Mechanisms involved in the cross-talk between humoral and mechanical cues underlying muscle wasting in cachexia

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THE LIST OF ABBREVIATIONS

ActIIBR : Activin IIB Receptor
AKT (PKB) : Protein Kinase B
Alk: Activin receptor-like kinase
AMPK: AMP-activated protein kinase
Ang II: Angiotensin II
ATP: Adenosine tri-phosphate
Bnip3: BCL2 Interacting Protein 3
CM : C26 cell conditioned medium
DRR: Distal Regulatory Region
EBS: Ets-binding site
Edl : Extensor digitorum longus muscle
ElF4E: Eukaryotic translation initiation factor 4E
ElF4EBP1: Eukaryotic translation initiation factor 4E-binding protein 1
ERK: Extracellular signal-regulated kinases
FGF: Fibroblast Growth Factor
FoxO: Forkhead box O
Fst: Follistatin
GSk3β: Glycogen Synthase kinase 3β
HGF: Hepatocyte growth factor
HSA: Human Skeletal Actin
IEG: Immediate Early Genes
IFN: Interferon
IGF-1: Insulin Growth Factor 1
IkB: Inhibitor of kappa B
IL: Interleukin
JAK: janus kinase
JNK: c-Jun N-terminal kinases
lc3: microtubule-associated protein1 light chain 3
LIMK: LIM domain kinase
LLC: Lewis Lung Carcinoma
MAFbx/atrogin-1: Muscle Atrophy F-box
MCK: Muscle Creatine kinase

MEF2: Myocyte Enhancer Factor-2
MEK: Mitogen-activated protein kinase/extracellular signal-regulated kinase
MRFs: Myogenic regulatory factors
MRTFs: Myocardin Related Transcription Factors
mTOR: mammalian target of rapamycin
MuRF1: Muscle RING finger 1
Myf5: Myogenic factor 5
MyoD: Myogenic Differentiation
NCAM (CD56): Neural Cell Adhesion Molecule
NFkB: Nuclear Factor-kappa B
NLS: Nuclear Localization Signal
Pax : Paired box protein
Peg3: Paternally-expressed gene 3 protein
Pl3K: Phosphoinositide 3-kinase
ROCK: Rho associated protein kinase
ROS: Reactive Oxygen Species
SC: Satellite cells
SRE: Serum Response Element
SRF: Serum Response Factor
STARS: Striated muscle activator of Rho signaling
STAT: Signal Transducers and Activators of Transcription
TCF: Ternary complex factor
TGF-β: Tumor Growth Factor β
TNFα: Tumor Necrosis Factor α

THE THESIS EXPLAINED

Cachexia is characterised by an increased level of pro-inflammatory cytokines and other humoral factors, which promote autophagy and proteolysis, leading to muscle fiber atrophy. Satellite cells (SC) myogenic potential is also hampered and contributes to muscle wasting. Clinical studies showed that exercise training improves quality of life and survival of cancer patients. In agreement, we proved that wheel running maintains body weight of C26 tumor bearing mice, due to the rescue of muscle fiber size. However the molecular pathway implicated in response to exercise by the skeletal muscle are unknown. Exercise could act thought on muscle homeostasis by affecting the levels of circulating factors or by a mechanotransduction response of muscle cells. The latter may involve Serum Response Factor (SRF) response, which is known to be induced by mechanical cues transduced by the actin network. To dissociate these pathways, we used an *in vitro* model, consisting of mixed cultures of C2C12 myotubes and myoblasts, treated with C26 tumor cells conditioned medium (CM) containing pro-inflammatory cytokines secreted by C26. Our results showed that CM had a negative effect on muscle cells. To study the interaction between humoral and mechanical cues, mixed cultures were incubated in absence or presence of CM and in absence or presence of cyclic stretch. We observed that the effects of CM are counteracted by cyclic stretch, suggesting that mechanical effect is sufficient to activate a mechanotransduction pathway with pro-myogenic effects; we had several evidences on SRF involvement in this response, associated to an increase of pro-myogenic factors in the culture medium potentially able to recruit satellite cells. We suggest that the positive effects of exercise on cancer patient and murine muscle may be due to a mechanical response of muscle fibers to induce myogenic differentiation of satellite cells via humoral factors secreted by myofibers. This study could be the basis for developing training protocols to counteract cachexia in cancer patients.

INTRODUCTION

1. Muscle mass homeostasis in the adult

The skeletal muscle represents *circa* 40% of the total body weight. In addition to function in locomotion, posture and body temperature regulation, skeletal muscle plays an important role in the regulation of metabolism.

1.1 Skeletal muscle regeneration

During embryogenesis and foetal development, progenitor cells are involved in myofiber formation: a first wave of cellular fusion forms embryonic muscle fibers, while a second wave of fusion forms larger and more definitive muscle fibers, the fetal musculature, in response to morphogenic factors such as TGF-β, HGF, etc. Muscle growth is ensured by an increase of fiber cytoplasmic volume due to an accumulation of contractile proteins and by fusion of satellite cells with fibers.

Satellite cells (SC) are quiescent cells and represent only 2 to 7% of the cells associated to muscle fiber. They are characterized by the expression of the transcription factors Pax7 and Pax3, as well as the myogenic regulator factor Myf5, adhesion proteins such as NCAM (CD56), M-cadherin, integrin α7 and β; in addition, SC express transmembrane proteins syndecan 3 and 4 and also nuclear membrane protein like laminin A/C, emirin. SC derive from the pax3- and 7- expressing dermomyotome cells. The latter is involved in bulk muscle formation dependent on myogenic regulator factor expression (MRF). However, a subpopulation skips differentiation and, following a proliferative phase, resides in a quiescent state underneath the muscle basal lamina and around muscle fibers (hence the name of satellite cells). In adult life SC

play a major role in muscle regeneration, as shown by the total lack of regeneration following SC ablation (Lepper et al., 2011, Murphy et al., 2011),

SC fate is deeply affected by their microenvironment (the niche): cell-dependent, paracrine and mechanical cues all can drive their activation or maintain their quiescent state. Paracrine signals of muscle fiber origin include follistatin, myostatin and Wnt *(Guerci et al., 2012, Le Grand et al., 2009, Tran et al., 2012*).

Following muscle fiber injury, satellite cells are stimulated by Insulin Growth Factors (IGF), Fibroblast Growth Factor (FGF) and other factors of muscle fiber origin. Upon activation, satellite cells express MyoD in addition to pax7 and Myf5 and undergo symmetric and/or asymmetric division (as summerized in Figure 1) becoming myoblasts and, then, myocytes. They fuse with damage muscle and express Mrf4 and myosin heavy chain (Figure 1). SC proliferative and regenerative capacity is impressive in mice 5-7. SC associated to the Edl muscle give rise to almost 300 nuclei in culture in a biologically relevant time frame (Zammit et al., 2002); *in vivo* 50 cycles of extensive injury are not enough to deplete the muscle of SC and hamper regeneration (Sadeh et al., 1985).

1.2 Muscle masse regulation

The great plasticity of skeletal muscle allows the organism to adapt to a changing demand during adult life: a decrease or a stop of mechanical stimulation, fasting or increased loading induce muscle fiber atrophy and hypertrophy. Both phenomena are primarily due to metabolic events within the fibers, but SC are also involved (Mitchell et al., 2004).

Muscle atrophy can be due to an increase of ubiquitin-proteasome activity and a deregulation of autophagy. The proteasome system involves ubiquitin ligases, such as MuRF1 and MAFbx/atrogin 1 which target proteins for degradation. MyoD is included among

MAFbx targets, suggesting a regulatory role for ubiquitin-ligases in myogenic differentiation, in addition to a catabolic function. MuRF induces troponin I, actin and myosin degradation (Clarke et al., 2007; Cohen et al., 2009; Polge et al., 2011). Autophagy includes micro autophagy, lysosome-mediated substrate degradation, macro-autophagy induced by fusion of autophagosome with lysosomes, and mitophagy, which participates to mitochondrial network remodeling. Multiple pathways affect autophagy, such as the IGF1/AKT/FoxO, inflammatory cytokine-dependent NF-kB activation, and ActIIBR-dependent signal transduction shared by other TGF-β-family members, such as Activin A and myostatin

Downregulation of the IGF1/AKT pathway is known to induce muscle atrophy. In particular, IGF1 downregulation induces AKT dephosphorylation in cancer cachexia (Costello et al., 2006); upon this condition, AKT cannot activate the mTOR complex, which, in turn, is not able to induce transcription factor activity for protein synthesis via phosphor-S6 kinase and EIF4EBP1 (the latter releases the initiation factor of traduction, eIF4E). The lack of AKT-dependent phosphorilation of the transcription factor FoxO1, 3 and 4, results in the nuclear translocation of these factors, which are then able to recognize target genes, such as MAFbx/Atrogin-1 and MURF1. Moreover, it was shown that FoxO3 is implicated in autophagy via the induction of gene expression like LC3 and Bnip3 (Romanello et al., 2015; Luo et al., 2014). A well known pro-atrophic pathway is the cytokine dependent NF-kB activation. NF-kB is maintained in inactive state by a binding with its inhibitor lkB. A increase of pro-inflammatory cytokines concentration, in particular TNFα (Tumor necrosis factor α), activates a kinase IKK which phosphorylates IkB, thus promoting its ubiquitinylation and degradation. In this way, NF-kB is released and can translocate to the nucleus to active the transcription of NF-kB dependent genes such as FoxO.

The role of TGF family members is also well known to play a pivotal role in muscle homeostasis. Myostatin and Activin A are able to

recognize the Activin A receptor type IIB, which dimerize with receptor type I, Alk4, Alk5 (Activin receptor-like kinase 4 or 5). Following dimerization, Alk phosphorylate the complex smad2/3, which upon smad4 recruitment translocates to the nucleus and represses the transcription of genes implicated in muscle differentiation, like the MRFs. In parallel, phosphor-smad2-3 induces phosphorylation of AKT, which allows FoxO nuclear translocation to induce the transcription of atrogenic genes for muscle atrophy.

Hypertrophy is a muscle response to environmental changes opposite of atrophy. In fact, the volume of myofiber can increase following mechanical cues or alteration of hormonal signalling. Hypertrophy is due to an increase of protein synthesis, the inhibition of proteolysis, and an increase in satellite cells fusion with myofibers.

The IGF1/AKT/mTOR pathway plays a primary role in muscle hypertrophy. In fact, IGF-1 activates the PI3K/AKT pathway. AKT activates mTOR by the formation of mTOR/raptor, complex which, in turn, activates phosphor-S6 kinase and EIF4EBP1, ultimately leading to increase protein synthesis. In fact, EIF4EBP1 releases eIF4E, as initiator of traduction factor. Furthermore, AKT represses GSK3β (Glycogen Synthase Kinase 3β), which inhibits the initiator of traduction factor eIF2B. In addition, AKT phosphorylates transcription factors of the FoxO family, thus hampering their translocation in nuclei and repressing the transcription of atrogenic genes.

Follistatin (Fst) is a TGF family member, whose principal function is the inhibition of myostatin and Activin A. Fst is an autocrine glycoprotein secreted by skeletal muscle (Hansen et al., 2016). In human, 2 isoforms of follistatin, Fst288 and Fst315, are the tissular and circulating forms, respectively. Fst can sequester Activin A and myostatin and prevents their interaction with Activin IIB receptor (ActIIBR). Fst has an affinity for Activin A of 1,7nM and for

myostatin of 12.3nM (Cash et al., 2009). So Fst counteracts muscle atrophy by preventing primarily Activin A as well as myostatin, from recognizing the Activin IIB receptor. Thus the downstream activation of the complex smad2/3 is not triggered by phosphorylation. Ultimately, smad2/3 remains in the cytoplasm and cannot repress MRFs expression.

From the aforementioned pathways, it is evident that extensive cross-talk exists among different pathways, which are mutually exclusive, leading to atrophy or hypertrophy.

2. Cancer effects on muscle mass

A tumor secretes many circulating factor promoting muscle wasting, like pro-inflammatory cytokines, but chemotherapy commonly used against cancer also negatively affects muscle mass, independently of tumor mass (Damrauer et al., 2008) adding up atrophying effects. Many studies demonstrate toxicity effects of chemotherapy *per se,* which worsens cancer cachexia in cancer patients (Andreyev et al., 1998).

Cachexia is a multifactorial syndrome associated to a chronic or acute disease like cancer and characterized by a body weight loss of at least 5% in one year on presence of illness and at least three of the following criteria: decreased muscle strength, fatigue, anorexia and abnormal biochemistry.

Indeed muscle atrophy is associated to muscle strength loss, due to sarcomere degradation in the myofibers. Anorexia may or may not be associated to cachexia; however, it further decreases muscle mass by a lack of nutriment to support protein homeostasis. When a patient enters this vicious cycle, fatigue contributes to further diminish physical activity and, thus,

contributes to muscle atrophy exacerbation, with negative consequences on the patient's quality of life.

