed that a lower proportion of cultured cells expressed p75NTR on the surface after exposure for 72 hours to IL-1α or IFN-γ (Figure 4.23B). These data indicate that an inflammatory microenvironment may lead to the reduction in the proportion of muscle precursor cells expressing p75NTR in vitro.

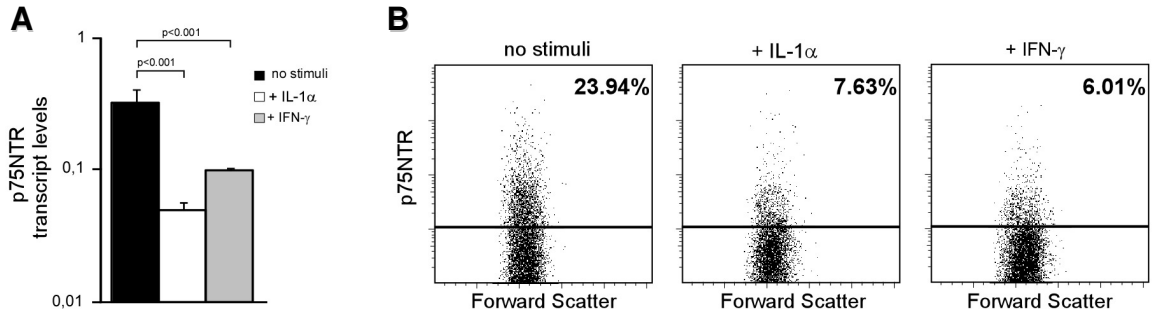


Figure 4.23. Downregulation of p75NTR transcript (A) and protein (B) levels in myoblasts exposed to IL-1α or IFN-γ.

Thus, we checked in vivo expression of p75NTR on CD56/NCAM positive satellite cells in muscle biopsies from patients affected by inflammatory myopathies. Double immunofluorescence and confocal imaging investigations showed that, differently from non-myopathic muscle, where almost all satellite cells expressed p75NTR (Figure 4.24A left panel) and maintained this expression despite ageing ($p = 0.54$, Figure 4.24B), in all inflammatory myopathy specimens the neurotrophin receptor was upregulated in mature fibers as previously described but less expressed on CD56 positive precursor cells (Figure 4.24A right panel). In fact, the percentage of p75NTR positive satellite cells ranged

between 21% and 66% in inflamed muscle (Figure 4.24B). Furthermore, this percentage decreased with ageing (p = 0.004, Figure 4.24B), suggesting a link between prolonged exposure to inflammatory stimuli and p75NTR expression on satellite cells.

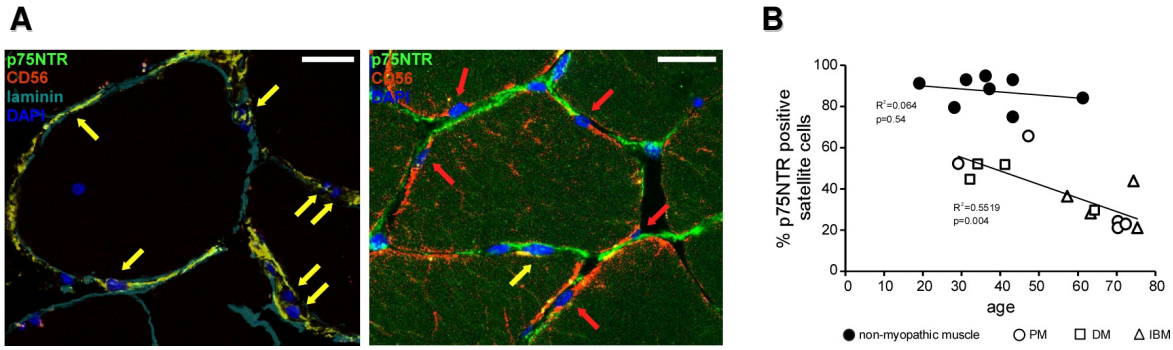


Figure 4.24. A: Representative confocal images for p75NTR and CD56 stainings in control (left panel) and PM (right panel) muscle. Yellow and red arrows indicate p75NTR positive or negative satellite cells respectively. Scale bar = 25 µm. B: Correlation between percentage of p75NTR positive satellite cells and ageing in non-myopathic tissues and in inflammatory myopathy biopsies.

DISCUSSION

Immune reactions play a central role in the pathogenesis of idiopathic inflammatory myopathies (Dalakas, 1991). In fact, though the inflammatory phase that follows tissue injury is important to remove necrotic material and to stimulate satellite cell activation (Cantini and Carraro, 1995; Merly et al., 1999), the chronic persistence of inflammatory cells and mediators may determine loss of muscle fibers and prevent appropriate tissue repair (Douglas et al., 2002). The presence of pro-inflammatory cytokines, such as IL-1, IL-2, IFN-γ and TNF-α has been well documented in inflammatory myopathies and considered detrimental for tissue homeostasis (Lepidi et al., 1998; Lundberg et al., 1997; De Bleecker et al., 1999; Kalovidouris et al., 1993). Satellite cells are likely critical targets for these mediators. In fact, several inflammatory cytokines, including IL-1α and IFN-γ, impair satellite cell proliferation and differentiation (Kalovidouris et al., 1993; Ji et al., 1998). As reported in PART 1 of this study, p75NTR positive myoblasts display an enriched repertoire of gene products involved in muscle differentiation and contraction, which confer enhanced myogenic properties when compared to p75NTR negative myoblasts. Furthermore, differentiation stimuli force p75NTR expression in p75NTR negative myoblasts, underlying the central role for this receptor in myogenesis. These data suggest that the loss of p75NTR may have direct functional consequences for myogenesis. So, we hypothesized that the inflammatory mediators may negatively modulate p75NTR expression in muscle precursor cells. Indeed, the *in vitro* experiments showed that both an innate (IL-1 α) and an adaptive (IFN-γ) immune factor reduced mRNA and protein levels of the neurotrophin receptor.