Cancer cachexia is characterized by an increased level of pro-inflammatory cytokines, such as Tumor necrosis factor α (TNFα), Interleukin 6 (Il-6), Interferon (IFN), due to secretion by the tumor, but also because of immune system and treatment reaction of the patients. In respect to myofibers much less known are the effects of the tumor on muscle stem cells. It was shown that the number of satellite cells increases in cachexia but their myogenic potential is inhibited *in vivo* in cachectic mice (He et al., 2013). This occurs in spite of the fact that satellite cells keep their myogenic differentiation capacity *in vitro*. This suggests that the microenvironment inhibits myogenic differentiation of satellite cells *in vivo*. It was shown that ratio between Pax7 and MyoD increases in cachexia, with Pax7 being inhibitory for satellite cell differentiation and muscle regeneration. NFkB is transcriptional factor which plays a role in proliferation, renewal and survival of cell (Peterson et al., 2011). In cancer cachexia, it was shown that high NFkB activity mediated Pax7 dysregulation (He et al., 2013) and inhibited myogenic differentiation likely by decreasing *in vivo* and *in vitro* MyoD RNA levels (Guttridge et al., 2000). Thus, in cachexia, NFkB activity increases and keeps satellite cells in an undifferentiated state, increases Pax7 expression, induces decrease of MyoD RNA and participates to protein degradation by increasing FoxO expression. Consistently, many studies demonstrated that NFkB activity is induced inflammatory factors like TNFα, angiotensin II (Ang II), TGF family members, as well as by Reactive oxygen species (ROS), which are increased in cancer cachexia. For what concerns the anti-differentiative and pro-catabolic effects of cytokines, many studies showed that TNFα is sufficient to induce myotube atrophy *in vitro* (Miller et al., 1988) and myofiber atrophy *in vivo* (Langen et al., 2006); in addition, TNF inhibits muscle regeneration (Coletti et al., 2005). Some groups showed that TNFα activates NFkB activity to block muscle

regeneration (Guttridge et al., 2000; Ladner et al., 2003), while others demonstrated the implication of a p53 cell death pathway including PW1/Peg3, Pax- and caspases-dependent signaling downstream TNFα (Moresi et al., 2008; Schwarzkopf et al., 2006). In cachexia, IL-6 level also increases, and many studies *in vivo* and *in vitro* demonstrated IL-6 activates JAK/STAT3 (Pelosi et al., 2014; Bonetto et al., 2012). Additional pathways downstream of IL-6 are the ERK and PI3K/AKT pathways (Munoz et al., 2013 and Zhang et al., 2013). *In vitro* studies show that IL-6 impairs myogenic differentiation by modulation of p90RSK/eEF2 and mTOR/p70S6K (Pelosi et al., 2014).

Ang II increases in animal model of cancer cachexia by inhibition of angiotensin converting enzyme (Sanders et al., 2005). An increase of Ang II in the microenvironment of satellite cells induces the activation of smad2/3 which plays a catabolic function by blockage of AKT/ m TOR signaling (Rommel et al., 2001). A study showed a further, intriguing link between Ang II and NFkB activation (Ruiz-Ortega et al., 2000), even though this was not in the context of cancer cachexia.

The smad2/3 complex can also be involved in response to increase levels of TGF family member, such as Myostatin. The latter inhibits MyoD expression in satellite cells (Langley et al., 2002). Elevated levels of myostatin and Activin A, TGF family members secreted by muscle and tumor (Chen et al., 2014; Han et al., 2013), correlate with cancer patients who develop cachexia (Bonetto et al., 2009; Chacon-Cabrera et al., 2014; Zhou et al., 2010). A study showed that myostatin gene inactivation prevents the severe loss of skeletal muscle mass in Lewis Lung carcinoma bearing mice. The inactivation of myostatin also reduced the growth of LLC tumors and increased survival of tumor bearing mice. This rescue can be explained by a decrease of genes expression involved in tumor metabolism and Activin signalling, thus a reduction of apoptosis activity (Gallot et al., 2014).

Other studies in tumor bearing mice have also demonstrated that the administration of Activin IIB Receptor preserves skeletal muscle mass (Benny Klimek et al., 2010), restores muscle strength (Busquets et al., 2012) and increases lifespan (Zhou et al., 2010). Targeting ActIIBR ligands can also affect Activins, including Activin A (Zhou et al., 2010). From the all above, it is clear that TGF family members have a key role in cancer cachexia; therefore, TGF family members response to physical exercise and a modulation of their concentration may affect and counteract cancer cachexia via training exercise.

3. Exercise effect on cancer cachexia

In clinical practice a multimodal intervention is often used against cachexia, based on the following main axes: nutritional support, appetite stimulants, anti-inflammatory agents, stimulation of pro-anabolic pathways and physical exercise. However, physical activity was proven sufficient for a positive clinical effect on survival and lifestyle on cancer patients.

3.1 Benefit of exercise practice during cancer cachexia

Recently, it was shown in tumor-bearing mice performing wheel running that natural killer cells were recruited to attack the tumor and decreased its mass, thus contributing to remove a primary cause of cachexia. Nonetheless, it is clear that exercise has direct effects on muscle cells and tissues, in both physiological and pathological conditions (Pigna et al., 2016; Padilha et al., 2017; Aversa et al., 2017).

Epidemiological studies and one clinical trial showed that physical activity that occurs post-diagnosis improves prognosis, rather that exercise habits established before disease (breast and colorectal cancers): indeed, physical activity after diagnosis lowers the risk of

both cancer-specific and overall mortality, ameliorates patients' quality of life and increases survival (Holmes et al., 2005; Meyerhardt et al., 2006; Irwin et al., 2008; Holick et al., 2008).

3.2 Molecular pathway involved in exercise response

It is worth noting that exercise used as a support to medical treatments may vary significantly in intensity and metabolic impact. Resistance exercise training is defined as multiple repetitions of static or dynamic muscular contractions performed against a high load or resistance. Resistance training increases muscle mass in healthy subjects and attenuates muscle wasting associated with ageing. Endurance exercise training consists of performing low-to-medium intensity exercise for long periods of time. Endurance training (such as running, cycling, or swimming) involves the use of several large groups of muscles and tends to be aerobic. Adaptations to endurance exercise include improved oxygen delivery to muscles and their increased oxidative capacity (Hakkinen et al., 2005).

The IGF1/AKT/FoxO pathway, known to induce muscle hypertrophy via protein synthesis and a reduction of protein degradation, is activated by both training types. Aerobic exercise induces mitochondrial biogenesis via AMPK activity, which, in turn, induces PGC1a activation leading to potentiated ATP synthesis. At day 1 after overload, which mimics resistance training, the mitogen-activated protein kinase (MEK)/extracellular signal-regulated kinase (ERK)-dependent pathway is activated. This pathway can activate mTOR to induce protein synthesis. However, ERK1/2 are also activated by endurance training via integrins like integrins $\beta 1$ and $\alpha 7$ which form a dimere, a mechanotransductor on cytoplasmic membrane.

Some studies showed that skeletal muscle secretes TGF family members in response of exercise.

As reported above, follistatin sequesters myostatin and Activin A and all are associated with the regulation of muscle growth (Lee et al., 2001; Bilezikjian et al., 2004). The mechanism of regulation of exercise induced follistatin secretion is not clear, but it was shown that exercise training increased follistatin expression in rat (Guizoni et al., 2013; Rashidlamir A et al., 2016). More recently, a study demonstrated that liver-dependent follistatin secretion increases in response to exercise in humans (Hansen et al., 2016). This is of particular relevance due to the key role of follistatin in muscle homeostasis and hypertrophy (Sepulveda et al., 2015; Winbanks et al., 2012; Zhu et al., 2011). In summary, these results suggest that follistatin could be involved in the beneficial effect of physical activity.

Since the maintenance of muscle mass is the primary goal to counteract cachexia, resistance training is often preferred to endurance exercise. Indeed, while resistance exercise induces an anabolic action, endurance exercise stimulates oxidative metabolic adaptations with little changes in muscle mass. Resistance exercise in healthy conditions stimulates the Akt/mTOR signaling pathway, a pathway that was shown to be unaffected or even hyperactivated in tumor-bearing animals (Penna et al., 2010), suggesting the uselessness of Akt stimulation to prevent muscle wasting in cancer cachexia. On the contrary, soft endurance exercise might counteract oxidative stress found in experimental cachexia (White et al., 2011). Finally, endurance exercise induces physiological adaptations resulting in the attenuation of the inflammatory response (Gleeson et al., 2011; Argiles et al., 2011). For these reasons, while fewer studies are available on endurance exercise, the latter can represent an innovative, more efficient intervention against cachexia, in respect to resistance exercise.

4. Serum response factor

4.1 SRF expression

The *SRF* gene is conserved from drosophila to humans (Affolter et al., 1994). The unique *SRF gene* is located on chromosome 6p2.1 in human and 17 in mouse. It has 7 exons separated by 6 introns and has 10 607 pair of bases. The transcription of SRF gene gives rise to two messager RNAs of 4,5 and 2,5kb, containing different 3' non-encoding regions (Chai et al., 2002).

The SRF protein has 508 amino-acids and a molecular weight of 67kDa. The N-terminal region contains nuclear localisation signal (NLS). The medium region is characterised by the presence of MADS domain/MADS box, which contains the dimerisation domain of SRF and a DNA fixation domain. In the C-terminal region SRF has the domain responsible for transcriptional activity (Chai et al., 2002; Johansen et al., 1993).

4.2 SRF target genes

Upon activation, SRF forms a homodimere and binds the specific sequence CC(A/T)GG, called CArG box (Minty et al., 1986; Treisman et al., 1987). SRF target genes are characterized by a promoter region containing one or many CArG boxes. In humans, almost 300 genes have a CArG box and are activated by SRF that is about 1% of genome (Modak et al., 2010). SRF gene itself has two functional CArG boxes in its promoter, thus it is its own target. In addition, SRF regulates the expression of immediate early genes (IEG) like c-fos, junB, erg-1 and erg-2, so called since their expression is quickly activated after a given stimulus. These genes regulate other genes involved in the cell cycle or coding for growth factors. SRF

regulates cell proliferation and growth via IEG. However, some studies shown SRF is not necessary for cell proliferation (Fleige et al., 2007; Koegel et al., 2009), suggesting a compensatory pathway to maintain cell growth in absence of SRF.

SRF can recognize many genes implicated in the organization and architecture of cytoskeletal, such as vinculin, profiling, tropomyosin, actinin α1 etc. (Chai et al., 2002; Sun et al., 2006). SRF also regulates the expression of genes involved in cell adhesion like integrin α1, 5, 9 and β1 (Miano et al., 2007).

SRF plays a pivotal role in muscle tissues. Indeed, SRF controls the expression of numerous muscle genes, including MyoD, actin α1 (skeletal muscle), myosin heavy chain, myosin light chain, troponin and many others (Chai et al., 2002; Sun et al., 2006).

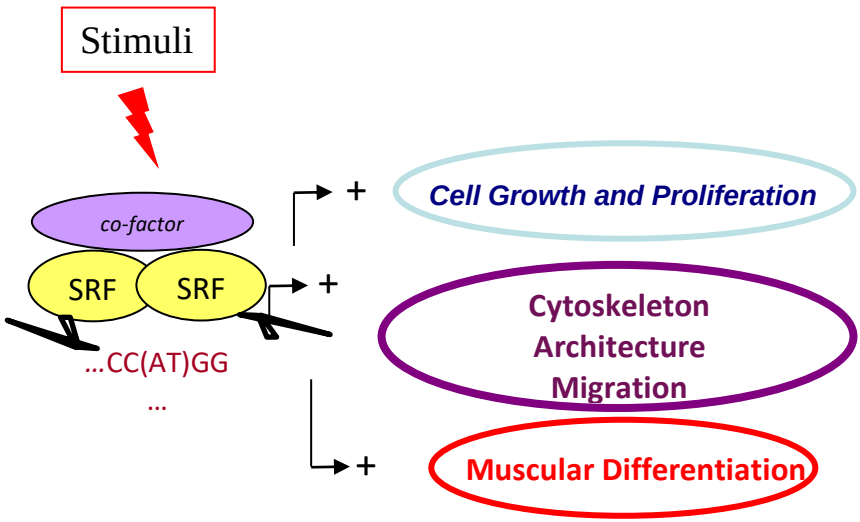


Figure 4: Roles of serum Response Factor from Chai J, 2002.

4.3 Regulation of SRF activity by Rho/actin/MRTFs pathway

SRF by itself is a low transactivator and it needs coactivators such as ternary complex factors (TCF) or Myocardin-related transcription factors (MRTFs) to induce the transcription of SRF target genes (Cen et al., 2003; Schroter et al., 1990). TCF family members are activated via phosphorylation by MAPK like ERK, JNK or p38. The phosphorylation of TCF induces the inhibition of interaction between it C domain and ETS domain, which, in turn, increases DNA affinity (Posern et al., 2006; Yang et al., 1999). TCF forms a complex with SRF and a Serum response element (SRE) in the promoter of target genes. In this way, the ETS domain recognizes an EBS region near of CArG box in promoter of SRF target genes.

MRTFs show high homology with myocardin, another cardiac-muscle specific SRF cofactor. The two MRTFs, MRTFA (MAL/MKL1/SAC) and MRTF-B (MAL16/MKL2), are ubiquitary. Their subcellular localization is regulated by actin dynamics. Some studies show that MRTFA is localized most of the time in the cytoplasm and that stimulation induces its accumulation in the nucleus (Miralles et al., 2003). Modulation of actin dynamics mediates signal-induced SRF transcriptional activity: stabilization of filamentous actin (F-actin) by the actin-binding drug, jasplakinolide, is sufficient to activate SRF without extracellular stimuli, but overexpression of actin inhibits SRF (Sotiropoulos et al., 1999). The Rho family of Ras-related GTPases (RhoA), controls the polymerization of the actin cytoskeleton in response to extracellular signals, but also myosin-based contractility, focal adhesion formation, transformation and cytokineses (Olson et al., 2010). RhoA regulates formines like mDia and ROCK activity (Rho-associated protein kinase). mDia controls profiline and stimulate

its polymerization. ROCK controls LIMK activity (LIM domain kinase), which induces actin polymerisation by repression of cofiline activity (Miralles et al., 2003; Pollard et al., 2003, dos Remedios et al., 2003). Profilin and cofilin regulate concentration of monomeric actin (Globular-actin/G-actin) and transport of G-actin between nuclei and cytoplasm. In fact, the association of cofilin with importin 9 allows the import of monomeric actin in the nucleus; on the contrary, interaction between profiling and exportin 6 induces G actin exportation to cytoplasm (Dopie et al., 2012). Striated muscle activator of Rho signalling (STARS) controls MRTFA localization directly by actin polymerisation and indirectly via the activation of RhoA (Kuwahara et al., 2005; Zheng et al., 2009).

Since activation of the Rho/actin pathway is sufficient to induce MRTFA nuclear import, it is worth knowing monomeric actin binds the RPEL (RPxxxEL) domain of MRTFA, which plays the role of nuclear localization signal (NLS) (Pawlowski et al., 2010). The importation of MRTFA requires importin α and β activity, which is inhibited by monomeric actin, due to a modulation of steric conformation of MRTFA which hide NLS during actin binding (Hirano et al., 2010). So, when MRTFA binds monomeric actin, NLS sequence is hidden for importin, and MRTFA cannot be imported in the nucleus. When actin is poorly polymerized, the cytoplasmic actin sequesters MRTFA in the cytoplasm (Posern et al., 2004). In addition, nuclear export of MRTFA is facilitated by nuclear G actin binding and nuclear G-actin prevents activation of SRF target genes by nuclear MRTFA (Vartiainen et al., 2007). In this manner, the actin-MRTFA-SRF circuit permits the specific modulation of gene expression, in a synergy with cytoskeletal dynamic (Olson et al., 2010). A recent study demonstrated that MICAL-2, an atypical actin-regulatory nuclear protein, regulates nuclear actin and promotes the depolymerization of nuclear actin upon redox stimuli and facilitates MRTFA-SRF-dependent gene expression (Lundquist et al., 2014). SRF activity is regulated by actin dynamics via MRTFs and SRF controls at the same time the transcription of actin and actin binding protein

(ABP), which, in turn, regulate polymerisation and depolymerisation of actin (Pipes et al., 2006; Olson et al., 2010).

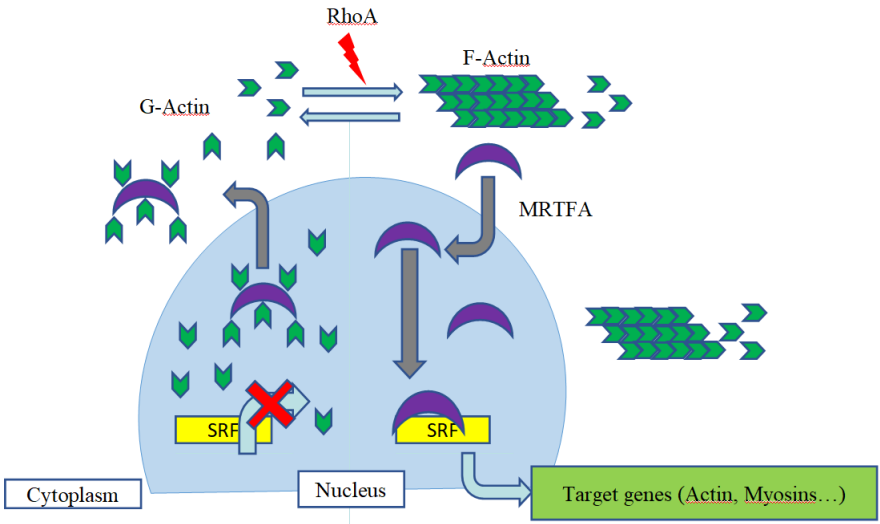


Figure 6: Regulation of SRF activity by Rho/actin/MRTFs pathway

4.4 SRF Knock-out in skeletal muscle cells

SRF is necessary for muscle cell proliferation and differentiation, as shown *in vitro*, by SRF knock-out in the myogenic cell line C2C12, by anti-sense RNA (Soulez et al., 1996). Consistently, the inhibition of SRF activity in C2C12 by antibodies or by competition with SRE, prevents myogenic regulator factor expression, MyoD (Gauthier-Rouviere et al., 1996).

SRF knock-out in embryonic skeletal muscle showed that SRF plays a key role in skeletal muscle development (Li et al., 2005). A SRF KO model used Cre recombinase dependent of myogenin promoter called Myo-Cre, thus SRF KO is possible in skeletal

muscle from E9.5 on. Another SRF KO model had Cre expression led by creatine kinase promoter (MCK), which permitted the late deletion of SRF In both models, there was a several muscle hyperplasia with a fail of hypertrophy capacity in skeletal muscle and finally the death of mice during perinatal period (Li et al., 2005).

To exclude a role of SRF in early myogenesis, a study with a Myf5-SRF conditional KO was done. However, this study showed a hypotrophic phenotype in Myf5-SRF conditional KO mice similar to the one of the MyoD-null mice. Thus, the hypotrophy could be due to SRF alteration involved in myogenic differentiation via MyoD. MyoD gene contains a SRF binding CArG-like element in distal regulatory region (DRR). This sequence is involved in the transcriptional activation of MyoD in myoblasts and during muscle regeneration (L'honore et al., 2003). MyoD DRR contains a binding site for SRF and also for Myocyte Enhancer Factor 2 (MEF2); MEF2 is a muscle transcription factor which controls myogenic program. So MyoD is controlled by both SRF and MEF2.

In postnatal muscle, muscle-fiber specific SRF deletion under the promoter of Human Skeletal Actin-Cre (HSA) induced a severe skeletal muscle mass reduction and hampered myogenic regeneration (Charvet et al., 2006). At birth, mice seemed normal but at 6 weeks of age 50% of the population died due to muscle growth anomaly: null mice displayed very small myofibers with larger interstitial space than control mice. Moreover, myofibers presented abnormal sarcomeric organisation and an accumulation of glycogen (Charvet et al., 2006). A more recent study in a murine model with tamoxifen-inducible SRF deletion showed that the muscle fiber specific SRF KO blocks hypertrophy due to overload and impairs satellite cell proliferation and recruitment to fuse with myofibers (Guerci et al., 2012). This work also showed that in response to increased workload, myofibers secrete IL-6 and IL-4 in a SRF-dependent manner. SRF leads to IL-6 and lL-4 expression via Cox2 and induces proliferation of satellite cells and their fusion with myofibers ultimately promoting hypertrophy (Guerci et al.,

2012).

The same laboratory showed that SRF is an important key mediator of mechanotransduction in skeletal muscle via the actin-MRTF-SRF axis. A deletion of MRTFA in satellite cells induces a downregulation of MyoD expression (Mokalled et al., 2012). Inhibition of this axis induced skeletal muscle atrophy (Collard et al., 2014). In addition, this laboratory showed that SRF is not indispensable for the MyoD expression and for proliferation of satellite cells. Rather, SRF plays an important role in the crosstalk between satellite cell and the myofiber. A recent study demonstrated that in myoblasts, the necessity of SRF expression in both partners to fuse, is symmetric fusion (unpublished results, personal communication) (Figure 9).

Surprisingly, a SRF deletion in adult mice does not alter mouse phenotype despite a high decrease of SRF until 5 months after tamoxifen injection. After this period, a premature ageing is observed with a decrease of myofiber size, disorganization of sarcomeres, and fast to slow fiber type transition (Lahoute et al., 2008). Worth nonetheless noting that in human and mouse, SRF expression in muscle decreases during ageing (Lahoute et al., 2008, Sakuma et al., 2008).

OBJECTIVES

Exercise increases life span in cancer patients and rescues muscle homeostasis in animal models of cancer cachexia. Exercise has pleiotropic effects, directly affecting the musculoskeletal apparatus but also the endocrine, immunological, cardiovascular and neurological apparatuses. In spite of the importance of muscle stem cell dysfunction for cachexia that we and others contributed to demonstrate, whether exercise directly affects muscle cells is not known; in addition, it has never been demonstrated whether secreted factors and/or mechanical stimulation, both phenomena associated to exercise, mediate exercise beneficial effects on muscle cell homeostasis and myogenic potential. Acquiring additional information on exercise targets would allow to improve exercise-based protocols against cancer cachexia.

Primarily, I strived to determine whether an *in vitro* model (consisting of cocultures of myotubes and myoblasts, in the absence or presence of tumor-derived factors) simulate myotube atrophy observed *in vivo* in the presence of cancer.

The major objectives of this thesis are the following:

1) To demonstrate whether a purely mechanical treatment is sufficient to counteract tumor cell negative effects on myotubes;

2) To identify which are the paracrine mediators of myoblast recruitment to myotubes, if any;

3) To demontrate SRF regulation and its role in mediating these responses. SRF seems to be an important actor of muscle plasticity and could play a role to prevent muscle atrophy in cancer cachexia by the way of physical exercise. This hypothesis arises from studies showing the pivotal role of SRF in muscle homeostasis and in interleukin-mediated satellite cell recruitment to myofibers.

RESULTS

5. C26 cell conditioned medium effects on C2C12 myotubes

5.1 C26 cells conditioned medium blocks myotubes growth while mechanical stimulation counteracts this negative effect

We established cell cultures containing a mixed population of myotubes and unfused myoblasts, obtained by 4d culturing in differentiation medium (DM). In the order to study the effects of tumor-derived factors on muscle cell culture, C26 cell conditioned medium was obtained and used to treat C2C12 mixed cultures. In preliminary experiments, two-day treatment with a DM containing 20% of C26 conditioned medium (CM) induced myotube atrophy and hampered myoblast differentiation (data not shown). Control medium or CM were, therefore, used to treat C2C12 mixed cultures, in the absence (static condition, SC) or presence (dynamic condition, DC) of mechanical stimulation consisting of daily sessions of longitudinal stretch for 6 hours per day, as described in M&M (Figure 9).

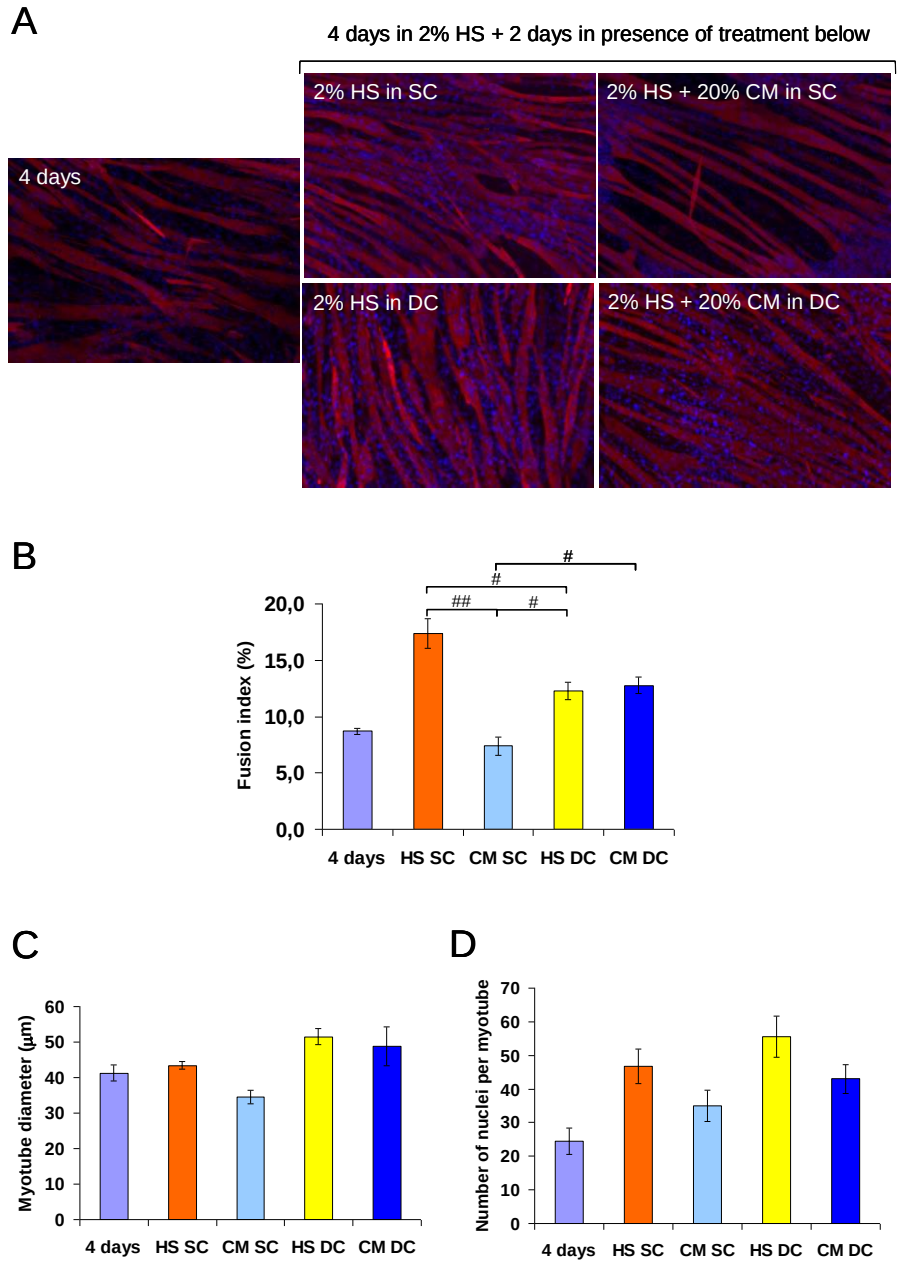


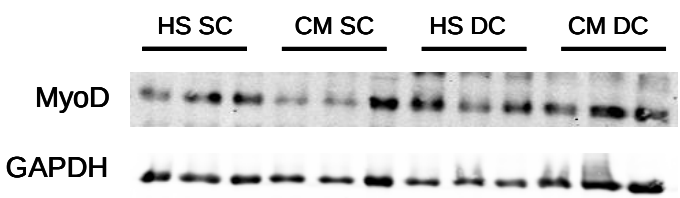
Figure 9: **Effects of C26 conditioned medium on myotube growth.** (A) Immunofluorescence of myosin heavy chain (red) and nuclei (blue) on C2C12 cell

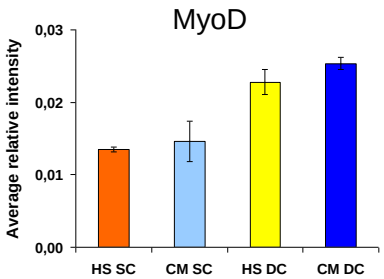
cultures: C2C12 are incubated for 4 days in DMEM + 2% HS to obtain a mixed culture of myotubes and myoblasts (4 days); additional 2 days culture in DMEM + 2% HS in the absence (2% HS in SC and 2% HS in DC) or presence (2% HS + 20% CM in SC and 2% HS + 20% CM in DC) of C26 CM (the latter represents 20% of the culture medium) cyclic mechanical stretch is applied as described in M&M (Dynamically stretched condition, DC) or not (statically stretched condition, SC), 2% HS in DC, 2% HS + 20% CM in DC, 2% HS in SC, 2% HS + 20% CM in SC, respectively. (B) The percentage of nuclei into myotubes (fusion index) (C) The myotube diameter and (D) the myotube nuclear size (number of nuclei per myotube) were measured on IF pictures (as in A). n=3. Statistical analysis demonstrated a significant effect of cyclic stretch on myotube diameter and a significant effect of conditioned medium on the number of nuclei per myotube by TWO-WAY ANOVA; For fusion index, by TWO-WAY ANOVA: F=24,73; df=1; p<0,001 for conditioned medium, F=30,22; df=1; p>0.001 for interaction; we used Tukey *post hoc* test, # p<0,05, ## p<0.01; For myotube diameter, by TWO-WAY ANOVA F=12,66; df=1; p>0,05 for cyclic stretch; in absence of interaction not able to use Tukey *post hoc* test to compare every group each others. For the myotube nuclear size, by TWO-WAY ANOVA: F=5,64, df=1, p<0,05 for conditioned medium in absence of interaction.