While expressed on a fraction of cultured myoblasts, p75NTR is displayed by the great majority of satellite cells *in vivo* in human healthy skeletal. Considering that regeneration takes place to some extent in inflammatory myopathies and is associated with p75NTR expression on newly forming fibers, we could expect maintained expression of p75NTR on the satellite cells. Here we report that a lower proportion of satellite cells in inflammatory myopathies bears p75NTR, and that this percentage decreases with ageing. Overall, we speculate that chronic exposure to inflammatory factors may be one of the causes for the reduction in the pool of p75NTR expressing satellite cells and that in the long term period this may lead to inadequate muscle repair.

PART 4:

p75NTR LIGANDS IN HUMAN SKELETAL MUSCLE: A ROLE FOR AUTOCRINE AND IMMUNE CELL DERIVED BDNF IN HUMAN MYOGENESIS AND MUSCLE REGENERATION

RESULTS

BDNF is the major neurotrophin produced in vitro by myoblasts and differentiating cells

First we analyzed BDNF, NGF and NT-3 expression in primary myoblast cultures. Quantitative RT-PCR revealed that the neurotrophin BDNF was expressed at higher levels that NGF and NT-3 in myoblasts under normal growth conditions (Figure 4.25A). Double immunofluorescence performed with a specific antibody showed BDNF protein in all myoblasts (Figure 4.25B).

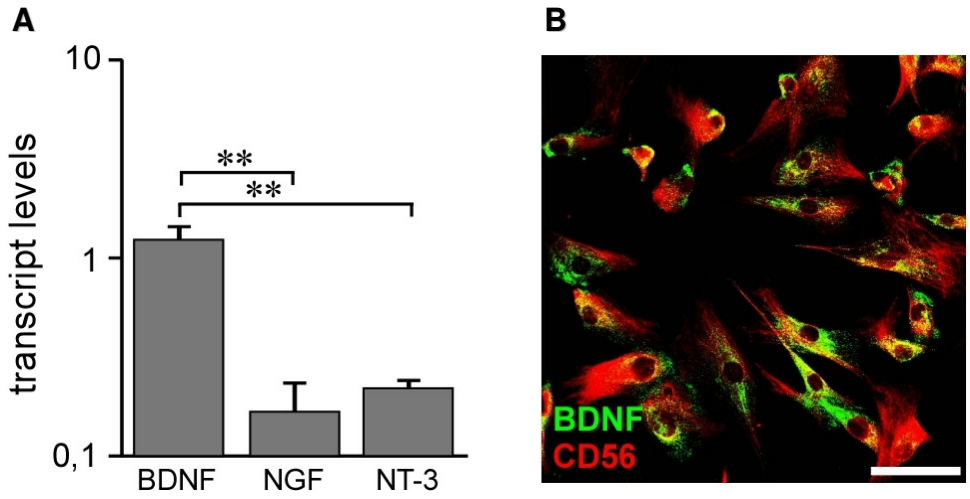


Figure 4.25. A: mRNA levels for BDNF, NGF and NT-3 in myoblast cultures in normal conditions. ** P<0.01. B: Double immunofluorescence for BDNF in CD56 positive myoblasts. Scale bar = 30 µm.

Then, we induced myoblast differentiation and fusion into multinucleated myotubes by the addition of specific culture medium, and monitored neurotrophins mRNA levels over two weeks. Quantitative Real-Time PCR performed on myotubes at day 13 showed that also in differentiating cells BDNF resulted the major expressed molecule (Figure 4.26).

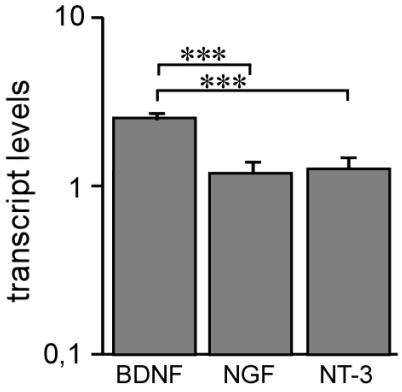


Figure 4.26. mRNA levels for BDNF, NGF and NT-3 in myotubes cultures at day 13 in normal conditions. *** P<0.001.

BDNF expression remained high during the first 10 days and increased slightly afterwards (Figure 4.27A, triangles). Double immunofluorescence confirmed the presence of BDNF in cultured myotubes (Figure 4.27B). As BDNF action may be mediated by two distinct receptors, we investigated the expression of TrkB and p75NTR at mRNA levels in parallel to BDNF. As previously described, p75NTR mRNA was expressed in undifferentiated myoblasts (Figure 4.27A, day 0, circles) and increased during myogenesis (Figure 4.27A, circles). In contrast, TrkB transcript levels were barely detectable both in myoblasts and in differentiated cultures (Figure 4.27A, squares).