Compared to standard conditions for myogenic differentiation that DM we observed atrophic myotubes in presence of C26 cell CM determined myotube atrophy, in the absence of mechanical stretch, in agreement with what previously reported (Pelosi et al 2014). When mechanical cues are applied , we observed myotube hypertrophy even in the presence of C26 CM, consisting of a higher myotube diameter associated to an increased number of myonuclei per myotube and higher fusion index (the percentage of nuclei in myotubes on the total number of nuclei) (Figure 9A). In particular, CM decreased by twice the fusion index in compared to controls and negatively affected myotube diameter; statistical analysis demonstrated the significance of these effects (Figure 9B and C). Cyclic stretch significantly affected myotube size in terms of nuclei per myotube, suggesting that it stimulated nuclear incorporation into myotubes, even in the presence of C26 CM, in addition to protein synthesis and true hypertrophy (Figure 9D).

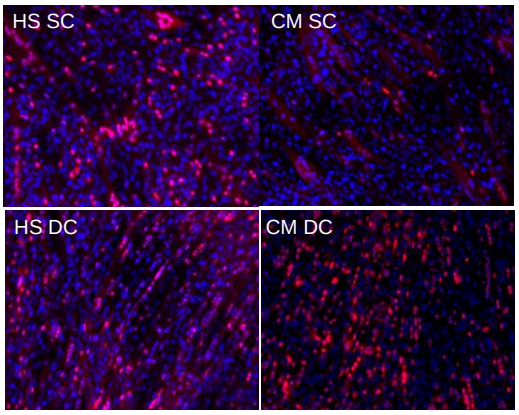
5.2 Mechanical cues rescue the myogenic differentiation program likely via MRFs activation

A





B



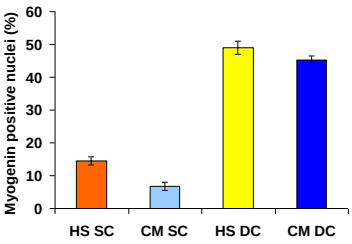


Figure 10: **Effects of mechanical cues on myogenic differentiation marker.** (A) Western-blot of MyoD expression in C2C12 cell cultures. n=3, however each lane contains protein extracts representing the pool of 3 independent samples from C2C12 cells after 6 days cultures in absence (HS) or presence of C26 CM (CM), in the absence (SC) or presence of (DC) of cyclic stretch for the last two days. In addition experiments were carried out in triplicate. The columns represent average relative intensity of myoD protein expression. Statistic analysis demonstrates a significant positive effect of cyclic stretch on MyoD protein expression ($F=9,47$; $df=1$; $p<0,001$ for cyclic stretch, absence of interaction by TWO-WAY ANOVA). (B) Immunofluorescence of myogenin (red) and nuclei (blue). n=6. Columns represent the percentage of myogenin positive nuclei from triplicate experiments. Statistic analysis demonstrates a significant positive effect of cyclic stretch on the percentage of nuclei which expressing myogenin ($F=20,61$; $df=1$; $p<0.01$ for conditioned medium and $F= 821,61$; $df=1$; $p<0,001$ for cyclic stretch, absence of interaction by TWO-WAY ANOVA).

The C26 CM-mediated inhibition of myoblast recruitment to myotubes does not seem to be associated to an overall decrease of MyoD and myogenin expression. However, in presence of cyclic stretch, MyoD protein expression and the number of myogenin positive nuclei increase significantly in absence or presence of CM. That suggests that increased MRF expression mediates its beneficial effects, ultimately resulting in myotube growth (Figure 10). Worth noting that the majority of MRFs RNA expression, including MyoD and myogenin, is negatively affected by the presence of CM, while cyclic stretch improves the level of RNA expression of these genes (Table 1); this confirms that cyclic stretch positive effects are associated to increased MRFs expression and suggests a certain stability of MyoD and myogenin at protein level, in the presence of C26 CM. Anyhow, as a result of C26 CM treatment, we observed a significant negative effect on myosin heavy chain (myh7) RNA expressions that demonstrated a late inhibition of the myogenic program, an effect counteracted by cyclic stretch.

	MyoD	Myf5	MRF4	myogenin	Myh7 (type I fibers)
HS SC	0.70 +/- 0.03 #	1.00 +/-0.14	1.59 +/-0.15	0.82 +/-0.09	1.39 +/-0.11
CM SC	0.54 +/-0.04##	0.82 +/-0.09	1.19 +/-0.14	0.49 +/-0.06	0.70 +/-0.10
HS DC	0.73 +/-0.07	0.93 +/-0.12	1.69 +/-0.28	0.87 +/-0.09	1.38 +/-0.16
CM DC	0.82 +/-0.07	1.06 +/-0.09	1.72 +/-0.30	0.82 +/-0.10	1.06 +/-0.08

Table 1: **Comparison by Q-PCR of MRFs expression implicated in myogenic differentiation**. Gene expressions, normalized by GAPDH and expressed as fold-change over the expression after 4 days of myogenic differentiation in horse serum 2%. C2C12 are incubated for 4 days in DMEM + 2% HS to obtain a mixed culture of myotubes and myoblasts; additional 2 days culture in DMEM + 2% HS in the absence (HS SC and HS DC) or presence (CM SC and CM DC) of C26 CM (the latter represents 20% of the culture medium) cyclic mechanical stretch is applied as described in M&M (Dynamically stretched condition, DC) or not (statically stretched condition, SC), HS DC, CM DC, HS SC, CM SC, respectively. .n=12. Statistical analysis (TWO-WAY ANOVA) demonstrates a positive effect of cyclic stretch on MyoD ($F=6,58$; $df=1$; $p<0,01$) and myogenin ($F=4,42$; $df=1$; $p<0,05$) gene expressions. Statistical analysis (TWO-WAY ANOVA) demonstrates a negative effect of C26 conditioned medium on myh7 ($F=14,87$; $df=1$; $p<0,001$) and myogenin ($F=3,97$; $df=1$; $p<0.05$) gene expressions. TWO-WAY ANOVA shows an interaction on MyoD ($F=4,38$; $df=1$; $p<0,05$) gene expression, which allows Tukey *post hoc* test #, $p<0.01$ between HS SC and CM SC, ## $p<0.01$ between CM SC and CM DC.

6. C26 cells conditioned medium and mechanical cues affect myogenic differentiation by modulating secreted factors

6.1 Mechanical cues restore Activin A and Follistatin balance favouring myogenic differentiation

With the aim to identify potential mediators of cyclic stretch beneficial effects against C26 CM, we investigated the secretion of protein known to be involved in myotube growth, such as TGF family members. Surprisingly, we found that Myostatin concentration decreased in the presence of CM, and increased in the presence of cyclic stretch independently of CM (Figure 11A).

This result was unexpected, knowing that myostatin is an atrophic factor which plays a key role in muscle homeostasis, blocking its growth; however, this result was observed at the point of the experiment, i.e. at day 6, when the final effects of the treatments were already achieved: the implications of this unexpected finding will be further discussed.

We also observed that C26 CM induced a high concentration of Activin A in the supernatant of C2C12 cultures after 6 days suggesting that C26 cells could inhibit myogenic differentiation by an activation of the Activin receptor 2B. Intriguingly, the Activin A-inhibitor follistatin (Fst) decreased in the same conditions, further suggesting a role for this pathway in the control of myotube size. In the presence of cyclic stretch, not only Activin A concentration in the supernatant is lowered, but also, quite surprisingly, follistatin concentration decreases (Figure 11B). However, since physiologically relevant is the ratio of follistatin on Activin A, the latter was calculated for the different experimental conditions. The ratio decreases dramatically in static condition in presence of CM, while in the presence of cyclic stretch, the ratio increases significantly in respect to CM alone, suggesting a potential rescue of myogenic differentiation and myotube homeostasis by cyclic stretch; the fact that the follistatin/Activin A ratio never reaches control levels in the presence of cyclic stretch suggests that mechanical cues have an effect on this ratio, likely acting on both follistatin and Activin levels (Figure 11C).

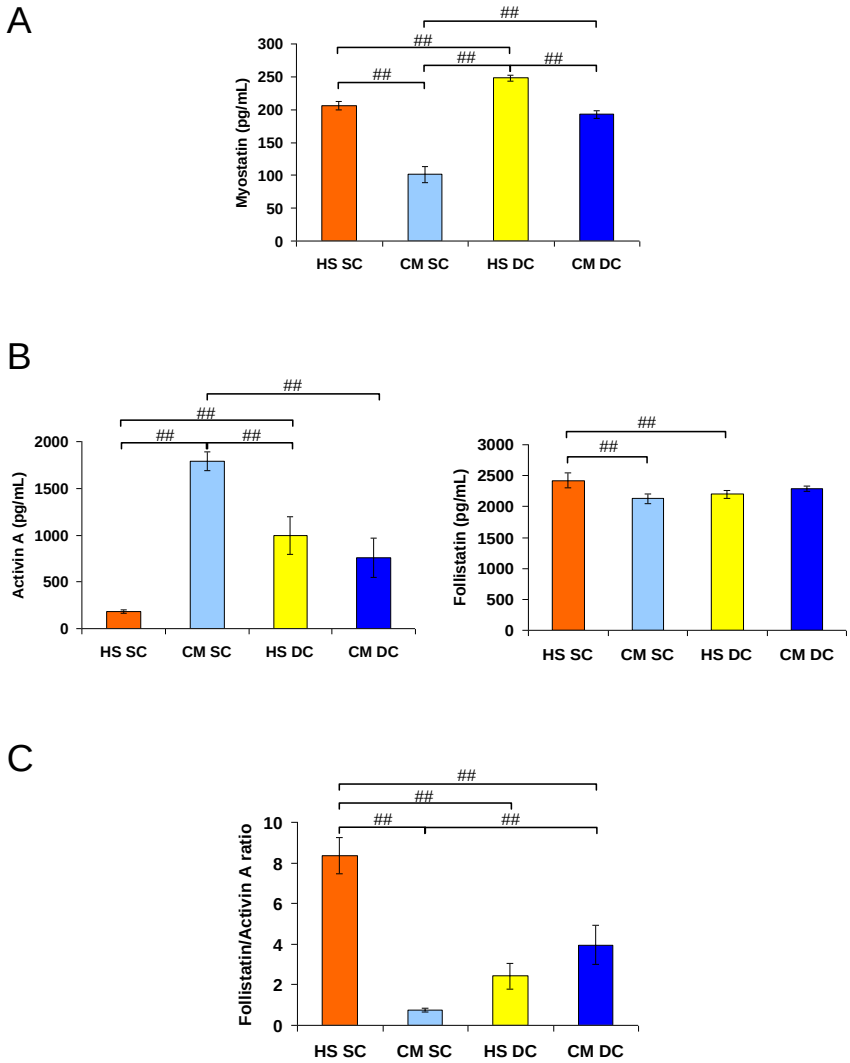


Figure 11: **Comparison of TGF family member secretion on C2C12 supernatant.** Myostatin (A), Activin A and follistatin (B) concentration (pg/mL) in supernatant by ELISA on C2C12 supernatant. C2C12 are incubated for 4 days in DMEM + 2% HS to obtain a mixed culture of myotubes and myoblasts; additional 2 days culture in DMEM + 2% HS in the absence (HS SC and HS DC) or presence (CM SC and CM DC) of C26 CM (the latter represents 20% of the culture medium) cyclic mechanical stretch is applied as described in M&M (Dynamically stretched condition, DC) or not (statically stretched condition, SC), HS DC, CM DC, HS SC, CM SC, respectively. n=10. Statistical analyses demonstrated a significant effect

of conditioned medium (F=101,11; df=1; p<0,0001), of cyclic stretch (F=70,77; df=1; p<0,0001) on myostatin concentration (A) by TWO-WAY ANOVA. In addition a significant interaction (F=9,66; df=1; p<0,01) permit to use Turkey *post hoc* test (## p<0,001). Statistical analyses (TWO-WAY ANOVA) demonstrated a significant effect of condition medium on Activin A concentration (F=19,71; df=1; p<0,001) by an interaction (F=35,42, df=1; p<0.001) permitted to use Tukey *post hoc* test (## p<0,01). Statistical analyses (TWO-WAY ANOVA) also demonstrated a signicant interaction (F=5,95; df=1; p<0,05) which permitted to use Tukey *post hoc* test (## p<0,01) (C) Follistatin on Activin A ratio was calculated with Follistatin and Activin A concentration (pg/mL) (B) Statistical analyses (TWO-WAY ANOVA) demonstrated a significant effect of conditioned medium on the ratio (F=17,02; df=1; p<0.001), a also a significant interaction (F=38,65; df=1; p<0,0001) permitted to use Tukey *post hoc* test (## p<0,01).

To confirm the modulation of ActIIBR downstream pathways, the ratio between phospho-smad2-3 and total smad2-3 was quantified in the different conditions. In particular, C26 CM increased smad2-3 phosphorylation in respect to controls, while mechanical stretch rescued smad activation.

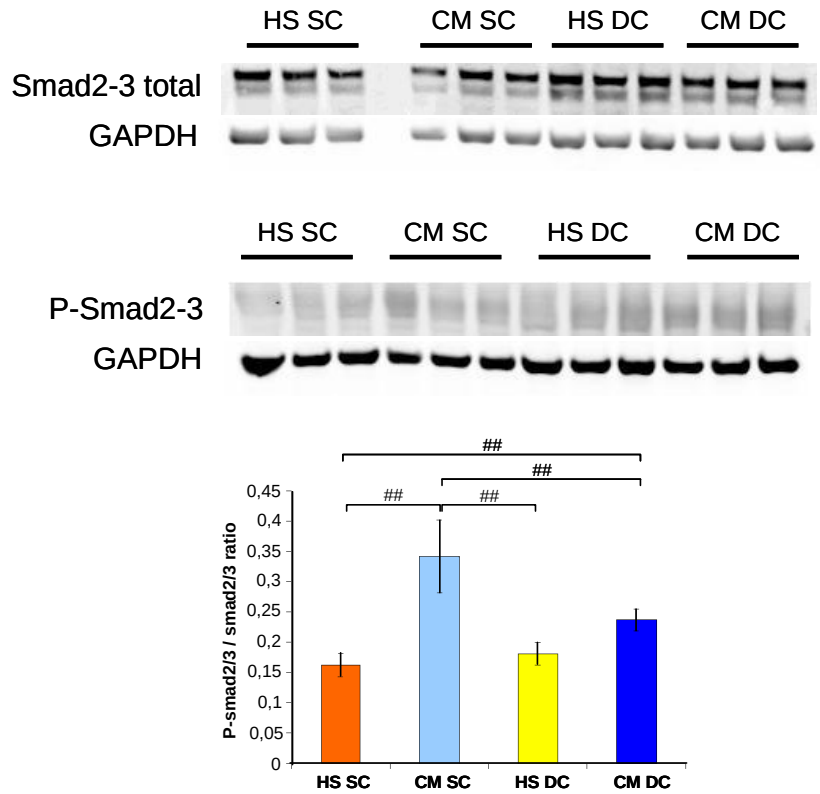


Figure 12: Effects of treatments on smad2-3 phosphorylation in presence of C26 CM. A) Western-blot of total and phosphorylated smad2-3 in C2C12 cell cultures. Each well contains protein extracts representing the pool of 3 samples from C2C12 cells after 6 days cultures in absence (HS) or presence of C26 CM (CM), in the absence (SC) or presence of (DC) of cyclic stretch for the last two days. In addition experiments were carried out in triplicate. The columns represent average relative intensity of smad2-3 total and phospho-smad2-3 protein expression. B) Columns represent the ratio of phosphorylated smad2-3 on smad2-3 total protein blot densities. TWO-WAY ANOVA demonstrates a significant effect of conditioned medium ($F=46,44$; $df=1$; $p<0,0001$), cyclic stretch ($F=6,25$; $df=1$; $p<0,01$), interaction ($F=12,36$; $df=1$; $p<0.001$). The latter permitted to use Tukey *post hoc* test, #$p<0,05$, ##$p<0,01$.

6.2 Follistatin is not sufficient to counteract myotube atrophy due to conditioned medium

To determine if follistatin is sufficient to restore the total myogenic capacity in presence of C26 CM, 4 d C2C12 cultures were treated with recombinant follistatin for 2 days, in the absence of mechanical stimulation. Preliminary data limited to three samples confirmed the negative effects of C26 CM compared to standard conditions, an effect not rescued by recombinant follistatin, regardless the parameter examined (Figure 13A-D). Based on the trend observed in figure 13B, we cannot exclude that follistatin *per se* might rescue fusion index, i.e. myogenic differentiation even in the presence of C26 CM. However, this pathway only contributes to the rescue of myotube homeostasis, since follistatin is definitely not sufficient to counteract C26 CM negative effects on myotube diameter or nuclear number.

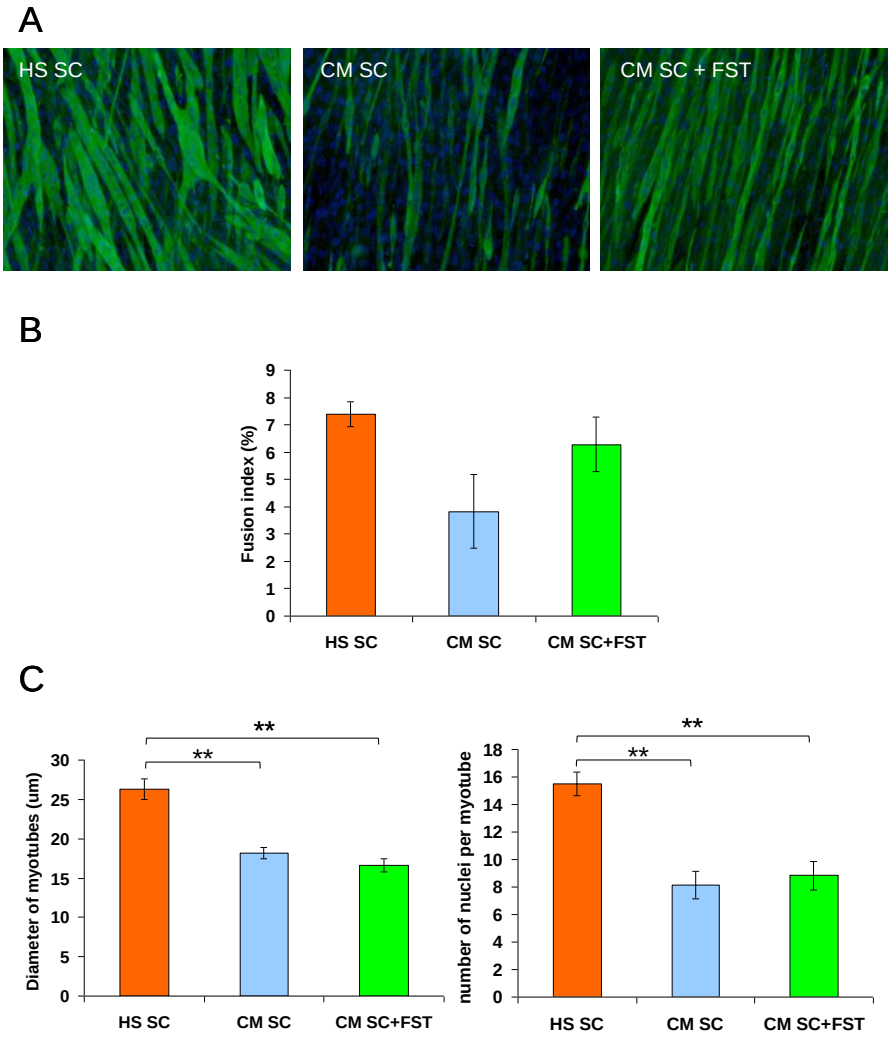


Figure 13: **Effects of follistatin on C2C12 mix culture in presence of CM.** (A) Immunofluorescence of myosin (green) and nuclei (blue) on C2C12 cell cultures, following 4 days in DMEM + 2% HS and additional 2 days in DMEM + 2% HS in absence (HS SC) or presence (CM SC, CM SC+FST) of C26 CM in statically stretched condition (SC); recombinant follistatin (FST 100ng/mL) was added in combination with CM. (B) The percentage of nuclei into myotubes (fusion index), (C) The myotube diameter and (D) the myotube nuclear size (number of nuclei per myotube) were calculated on IF pictures . n=3; **, $p<0.01$ by ONE-WAY ANOVA (for myotube diameter $F=28,92$, $df=2$, $p<0,001$; for number of myonuclei, $F=22,93$, $df=1$, $p<0,001$) followed by Tukey *post hoc* test,

7. SRF activity counteracts the negative effect of C26 cells conditioned medium on myogenic differentiation

7.1 Mechanical cues activate the Rho/actin/MRTFs pathway

We have shown that an increase in the follistatin/Activin A ratio could counteract myotube atrophy, even though it is not sufficient for a full restoration of myotube homeostasis in the presence of CM. For this reason, we explored another pathway, which is directly stimulated by mechanical cues in skeletal muscle and has been shown to recruit satellite cells to myofibers via IL-4: the Rho/actin/MRTFs pathway involving SRF transcriptional activity.

A precocious sensor of mechanical stretch in muscle is Striated Activator of RhoA signalling (STARS). The latter is localized on sarcomeric actin and responds to mechanical cues on the actin network by an induction of F-actin polymerisation. Indeed, we found that STARS RNA was downregulated by C26 CM, but cyclic stretch counteracts this effect and induces STARS expression to higher levels even compared to controls (Figure 14A). STARS regulates localization of the SRF cofactor MRTF-A via RhoA. Indeed, RhoA RNA expression was significantly upregulated by cyclic stretch associated to a similar modulation of RhoA protein (Figure 14B and 14C). Since STARS and RhoA activation induce actin polymerization, we purified monomeric and filamentous actin and calculated the F-actin to G-actin ratio, in the various experimental conditions. We observed a significant effect of cyclic stretch on F- to G-actin ratio, suggesting actin polymerisation in response to cyclic stretch both in presence and absence of C26 CM (Figure 14D).

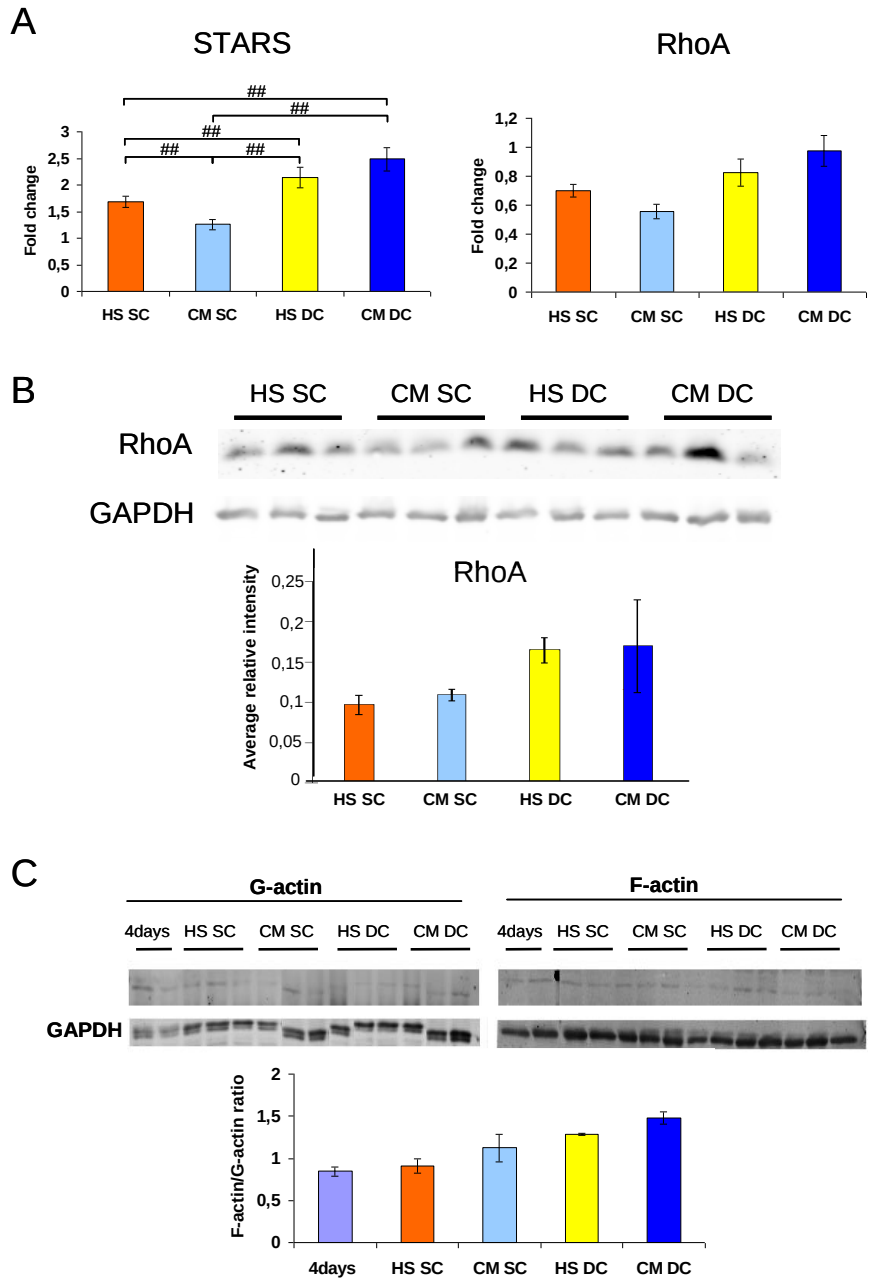


Figure 14: **Analysis of the STARS/RhoA/Actin axis in C2C12 cultures.** (A) STARS and RhoA gene expression normalized by GAPDH and expressed as fold-change

over cell cultures after 4 days of myogenic differentiation in horse serum 2%. C2C12 are incubated for 4 days in DMEM + 2% HS to obtain a mixed culture of myotubes and myoblasts; additional 2 days culture in DMEM + 2% HS in the absence (HS SC and HS DC) or presence (CM SC and CM DC) of C26 CM (the latter represents 20% of the culture medium) cyclic mechanical stretch is applied as described in M&M (Dynamically stretched condition, DC) or not (statically stretched condition, SC), HS DC, CM DC, HS SC, CM SC, respectively. n=12. Statistical analysis (TWO-WAY ANOVA) demonstrates significant effect of cyclic stretch ($F=24,02$; $df=1$; $p<0,0001$) and an interaction ($F=6,09$; $df=1$; $p<0,01$) on STARS gene expression. The interaction permits to use Tukey *post hoc* test (# $p<0,05$; ## $p<0,01$). TWO-WAY ANOVA demonstrates a significant effect of cyclic stretch ($F=9,38$; $df=1$; $p<0,01$) on RhoA gene expression in absence of interaction. (B) Western-blot of RhoA protein in C2C12 cell cultures. n=3, however each well contains protein extracts representing the pool of 3 samples from C2C12 cells after 6 days cultures in absence (HS) or presence of C26 CM (CM), in the absence (SC) or presence of (DC) of cyclic stretch for the last two days. In addition experiments were carried out in triplicate. The columns represent average relative intensity of RhoA protein expression. Statistic analysis (TWO-WAY ANOVA) demonstrates a significant positive effect of cyclic stretch ($F=20,72$; $df=1$; $p<0,001$) on RhoA protein expression. (C) Western-blot of Filamentous actin (F-actin) and Globular actin (G-actin) protein expression in C2C12 cell cultures. Columns represent the ratio between F-actin and G-actin protein expression. Statistic analysis (TWO-WAY ANOVA) demonstrates a significant positive effect of cyclic stretch ($F=9,59$; $df=1$; $p<0,001$) on this ratio.