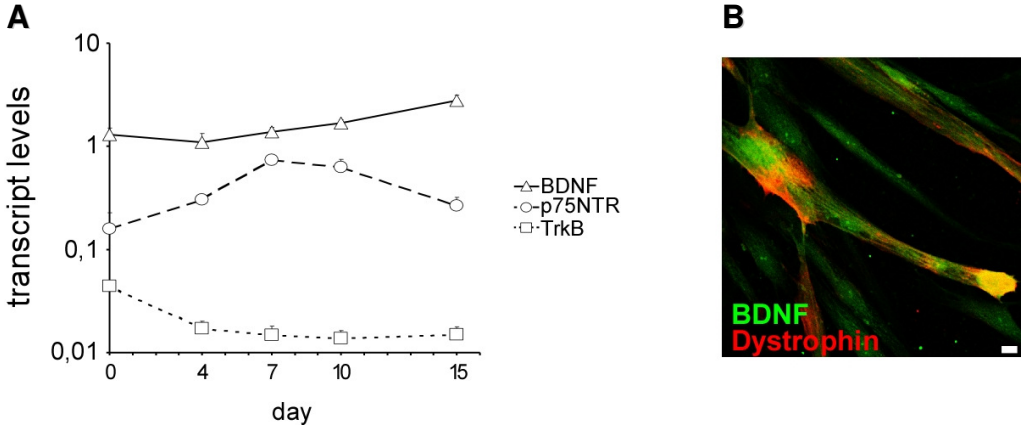


Figure 4.27. A: Regulation of BDNF, p75NTR and TrkB mRNA levels upon differentiation. Quantitative real-time PCR was performed for each timepoint. B: Double immunofluorescence for BDNF and dystrophin in mature myotubes induce to differentiate for 10 days. Scale bar 30 µm.

Overall, human muscle cells produce BDNF in vitro and may respond to it through p75NTR.

BDNF is displayed by satellite cells and mature myofibers in vivo

As p75NTR is a marker for satellite cells in vivo and myoblasts strongly produce BDNF in vitro, we checked expression of the neurotrophin in satellite cells in situ. Double immunofluorescence on non-myopathic tissues showed that satellite cells stained positive for p75NTR and BDNF (Figure 4.28A-B). Accurate quantification revealed that more than 80% of satellite cells produced the neurotrophin (Figure 4.28C).

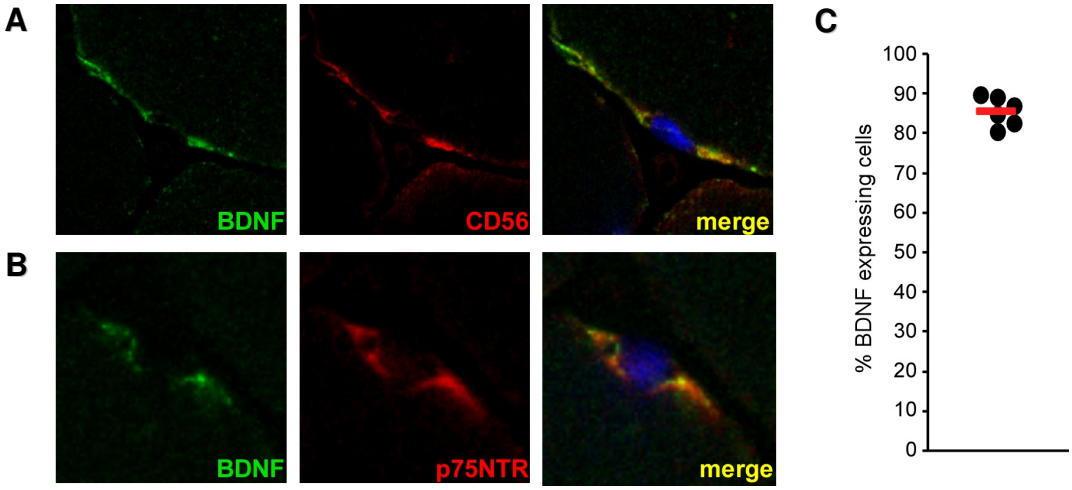


Figure 4.28. A: BDNF immunoreactivity in a CD56/NCAM positive satellite cell of adult skeletal muscle. B: Double immunofluorescence for the coexpression of BDNF and p75NTR in satellite cells. C. Percentage of positive satellite cells expressing BDNF in human adult skeletal muscle. Circles represent distinct tissue samples, horizontal bar represents average.

Then we analyzed BDNF expression and localization in mature muscle. First of all, we checked transcript levels in infant and adult healthy specimens. Total RNA was extracted by muscle biopsies and retro-transcribed. BDNF mRNA was detected at high levels in human muscle (Figure 4.29A). Interestingly its expression was lower in adult muscle (> 18 years old) than in infant muscle (< 10 years old), indicating a decrement in BDNF production upon aging. In harmony with in vitro observations on myotubes, immunohistochemistry on muscle biopsies confirmed that all mature myofibers were immunoreactive for this neurotrophin (Figure 4.29B).

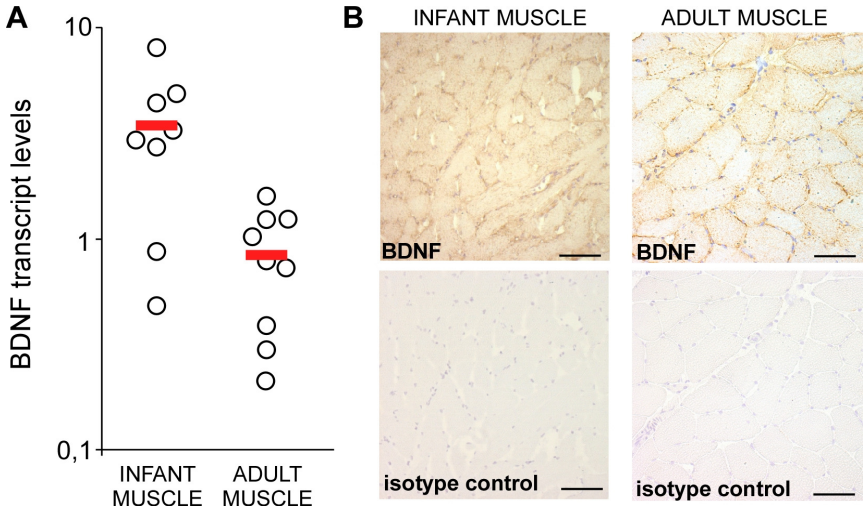


Figure 4.29. A: BDNF mRNA levels in infant and adult healthy muscle. B: Immunohistochemistry for BDNF and relative isotype control in human infant and adult skeletal muscle. Scale bar 100 µm.