7.2 MRTF-A translocation to the nucleus is stimulated by mechanical cues

An expected output of STARS/RhoA/actin pathway activation is MRTF-A translocation to the nucleus. Given the major role played by MRTF-A as SRF transcriptional cofactor, we decided to look in detail the regulation of MRTF-A expression and activity. We found that MRTF-A RNA is upregulated in presence of cyclic stretch independently of CM presence (Figure 15A). To verify if the increased MRTF-A expression could resultat in nuclear accumulation of this important pro-myogenic transcription co-factor, we infected C2C12 cultures at 4 days with an adenovirus overexpressing MRTF-A in association with GPF. By using this

approach, we observed that C26 CM prevented MRTFA localization in the myonuclei, and that the presence of mechanical cues induced nuclear translocation of MRTF-A, irrespective of any C26 CM treatment. The percentage of MRTF-A positive nuclei increased strongly up to 70% of the myonuclei of infected myotubes, suggesting that mechanical cues are a major regulator of MRTF-A activation (Figure 15B).

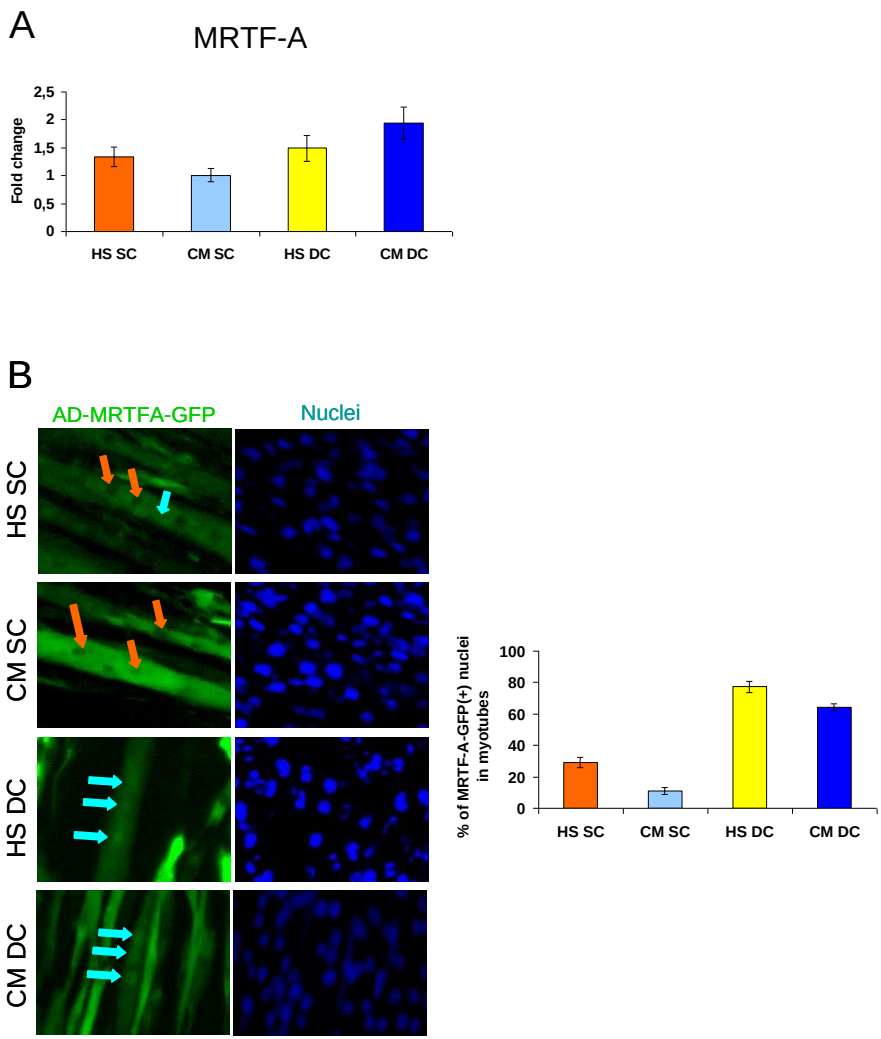


Figure 15: **MRTF-A expression on C2C12 cultures after 6 day**s. (A) MRTF-A gene

expression by Q-PCR; Gene expression is normalized by GAPDH and expressed as fold-change over the expression after 4 days of myogenic differentiation in horse serum 2% (4 days). C2C12 are incubated for 4 days in DMEM + 2% HS to obtain a mixed culture of myotubes and myoblasts; additional 2 days culture in DMEM + 2% HS in the absence (HS SC and HS DC) or presence (CM SC and CM DC) of C26 CM (the latter represents 20% of the culture medium) cyclic mechanical stretch is applied as described in M&M (Dynamically stretched condition, DC) or not (statically stretched condition, SC), HS DC, CM DC, HS SC, CM SC, respectively. n=12. Statistic analysis (TWO-WAY ANOVA) demonstrates a significant positive effect of cyclic stretch ($F=6,08$, $df=1$, $p<0,01$) on MRTF-A gene expression. (B) Infection of C2C12 cultures at 4 days with Adenovirus-MRTF-A-GFP; MRTF-A GFP expression (green) and nuclei (blue); MRTF-A-GFP positive nuclei (blue arrow); MRTF-A-GFP negative nuclei (orange arrow). Columns represent the percentage of MRTFA-GFP positive nuclei. n=3. Statistic analysis (TWO-WAY ANOVA) demonstrates a significant negative effect of condition medium ($F=14$, $df=1$, $p<0,001$) and positive effect of cyclic stretch ($F=284,06$; $df=1$; $p<0,0001$) in absence of interaction on the percentage of MRTFA-GFP positive nuclei.

7.3 SRF expression increases in presence of mechanical cues

We observed that SRF RNA and protein expression are both upregulated in response to mechanical cues, which is in agreement with the literature (Figure 16A).

This prompted us to look at known targets of SRF/MRTF-A transcription complex. We decided to focus on IL-4 concentration in supernatant of C2C12 culture in response to treatments, since IL-4 was previously shown to mediate myoblast recruitment in response to SRF activation. As expected, we found a significant increase of IL-4 concentration in the supernatants in response to mechanical stretch, independently of the presence of C26 CM (Figure 16B). This suggested that IL-4 is secreted by myotubes in response to workload via SRF activity. Integrin $\beta 1$ is a direct SRF target gene and we found that integrin RNA expression is also significantly upregulated in presence of cyclic stretch with or without CM (Table 2). Interestingly, integrin $\beta 1$ forms a dimer with

integrin α7, and this dimer is a mechanoreceptor which mediates mechanical cues inside muscle cells.

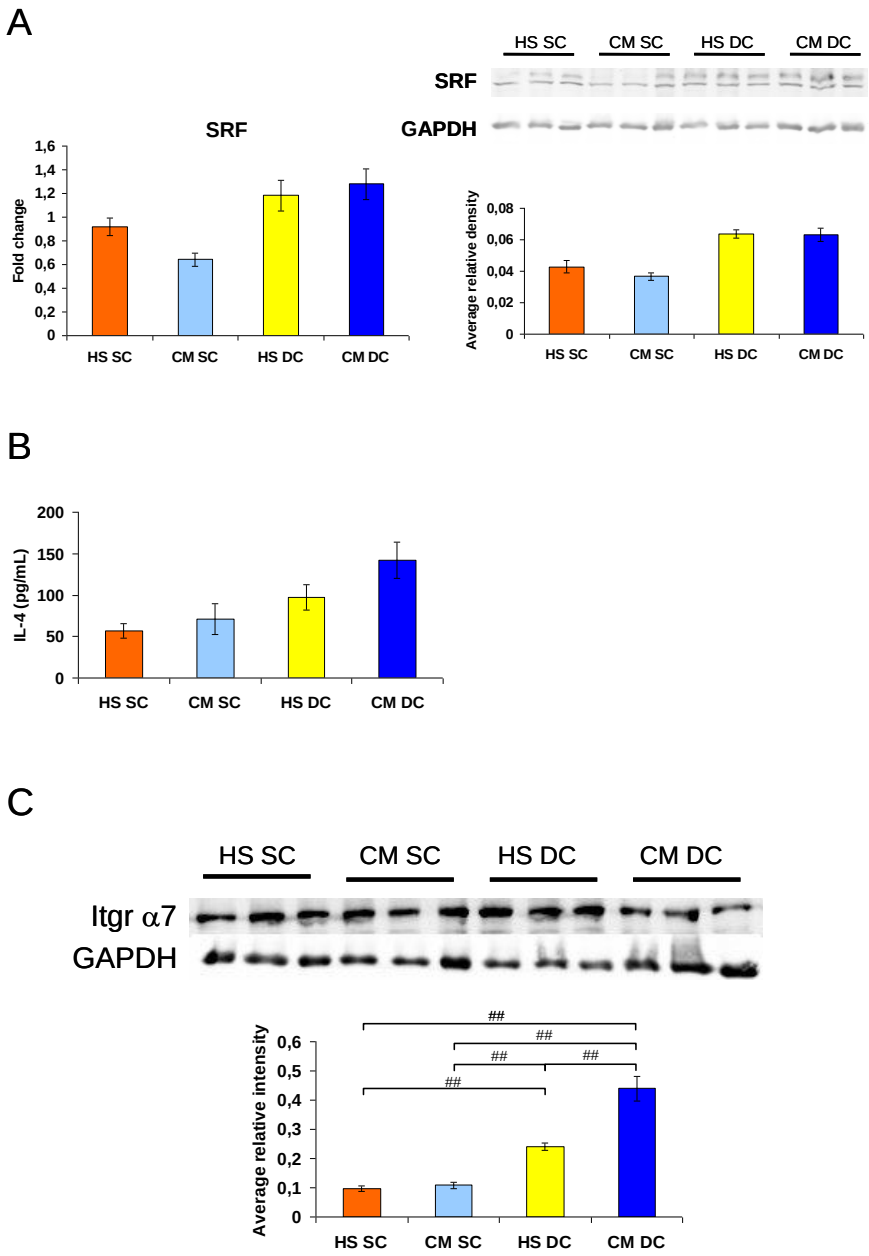


Figure 16: **SRF expression and transcriptional activity.** (A) SRF gene expression is normalized by GAPDH and expressed as fold-change over the expression after 4 days of myogenic differentiation in horse serum 2% (4 days). C2C12 are incubated for 4 days in DMEM + 2% HS to obtain a mixed culture of myotubes and myoblasts; additional 2 days culture in DMEM + 2% HS in the absence (HS SC and HS DC) or presence (CM SC and CM DC) of C26 CM (the latter represents 20% of the culture medium) cyclic mechanical stretch is applied as described in M&M (Dynamically stretched condition, DC) or not (statically stretched condition, SC), HS DC, CM DC, HS SC, CM SC, respectively. n=12. Statistic analysis demonstrates a positive effect of cyclic stretch ($F=15,56$; $df=1$; $p<0,001$) on SRF gene expression.; Western-blot of SRF protein in C2C12 cell cultures. n=3, however, each well contains protein extracts representing the pool of 3 samples from C2C12 cells after 6 days cultures in absence (HS) or presence of C26 CM (CM), in the absence (SC) or presence of (DC) of cyclic stretch for the last two days. In addition experiments were carried out in triplicate. The columns represent average relative intensity of SRF protein expression. Statistic analysis (TWO-WAY ANOVA)demonstrates a significant positive effect of cyclic stretch ($F=47,10$; $df=1$: $p<0,001$) on SRF protein expression. (B) IL-4 concentration in C2C12 culture supernatants by ELISA. n=10. Statistic analysis (TWO-WAY ANOVA) demonstrates a significant positive effect of cyclic stretch ($F=11,1$; $df=1$; $p<0,01$) on IL-4 concentration in absence of interaction. (D) Western-blot of Integrin $\alpha 7$ protein expression in C2C12 cell cultures n=3, however, each well contains protein extracts representing the pool of 3 samples from C2C12 cells. TWO-WAY ANOVA demonstrates a significant effect of cyclic stretch ($F=136$; $df=1$; $p<0,0001$) with an interaction ($F=24$; $df=1$; $p<0,001$) permitting Tukey *post hoc* test (## $p<0,001$.

The analysis of the expression of additional SRF target genes showed that Cox2 and vinculin expressions is downregulated by the presence of C26 CM, an effect significantly counteracted by mechanical cues (Table 2).