In contrast, the receptor TrkB was displayed neither by satellite cells nor by myofibers but was restricted to vessels (Figure 4.30).

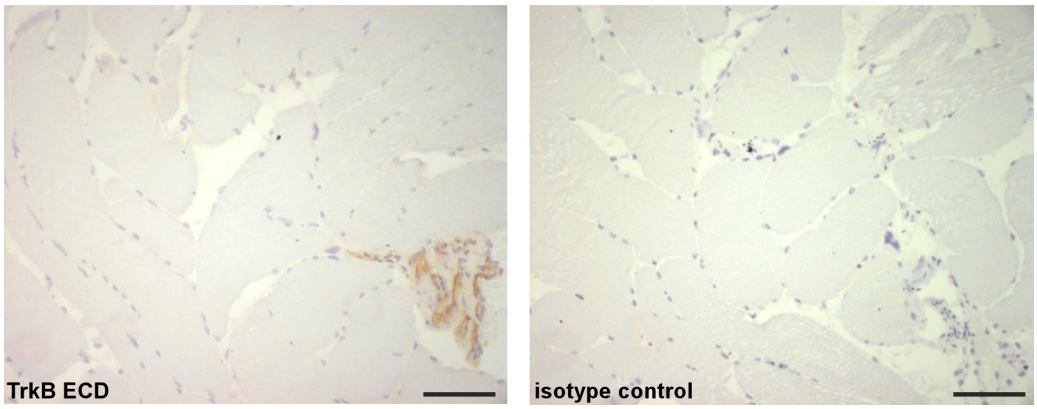


Figure 4.30. Immunohistochemistry for TrkB and relative isotype control in human adult skeletal muscle. Scale bar 100 µm.

These in vitro and in vivo observations suggest that BDNF may play a homeostatic role in human skeletal muscle and that p75NTR is the only available receptor for BDNF signalling in human muscle precursor cells.

BDNF regulates human myogenesis in vitro

We performed functional in vitro studies to evaluate the involvement of BDNF in human myogenesis. For this purpose we induced myoblast differentiation while hampering BDNF expression or activity. First, we reduced BDNF mRNA availability by specific RNA interference in undifferentiated myoblasts (Figure 4.31A), and then we induced in vitro differentiation for 6 days. Importantly, myoblasts transfected with specific BDNF siRNA showed lower fusion index than control cultures (*p value* = 0.03, Figure 4.31B). Second, we blocked BDNF protein activity in differentiating cultures by adding a specific neutralizing antibody. Parallel cultures where myoblasts were induced to differentiate in the presence of an isotype antibody were used as controls. At day 6 of differentiation myogenesis levels were significantly reduced in cultures exposed to the α-BDNF antibody (*p value* = 0.02) (Figure 4.31C).

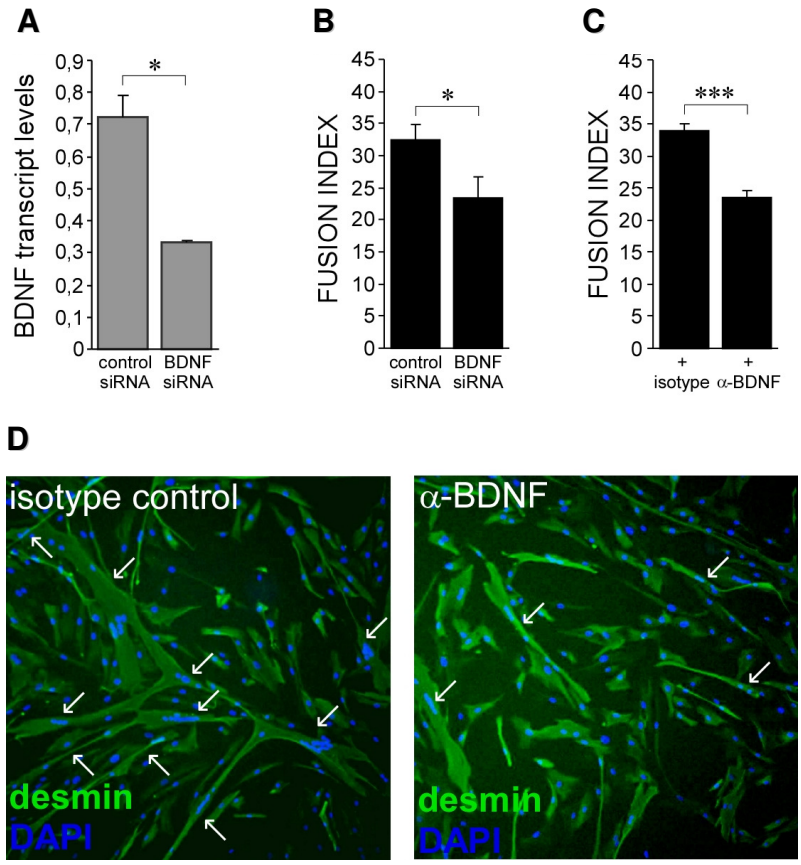


Figure 4.31. A: BDNF mRNA levels in myoblasts after silencing. B: fusion index in myotube cultures after BDNF silencing. C:Fusion index in a blocking experiment with anti-BDNF antibody or isotype control administered during cell differentiation. D: Representative stainings for desmin and DAPI in a blocking experiments. Arrows indicate myotubes (cells with more than 2 nuclei). * $P < 0.05$, *** $P<0.001$

Figure 4.31D report a representative staining for desmin in myotube cultures exposed to α-BDNF antibody or isotype control. White arrows highlight the higher number of multinucleated myotubes in control cultures than in antibody-treated cultures. In conclusion, the functional assays established that blockade of BDNF either at the transcript or at protein level impairs human myogenesis, demonstrating a role for BDNF in this muscle cell function.

In inflammatory myopathies immune cells are preferentially located near regenerating fibers and produce BDNF

We extended immunohistochemical analysis for BDNF to muscle biopsies from patients affected by inflammatory myopathies. Pathological conditions did not alter the signal for the neurotrophin in mature (CD56 negative) myofibers, while a number of CD56 positive regenerating fibers displayed enhanced immunoreactivity (Figure 4.32A,C). Importantly, the same fibers expressed the p75NTR receptor (Figure 4.32D), suggesting that the autocrine usage of BDNF is necessary for regeneration in vivo.

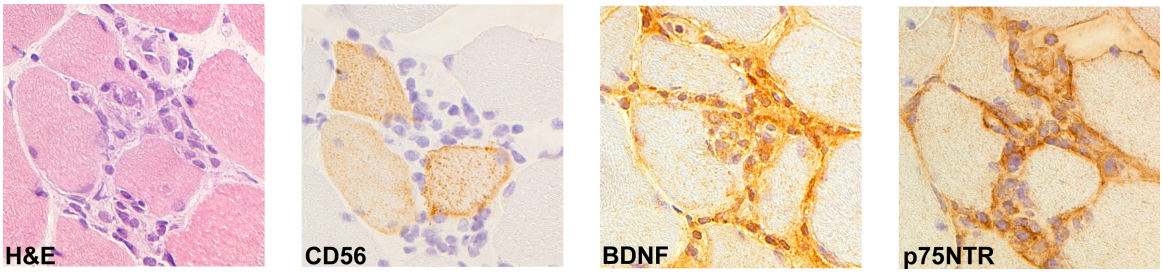


Figure 4.32. Immunohistochemistry for CD56 (B), BDNF (C), p75NTR (D), and haematoxilin-eosyn (A) in a DM biopsy. Scale bar 10 μm.

As expected, inflamed tissues were characterized by massive immune cell infiltration. We noticed that immune cells were not homogenously distributed within the tissue, but were mostly organized into clusters. Such clusters were topologically juxtaposed near regenerating fibers (Figure 4.33).

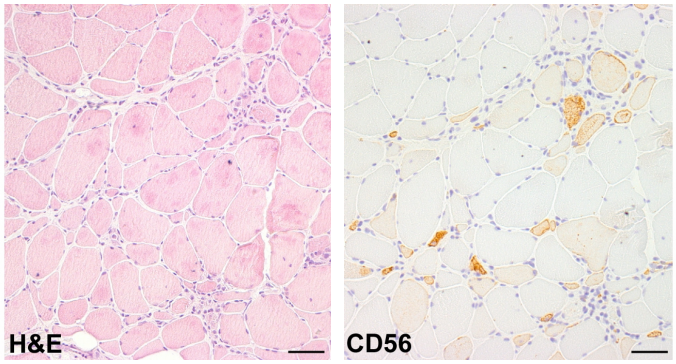


Figure 4.33. Immunohistochemistry CD56 and haematoxilin-eosyn in a PM biopsy. Scale bar 100 μm.

To quantify this observation we counted the number of immune cells in the vicinity of regenerating fibers or of mature myofibers in 8 biopsies from inflammatory myopathy patients. Such analysis confirmed that immune cells were preferentially located near newly forming fibers (p value = 0.027, Figure 4.34).

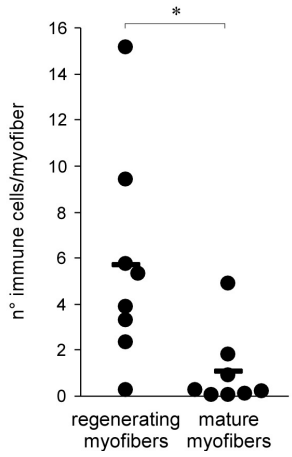


Figure 4.34. Number of immune cells localized in the proximity of regenerating or mature myofibers. Circles represent distinct tissue samples, horizontal bar represents average. * $P < 0.05$.

As inflammatory myopathies are considered autoimmune disorders of skeletal muscle, the possibility that infiltrating immune cells are pathogenic exists. In fact, it was shown that some immune cells, mainly located within myofibers, are activated and secrete cytolitic factors (Orimo et al, 1994). To further characterize the inflammatory infiltrates in inflamed muscle we checked the expression of the proliferation marker Ki-67 and of the cytolitic factor perforin. We found that T cells or macrophages positive for Ki-67 or

perforin were extremely rare, mainly located in direct contact with mature myofibers and not in the vicinity of CD56 positive regenerating fibers (Figure 4.35).