	Sk actin	cox2	vinculin	Integrin β1	Integrin α7
HS SC	1.11 +/-0.13	0.67 +/-0.06	0.83 +/-0.07	0.96 +/-0.07	0.10 +/-0.07*
CM SC	0.93 +/-0.16	0.47 +/-0.03	0.59 +/-0.09	0.64 +/-0.07	0.11 +/-0.03##
HS DC	1.00 +/-0.17	0.93 +/-0.04	0.89 +/-0.05	0.99 +/-0.10	0.24 +/-0.10**
CM DC	0.91 +/-0.13	0.67 +/-0.20	0.66 +/-0.04	0.83 +/-0.06	0.44 +/-0.12

Table 2: **Comparison by Q-PCR of SRF target gene expression**. Gene expressions, normalized by GAPDH and expressed as fold-change over the expression after 4 days of myogenic differentiation in horse serum 2%. C2C12 are incubated for 4 days in DMEM + 2% HS to obtain a mixed culture of myotubes and myoblasts; additional 2 days culture in DMEM + 2% HS in the absence (HS SC and HS DC) or presence (CM SC and CM DC) of C26 CM (the latter represents 20% of the culture medium) cyclic mechanical stretch is applied as described in M&M (Dynamically stretched condition, DC) or not (statically stretched condition, SC), HS DC, CM DC, HS SC, CM SC, respectively. n=12. Statistical analysis (TWO-WAY ANOVA) demonstrates a positive effect of cyclic stretch on cox2 ($F=23,31$; $df=1$; $p<0,001$),Integrin α7 ($F=10,81$; $df=1$; $p<0,001$) gene expressions. Statistical analysis (TWO-WAY ANOVA) demonstrates a negative effect of C26 conditioned medium on cox2 ($F=22,75$; $df=1$; $p<0,0001$), Vinculin ($F=15,06$; $df=1$; $p<0,001$), Integrin b1 ($F=10,59$; $df=1$; $p<0,01$) gene expression TWO-WAY ANOVA also demonstrates an interaction on integrin a7 ($F=5,14$; $df=1$; $p<0,05$) gene expression which allows to apply Tukey Post hoc test: *, $p<0.05$ between HS SC and CM DC, ** $p<0.05$ between HS DC and CM DC, ## $p<0.01$ between CM SC and CM DC.

7.4 A knock-down of SRF is sufficient to block the myogenic differentiation rescue associated to mechanical cues

In the order to determine is SRF is necessary to fully counteract the negative effects of C26 CM on C2C12 myotubes in response to dynamic stretch, we performed a transfection of siRNA against SRF RNA to perform a loss of function experiment. The extent of SRF inhibition was measured and is reported in figure 17D.The inhibition of SRF expression in dynamic conditions in presence of

CM prevented the rescue by mechanical cues: indeed we noticed a decrease in the fusion index and myotube size in the absence and in the presence of C26 CM in presence of cyclic stretch (Figure 17A). This confirmed that mechanical cues capacity to counteract the effects of CM was dependent on SRF expression (Figure 17B and C). All together, these data indicated that SRF is necessary rescues myogenic differentiation, even though it does not necessarily participate in myotube hypertrophy in response to mechanical cues.

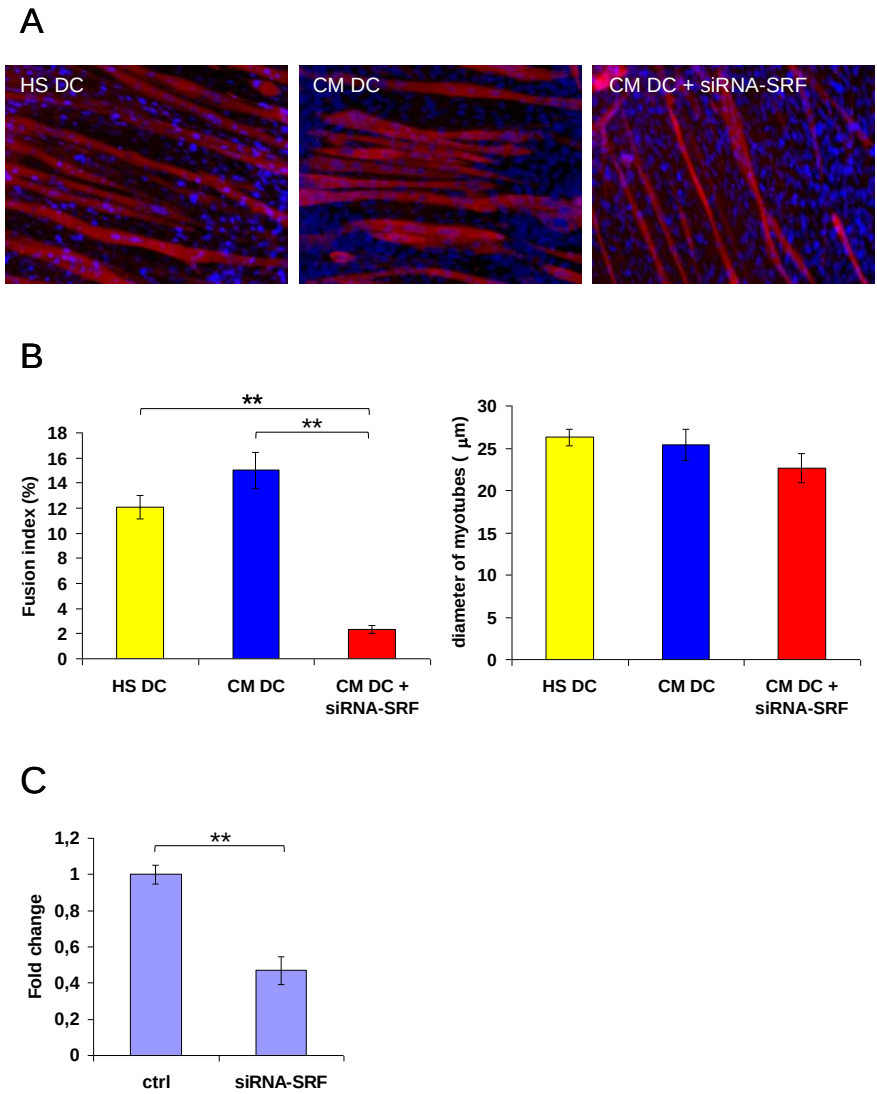


Figure 17: **SRF Knock-down on C2C12 mix culture in presence of statically stretched condition.** (A) Immunofluorescence of myosin (red) and nuclei (blue) on C2C12 cell cultures: C2C12 are incubated for 4 days in DMEM + 2% HS to obtain a mix culture of myotubes and myoblasts; additional 2 days culture in DMEM + 2% HS in absence (HS SC) or presence (CM SC, CM SC+siRNA-SRF) of C26 CM (the latter represents 20% of the culture medium) in dynamically stretched condition (DC). (B) The percentage of nuclei into myotubes (fusion

index). The myotube diameter were calculated on IF pictures (A). (C) siRNA-SRF efficiency is quantified by Q-PCR on C2C12 mix cultures at 6days on plastic plates. n=3; **, $p<0.01$ (ONE-WAY ANOVA followed by Tukey *post hoc* test $F=42,38$, $df=2$, $p<0,001$).

DISCUSSION

Previous works demonstrated that physical exercise prevents muscle wasting in cancer cachexia (Pigna et al., 2016; Padilha et al., 2017; Aversa et al., 2017; Holmes et al., 2005; Meyerhardt et al., 2006; Irwin et al., 2008; Holick et al., 2008). Additional studies showed a several increase of Activin A in plasma of cancer patient (Bonetto et al., 2009; Chacon-Cabrera et al., 2014; Zhou et al., 2010; Chen et al., 2014; Han et al., 2013). While other studies showed evidences the pivotal role of SRF in muscle plasticity and in muscle hypertrophy in response to workload (Guerci et al., 2012). Therefore, we wanted to determine if SRF is involved in the prevention of muscle atrophy due to factors of possible tumor origin, such as Activin A or myostatin. Our *in vitro* model of myotubes/myoblast mixed cultures treated with a tumor CM, and subjected or not to mechanical stretch, allowed us to control and separate the effects of chemical factors from those depending on mechanical stimulation and to evaluate the contribution of each pathway in the rescue of myotube.

Activin A is involved in the atrophy of myotubes by activation of smad2-3 pathway.

Myostatin is an atrophying factor and the regulation of its expression and secretion seemed to be an ideal candidate mechanism to explain both the negative effects of C26 CM and the exercise-mediated rescue. Surprisingly, we observed an increase of myostatin concentration in the supernatant in dynamic stretched condition, which was paradoxal response. We explain, the increase of myostatin concentration in presence of cyclic stretch by the fact that , myostatin is secreted by the myofibers themselves in a mass dependent manner (to block the increase of myofiber and limit an over response of myofibers to mechanical stimuli as it occurs *in vivo*); in conditions of increased myotube mass, such as upon

dynamic stretch, we think that these hypertrophic myotube secrete a higher amount of myostatin, independently of the presence of other factors present in the C26 CM. Differently form myostatin, Activin A concentration in the cell supernatant increased in response to the presence of CM. In fact, an increase of Activin A concentration induces an inhibition of myoblast differentiation in skeletal muscle (Trendelenburg et al 2012). In agreement, the C26 CM and mechanical cues differentially regulated Activin A concentration, suggesting the importance of-dependent pathways ActIIBR. When we put the concentration of follistatin in the picture, we found that secretion of follistatin decreased in presence of CM and increased upon mechanical stretch. We calculated Follistatin/Activin A ratio. By taking into account the information that Fst and Activin A are an affinity to Kd=1,7nM (Cash et al., 2009), when the ratio is down to 1,7; the activity of ActIIBR go to myogenic inhibition , and more the ratio is high, more myogenic activation is induced. Thus, we found that this ratio increases strongly in presence of stretch, suggesting that the Activin A present in the C26 CM could be inhibited by a very significant increased in follistatin. In agreement, in the presence of C26 CM, we observed a high phosphorylation of smad2-3, showing the actual activation of ActIIBR by factors secreted by tumor cells. Mechanical cues may prevent the activity of phosphorylated complex smad2-3, ultimately resulting in the promoter of smad target genes like myoD, myogenin and others MRFs involved in the progression of the myogenic program. We had conscience that cachexia is a multifactoral syndrome which is generated by multifactors. Theses results permit to say that skeletal muscle atrophy uses ActIIBR, which phosphorylates smad2-3 complex and inhibits myogenic program. Thus, mechanical stretch counteracts CM effects and generates a decrease of smad2-3 activity even in presence of CM, which could suggest a response to mechanical stretch via this molecular pathway. With the aim to clarify whether this molecular pathway is sufficient for the prevention of myotube atrophy, we treated C2C12 cultures with recombinant follistatin.

Surprisingly, the addition of follistatin on C2C12 cultures did not significantly improve the fusion index and the myotubes size was not affected by follistatin supplementation.

Based on the all above, mechanical stretch seems to have a benefit effect to counteract atrophic effect induced by Activin A by increasing follistatin concentration as compared to static stretched conditions. Worth noting, it was shown that C26 cell conditioned medium presents cytokines secreted by tumor like TNF-α and IL-6, which, in turn, induce Activin A secretion by skeletal muscle (Trendelenburg et al 2012).

SRF plays a role in mechano-transduction in response to cyclic stretch.

We demonstrated that mechanical cues induce an increase of SRF protein expression. C26 CM seemed to not alter SRF expression and function in our experimental model, and only mechanical cues increased SRF expression and activity, supporting the notion that SRF is a transcriptional factor, which is activated in the presence of mechanical stimulation. Its co-factor MRTF-A increased too in presence of mechanical stimuli and translocated to myonuclei, likely promoting the transcriptional activity of SRF (Wang et al 2003). In addition, we demonstrated by infection with AD-MRTFA-GFP that mechanical cues induce nuclear translocation of MRTFA independently of C26 CM presence.

SRF is known to promote the increase of muscle mass by myoblasts recruitment, which fuse with myofibers (Guerci et al 2012); SRF promotes cell growth too (Wallace et al 2016). The fact that SRF pathway is activated when the number and the size of myotubes increase, this suggests that SRF have the same effect *in vitro.* It looks like SRF could play an essential role in the preservation of muscle homeostasis in presence of mechanical stimulation. The fact that mechanical stimulation is sufficient *per se* to induce SRF expression and transcriptional activity, suggests

that this cue can be responsible for a direct effect of exercise on differentiated muscle cells and myoblasts. As a result, SRF can be considered as a key factor for muscle response to exercise *in vivo*.

The data about upstream signals promoting MRTF-A /SRF transcriptional activity, suggest a model whereby the SRF pathway is activated by a modulation of actin network. In fact, the actin-network responses to mechanical cues are characterised by F-actin polymerisation due to STARS and RhoA activation. As a consequence, MRTF-A is imported in nuclei to co-activate SRF target genes, which are involved in myogenic differentiation and myoblast recruitment to myotubes (Wallace et al., 2016).

To explain the molecular mechanisms involved in myoblast recruitment and fusion with myotubes, we propose that SRF target genes (Cox2, integrin β1, IL-4) play a major role. In particular, integrins may stabilize cell-ECM interactions, further reinforcing adaptative responses to an elastic substrate, which is moving upon dynamic stretch, and reinforce at the same time intracellular signalling in response to mechanical cues. In addition, we observed a positive effect of cyclic stretch on IL-4 secretion by myotubes. In fact, SRF, via Cox2, is already known to induce IL-4 secretion by myotube to promote the fusion of myoblasts with myotubes (Guerci et al, 2012). The fact that we observe the same gene regulation in our model suggests that SRF induces IL-4 secretion via Cox2 in response to cyclic stretch. In turn, IL-4 recruits myoblasts and increases myotube diameter and dimensions counteracting the negative effects induced by the proinflammatory cytokines present in C26 CM.