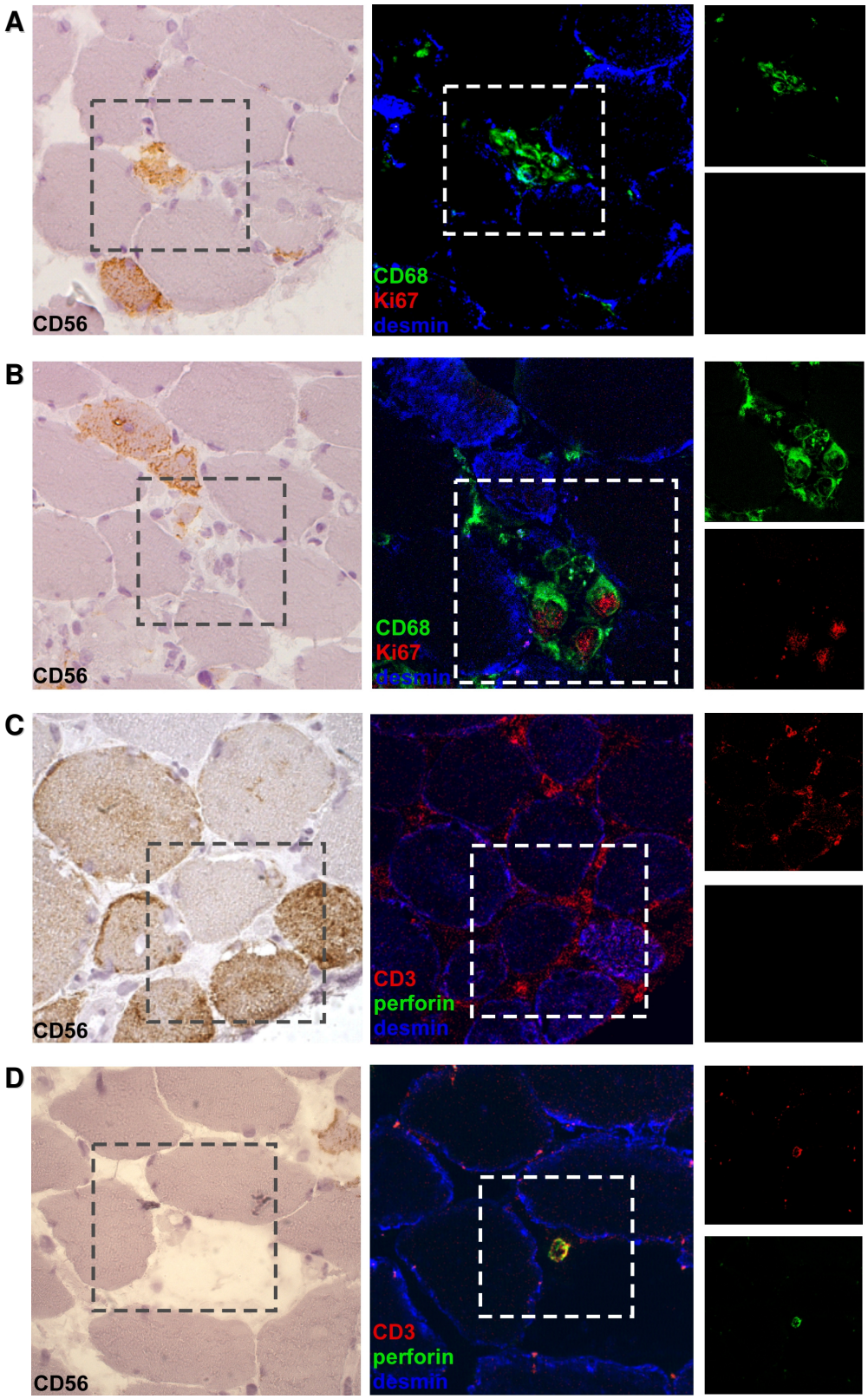


Figure 4.35. Immunofluorescence for CD3 or CD68 and Ki67 or perforin. Ki67 positive macrophages or perforin releasing T cells are localized near degenerating fibers (B and D) and not near regenerating fibers (A and C)

In contrast, immunohistochemistry for BDNF revealed plenty of immune cells strongly positive for the neurotrophin and frequently localized near p75NTR-expressing regenerating myofibers (Figure 4.32B-D). Accurate quantification of this observation confirmed that, while immune cells located near mature myofibers showed rarely staining for the neurotrophin, those cells in the vicinity of regenerating fibers often expressed BDNF (*p value* = 0.029, Figure 4.36).

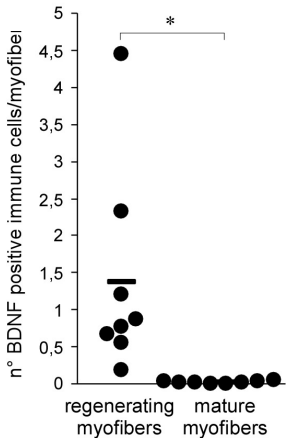


Figure 4.36. Number of BDNF positive immune cells localized in the proximity of regenerating or mature myofibers. Circles represent distinct tissue samples, horizontal bar represents average. * $P < 0.05$.

Fine histological characterization by double immunofluorescence and confocal microscopy showed that BDNF was produced by T lymphocytes (both CD4+ and CD8+ cells Figure 4.37A-B) and macrophages (Figure 4.37C) but not by B cells (not shown).

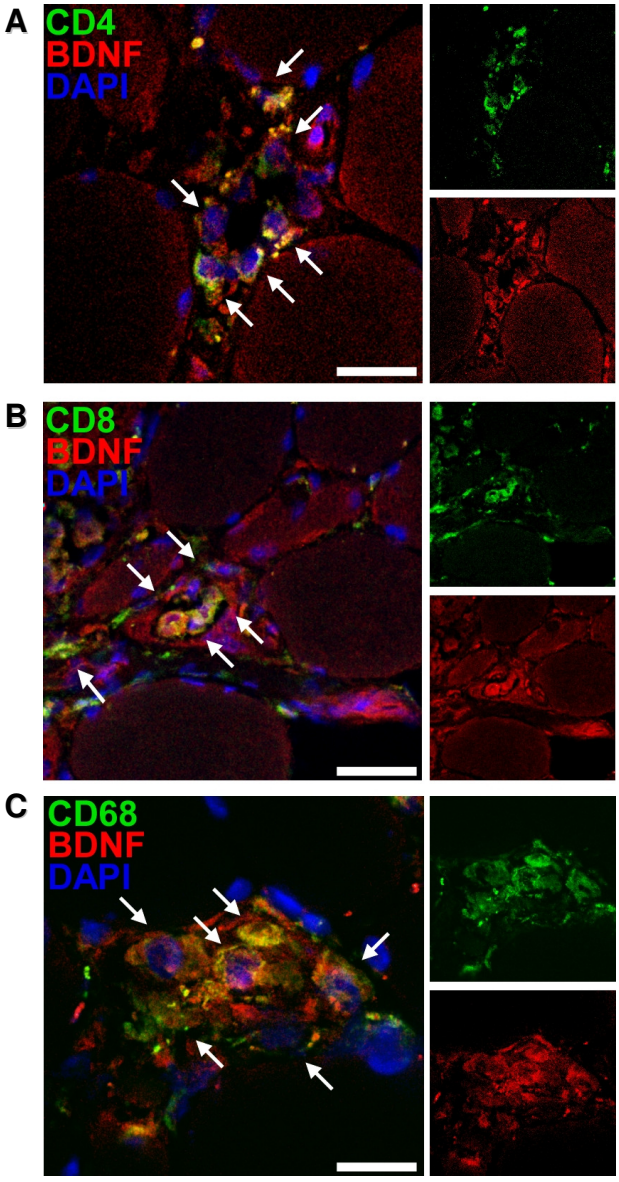


Figure 4.37. Double immunofluorescence for BDNF and CD4 (A), CD8 (B) or CD68 (C) in human myopathic muscle. Staining were performed on specimens with PM or IBM. Arrows indicate positively stained cells. Scale bar 30 μm.

In summary, in inflamed tissue both myocytes and immune cells contribute to BDNF production. The preferential distribution of BDNF producing immune cells around p75NTR positive regenerating fibers strongly suggests that such immune cells are not pathogenic but rather sustain skeletal muscle repair.

DISCUSSION

In this part of the study we identified the neurotrophin BDNF as the major neurotrophic factor produced by both skeletal muscle precursor cells and mature myofibers. In inflammatory myopathies several immune cells infiltrating skeletal muscle may contribute to BDNF production and preferentially accumulate near p75NTR positive regenerating fibers. Furthermore BDNF may positively regulate human myogenesis, suggesting this molecule as a candidate ligand to mediate p75NTR action in human muscle.

Human skeletal muscle regeneration requires the activation of satellite cells which is regulated by stimuli derived from the microenvironment, such as growth factors. Recent reports on rodent muscle highlighted the role of the neurotrophin BDNF in this process (Mousavi and Jasmin, 2006; Clow and Jasmin, 2010). In fact, BDNF is expressed in rodent satellite cells (Mousavi and Jasmin, 2006) and regulates myogenesis in vivo (Clow and Jasmin, 2010). In our study the fine characterization of the sources of BDNF in human skeletal muscle revealed that this neurotrophin is expressed by both precursor and differentiated cells. Quantification of satellite cells expressing the neurotrophin showed that the great majority of precursors is BDNF positive. This observation is in harmony with the in vivo expression of the p75NTR receptor. Consistent with in vivo data, high levels of BDNF were found in primary myoblasts and in multinucleated myotubes in vitro. Regarding the receptor TrkB, it has been detected at mRNA or protein level in total extracts from animal muscle (Ip et al., 2001; Funakoshi et al., 1993; Gomez-Pinilla et al., 2002), however its presence in muscle cells has not been described so far. Based on our data, the receptor TrkB is absent in human myocytes in vitro and in vivo, while p75NTR appears on BDNF expressing satellite cells and regenerating fibers as well as in cultured cells. These evidence indicate that the neurotrophin BDNF is commonly produced by human muscle cells and that muscle cells may be not only the source but also the target of BDNF due to the expression of the p75NTR receptor. As the neurotrophin receptor p75NTR is a key regulator of human myogenesis, we hypothesized that myoblast responses to BDNF could modulate skeletal muscle cell differentiation. Functional experiments conducted in the in vitro model of differentiation showed that the reduction in BDNF transcript levels or in protein availability hampered cell fusion. This is in accordance with the in vivo data obtained in a transgenic mouse model where BDNF

depletion was achieved in muscle precursor cells (Clow and Jasmin, 2010). Overall, these observations demonstrate that BDNF is essential for skeletal muscle differentiation and that its action is presumably mediated by the receptor p75NTR.

The characterization of muscle tissues from patients affected by inflammatory myopathies for BDNF protein expression led to novel additional observations. We showed here that in inflammatory myopathies immune cells are not evenly distributed within the tissue, but that they accumulate in regenerating areas. This might indicate an exacerbated immune response against newly-forming fibers. Several reports demonstrated that T cells in contact with myofibers are activated and clonally expanded *in situ*, indicating a local antigen-driven immune response. Other T lymphocytes near but not in contact with myofibers are not clonally expanded (Dalakas, 2001) and have been considered together with the innate immune cells as bystander inflammatory cells. Consistent with previous observations, in our specimens the activated T cells were located within muscle fibers but extremely rare. In contrast, T lymphocytes (and macrophages) were numerous in regenerating areas and they were not invading muscle fibers. Furthermore, these T cells were not in a proliferative state and did not produce cytolitic factors, dampening the hypothesis of an active immune response in such regions.

Opposed to the evidence about the involvement of immunity in the etiopathogenesis of inflammatory myopathies, some authors evidenced a crosstalk between immune and muscle cells which positively regulates muscle homeostasis and repair. For example, macrophages seem to play a primary role in muscle regeneration as they may regulate re-activation of muscle satellite cells. In vitro study on human muscle satellite cells demonstrated that myoblasts attract monocytes producing specific chemoattractants as monocyte chemoattractant protein-1, MP-derived chemokine and VEGF, and that the co-colture with macrophages increased myoblasts proliferation (Chazaud et al., 2003). Suppression of macrophages proliferation and differentiation, obtained with blockade of specific receptor, in mice after damage induction inhibits satellite cell proliferation and myotube formation (Segawa et al., 2008), indicating a clear role for these cells in promoting muscle regeneration after injury. Different activation states have been described for macrophages in vitro. Classical activation (M1) induces production of proinflammatory cytokines and reflects the primary state of macrophage activation upon tissue injury. Infact in human dystrophic muscle macrophages with inflammatory phenotype express TNF-a and IL-1 (Arnold et al., 2007) and are prominently concentrated in necrotic foci (Desguerre et al., 2009). Opposite, an anti-inflammatory

profile (M2) is associated to the phase of tissue repair with secretion by macrophages of IL-10 and IL-13 (Tidball, 2005; Arnold et al., 2007).

Here we would like to propose BDNF as a crosstalk factor between immune and muscle cells. In fact, we demonstrated the preferential accumulation of BDNF releasing immune cells near regenerating fibers. Immunofluorescence experiments combined with confocal microscopy revealed that both (CD4+ and CD8+) T lymphocytes and macrophages contributed to BDNF production. These findings are not entirely surprising as BDNF production by human thymocytes and circulating immune cells has been previously described (Berzi et al., 2008; Kerschensteiner et al., 1999; Besser and Wank, 1999). It becomes important however to consider that the localization of BDNF producing immune cells was preferentially within regeneration areas and that such cells were not activated and did not produce cytolitic factors. These observations support the hypothesis that protective immune responses may also take place in inflamed muscle and that BDNF may be a mediator for regeneration.

5. CONCLUSIONS

In this study we investigate the role of the neurotrophin receptor p75NTR and its ligand BDNF in several function of human skeletal muscle.

Some of the discussed data are already published in Colombo et al., 2011a; Colombo et al., 2011b; Colombo et al., 2011c.

In summary, regarding p75NTR, we demonstrate that:

1. p75NTR is a marker for satellite cells and regenerating fibers in vivo, since it is expressed by the great majority of muscle precursor cells in human adult skeletal muscle, and it is displayed on regenerating fibers in inflamed and dystrophic muscle (Colombo et al., 2011a).
2. It is expressed in vitro by cultured myoblasts. Its expression is transiently enhanced during differentiation from mononucleated cells to myotubes (Colombo et al., 2011a).
3. p75NTR defines differentiation-oriented muscle precursor cells. In fact p75NTR-expressing cells bears a transcriptional repertoire associated with muscle development and maturation. Gene products such as dysferlin, myogenin, actin, titin, myosin were enriched in p75NTR positive myoblasts (Colombo et al., 2011a).
4. p75NTR promotes precursor cell differentiation. In vitro experiments, such as receptor blockade and gene silencing, demonstrate that p75NTR induces specifically myogenesis, while not affecting other cell function as cell cycle entry and proliferation (Colombo et al., 2011a).
5. p75NTR signalling controls dystrophin expression, since this muscle protein starts to appear exclusively on p75NTR expressing myotubes during the first phases of differentiation, and p75NTR silencing leads to specific reduction of dystrophin expression on myotubes (Colombo et al., 2011a).
6. p75NTR is up-regulated in all inflammatory myopathies but not in BMD, both at transcript and protein level, suggesting a role for inflammatory mediators in induction of the neurotrophin receptor (Colombo et al., 2011b).
7. In inflamed muscle p75NTR is localized on distinct cell types. It is expressed in fibrotic areas at perimysium and endomysium, in mature skeletal muscle fibers at the cytoplasmic and membrane level and on immune cells (T cells, B cells, macrophages) (Colombo et al., 2011b).

8. Inflammatory factors such as IL-1 can induce p75NTR on myotubes and p75NTR silencing in differentiated myocytes exposed to inflammatory stimuli enhances cell apoptosis, indicating that neurotrophin signalling through p75NTR may mediate a protective tissue response to inflammation-induced injury in muscle (Colombo et al., 2011b).
9. On the contrary exposure to inflammatory stimuli decreases transcript and protein levels of p75NTR in muscle satellite cells and histological investigations show a reduction in the pool of p75NTR expressing satellite cells in inflammatory myopathies. These evidence suggest that chronic exposure to inflammatory factors may be one of the causes for the reduction in the pool of p75NTR expressing satellite cells and that in the long term period this may lead to inadequate muscle repair (Colombo et al., 2011c).

p75NTR can be activated by NGF, NT-3 and BDNF. Here we identify BDNF as the candidate molecule engaging p75NTR in human muscle.
In particular we report that:

1. BDNF is the major neurotrophin produced by myoblasts and myotubes in vitro
2. It is displayed by muscle satellite cells in vivo and mature myofibers. Its expression in skeletal muscle decreases upon aging.
3. In vitro and in vivo analyses reveal that TrkB, the high-affinity receptor for BDNF, is not expressed neither in muscle satellite cells nor in mature fibers, indicating that p75NTR is the only available receptor for BDNF signalling.
4. BDNF regulates muscle satellite cell differentiation. In fact BDNF gene silencing or protein blockade in myoblast cultures hamper myogenesis in vitro.
5. In inflammatory myopathies, immune cells are preferentially localized near p75NTR positive regenerating fibers and produce BDNF. Moreover, such cells are not activated and do not produce cytolitic factors.

Overall, we identify p75NTR as a novel marker for human differentiation-prone muscle precursor cells and demonstrate its involvement in myogenesis. It promotes resistance to inflammatory mediators suggest that neurotrophin signalling through p75NTR may support a tissue protective response to inflammation in skeletal myofibers. Moreover we

speculate that chronic exposure to inflammatory factors may cause the reduction in the pool of p75NTR expressing satellite cells and that in the long term period this may lead to inadequate muscle repair. The BDNF-p75NTR axis is present in human muscle precursor cells and supports myogenesis. BDNF is an autocrine factor produced by muscle and immune cells in inflamed muscle. Furthermore, the preferential localisation of BDNF producing immune cells near p75NTR positive regenerating fibers suggests that both muscle and immune cell derived BDNF is available to support the regeneration of p75NTR expressing fibers.

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