In conclusion, we propose the following model for the differential modulation of myotube homeostasis by atrophying factors of tumor origin and cyclic stretch. C26 tumor cells induce Activin A increase and follistatin decrease affecting the milieu to which muscle cells are exposed, promoting pro-atrophy conditions. This likely activates Activin receptor-like kinase (Alk) via ActIIBR and

induces phosphorylates smad2-3 complex. Its nuclear translocation represses the transcription of myogenic genes, what could explain myotube atrophy that we observe in presence of CM. Mechanical cues can prevent the previously signalling by diminishing secretion of Activin A and increasing follistatin secretion. However, a supplementation with follistatin is not sufficient to restore the full hypertrophy capacity of myotubes, thus, this pathway could just contribute to limit the C26 dependent myotube atrophy. Mechano-transduction induced by cyclic stretch, counteracts atrophic effects of CM, by the activation of SRF via a modulation of actin network which responses to mechanical cues applied on cell. STARS, RhoA and the polymerisation of F-actin play a key role in nuclear translocation of MRTF-A and activation of SRF transcriptional activity. Indeed, SRF activation is necessary to mediate the beneficial effects of mechanical cues, as shown by the loss of function experiments. This general model has been summarized in Figure 20. Our study permits a better understanding of the mechanisms underlying cachexia.

We have confirmed the importance of myoblast recruitment and fusion with myotubes to maintain muscle homeostasis. In addition, we have shown that purely mechanical cues play a major role, among the numerous responses to exercise *in vivo*, to counteract muscle atrophy in the presence of tumor derived factors. Finally, our study suggests that the targeting of ActIIBR and the stimulation of SRF pathway by exercise or pharmacological treatments, turn out to be some relevant approaches to treat cachexia.

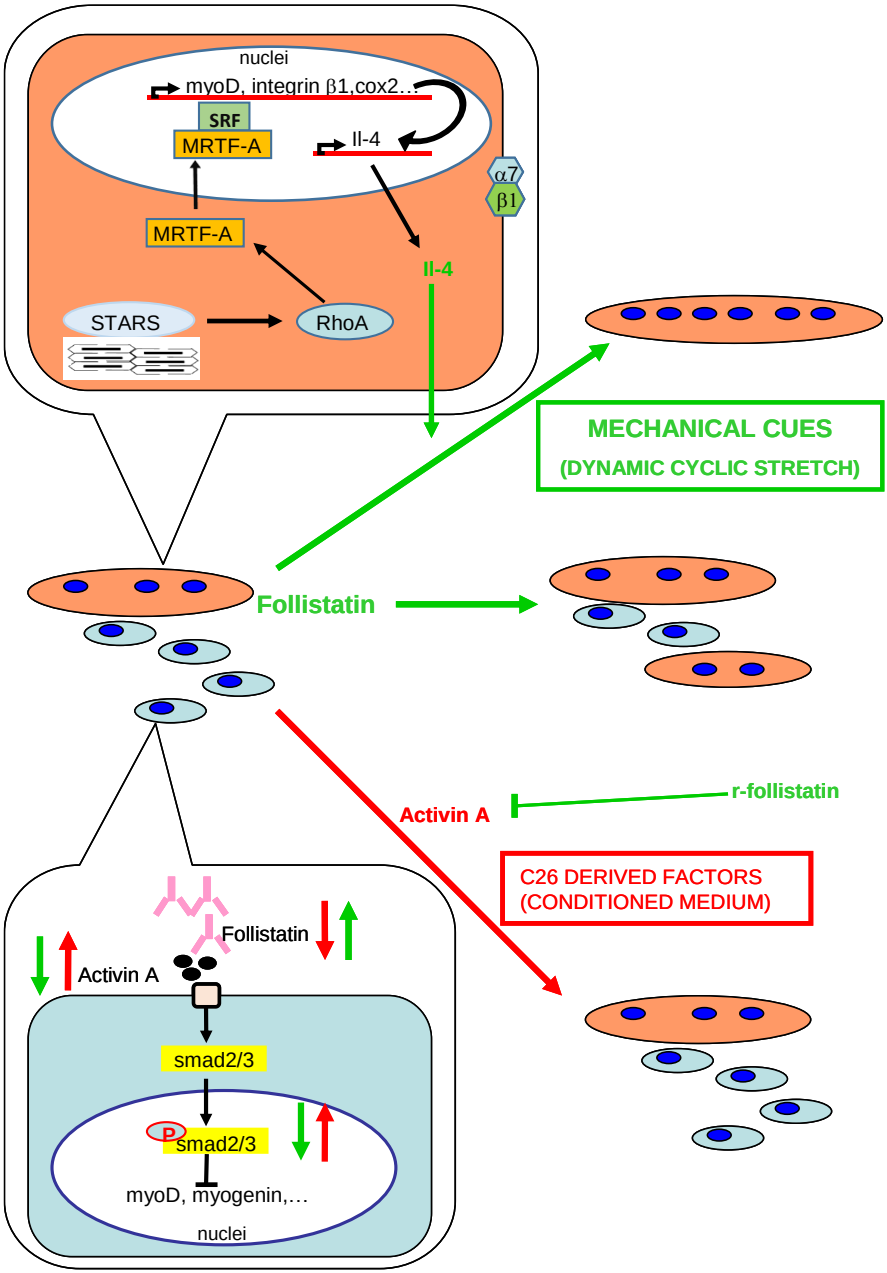


Figure 18: **Graphical abstract of mechanical cue responses on SRF and ActIIBR pathways to counteract the negative effects of CM.**

MATERIAL AND METHODS

8. Cell culture

8.1 C2C12 cell culture

C2C12 are mouse myoblasts, cultivated in DMEM with 15% FBS, 4.5g/L of glucose, 2mM of L-glutamine, 100ug/mL of penicillin-streptomycin (Sigma, Saint Louis, MO), at 37°C, 5% of CO2. C2C12 are seeded to obtain 80% of confluence and switched to differentiation medium with 2% horse serum, with or without 20% of C26 cell conditioned medium.

8.2 C26 conditioned medium protocol

C26 cell conditioned medium is obtained by cultivating the C26 cell line for 2 days in DMEM and 1% of penicillin-streptomycin without serum. The supernatant is taken after the 2 days of culture and mixed to DM to obtain a final concentration of 2% horse serum and 20% of C26 cells conditioned medium. The control medium contains 2% of horse serum and 98% of DMEM which was incubated for 2 days at 37°C in the absence of C26 cells.

8.3 Flexcell

Flexcell uses special multiwell plate with a silicone membrane which can be stretched when vacuum is applied under this membrane. In this way, C2C12 cells on this membrane are also stretched. C2C12 myoblasts are seed at 80% of confluence on the stretched area (3.89cm2) of each well. After 1 days of adhesion, growth medium is switched with differentiation medium for 4 days. During this time the vacuum is constantly applied to stretch the membrane to 10%, in order to increase the membrane stiffness. This has been shown it was necessary to allow a proper

differentiation of the C2C12 cells in preliminary experiments (data not shown). After 4 days, in this condition we obtain a coculture of myotubes and myoblasts. From this moment myogenic differentiation continues with 2% of horse serum in the presence or absence of 20% C26 cells conditioned medium and we apply or not mechanical cues by a release of vacuum under silicone membrane twice a day with the following stretching cycles: 2 hours of treatment with stretch and relaxation cycles at a frequency of 0,5 Hz (10% of stretch). Between each dynamic stretch treatment 3 hours of pause are planned. In static stretch conditions the membrane is continuously stretch for the additional 2 days of culture.

9. Infection by adenovirus MRTF-A-GFP

C2C12 myoblasts are cultured for 3,5 days in antibiotic free differentiation medium, and then treated overnight with adenovirus MRTFA-GFP from Vartiainen MK, et al., 2007, with concentration of 100 MO. Following supernatant removal, cells are switched to the different treatment described above.

10. Cell treatment by recombinant follistatin

C2C12 mixed cocultures of myoblasts and myotubes obtained as above are treated with 100ng/mL of recombinant follistatin (Fst288, Sigma) in presence of 2% horse serum and 20% C26 cells conditioned medium without mechanical cues.

11. SRF Knock-down

C2C12 myoblasts are cultured for 3,5 days in antibiotic free differentiation medium and then transfected with siRNA-SRF (Medjkane S et al., 2009) in presence of lipofectamine 2000 and RNAmax (Life technologies) overnight. Following supernatant removal, cells are swiched to the different treatment described above.

12. Immunofluorescence

For immunofluorescence analysis, we fix the samples with formaldehyde 3.7% in PBS for 10min and we rinse with PBS. We use a blocking/permeabilizing buffer containing 1% bovine serum albumin (BSA), 10% goat serum and 0,2 % Triton in PBS. We use antibodies anti-myosin heavy chain (Sigma), or F5D anti-myogenin. The secondary antibody is the anti-mouse Alexafluor 555 (Molecular probes, Invitrogen), while Hoescht is used for nuclear staining.

13. RNA extraction, c DNA synthesis and Q –PCR

Total RNA is isolated from C2C12 samples, with Trizol® reagent (Invitrogen) following the manufacturer's recommendations and homogenized. RNA concentration is determined by measuring the absorbance in 260nm/280nm in a NanoDrop spectrophotometer. Then, cDNA synthesis is carried out using the High capacity applied Reverse Transcription Kit (Biosystem). Lightcycler 480 is used to detected SYBR Green signal in Q-PCR. The mRNA levels are determined by the comparative Ct method. For each sample, a ΔCt value is obtained by subtracting GAPDH gene values from those of

the gene of interest. The average ΔCt value of the control group is subtracted from the sample to derive a −ΔΔCt value. The expression of each gene is evaluated by 2−ΔΔCt, according to Livak and Schmittgen (Livak KJ et al., 2001).

List of primers used in this study:

MyoD	R: TGG-CAT-GAT-GGA-TTA-CAG-CG F: GAG-ATG-CGC-TCC-ACT-ATG-CT
Myf5	R: CAT-CCG-CTA-CAT-TGA-GAG-CCT-C F: TAC-ATC-AGG-ACA-GTA-GAT-GCT-GTC
MRF4	R: GGG-AGT-TTG-CGT-TCC-TCT-GA F: GAA-GGA-GGA-GCA-AAC-GTG-GA
Myogenin	R: TAT-CCT-CCA-CCG-TGA-TGC-TG F: GCA-CTG-GAG-TTC-GGT-CCC-AA
Myh7	R:GGA- GCG-CAA-GTT-TGT-CAT-AAG-T F: CTC-AAG-CTG-CTC-AGC-AAT-CTA-TTT
IGF-1	R: CCA-CCT-TCT-GGA-GAA-TCC-AA F: AAC-RGC-ACC-AGC-CCA-TTT-AG
IGF-1Ea	R: TGT-GGC-ATT-TTC-TGC-TCC-GTG-G F: TGA-CAT-GCC-CAA-GAC-TCA
IGF-1Ec/MGF	R: GGT-GAT-GTG-GCA-TTT-CCT-GCT F: AGC-TGC-AAA-GGA-GAA-GGA-AAG-GAA-G
STARS	R: TGT-GGC-GAG-CCA-TTG-TGC-GG F:GAA-AGG-GCC-AAG-CGA-GCG-GA
RhoA	R: CTT-CTC-AGA-TGC-AAG-GCT-CA F: TCC-GTC-GGT-TCT-CTC-CAT-AG
MRTF-A	R: ACC-TTT-GGC-TTC-AGC-TCC-TT F: ACC-TCT-GCT-GCC-CCC-AAG-CC
SRF	R: GCT-GTG-TGG-ATT-GTG-GAG-GT F: CAC-CTA-CCA-GGT-GTC-GGA-AT

Skeletal actin	R: AGA-GCC-GTT-GTC-ACA-CAC-AA F: CGT-GAA-GCC-TCA-CTT-CCT-ACC
Cox2	R: GCT-CGG-CTT-CCA-GTA-TTG-AG F: GTG-GAA-AAA-CCT-CGT-CCA-GA
Vinculin	R: AAG-AAA-TAG-GGG-GAG-CCT-GA F: AGG-CCT-TCT-TCC-TGG-ATG-TT
Integrin β1	R: CCA-CCT-TCT-GGA-GAA-TCC-AA F: AAC-TGC-ACC-AGC-CCA-TTT-AG
Integrin α7	R: GCA-GCT-GAA-CAC-CAC-ACA-CT F: AGA-AGG-TGG-AGC-CTA-GCA-CA
GAPDH	R: ACA-CAT-TGG-GGG-TAG-GAA-CA F: AAC-TTT-GGC-ATT-GTG-GAA-GG

14. ELISA

In order to evaluate the concentration of factors secreted by the cell cultures in every condition, ELISA quantikine kits MG100 Mouse/Rat IGF-1, DAC00B Human/Mouse Activin A, DGDF80 gdf-8/myostatin, DFN00 from R&D systems are used.

15. Western Blot

15.1 Western Blot

Samples are treated with lysis buffer RIPA which contains Tris-Cl 50 Mm pH=7.5, 150Mm NaCl, 1% NP40, 0.5% desoxyclorate de sodium, EGTA 20mM, DTT 1mM, and an protease inhibitor cocktail. Proteins are denatured with a Bolt kit (Molecular probes, Invitrogen). The running period is 20 min at 200V with the Blot kit, and the transfer period is 20 min at 100V. Membrane of nitrocellulose is incubated with blocking buffer TBS-Tween with 5% not fat milk. The primary antibodies anti-MyoD, F5D (anti-

myogenin), MF20 (anti-myosin), anti-smad2-3 (Cell signalling), anti-phospho-smad2-3 (Cell signalling), anti-integrin α7 (Cell signalling), anti-SRF (Santa Cruz), anti-RhoA (Cell signalling), anti-GAPDH (Sigma) are incubated with TBS-Tween 5% of BSA. The secondary antibodies are fluorescence antibodies anti-mouse and anti-rabbit which are detected by Odyssey system. Each lane containes protein extracts obtained by pooling three independent sample (cell culture wells).

15.2 Western Blot of G and F actin

The F on G actin ratio is evaluated accordingly to the method by Roan et al., 2014. Following F and G actin separation, the samples are running gels with 8% acrylamid, for 1hour at 200V. Transfer period on nitrocellulose membrane is 1hour at 100V. Membranes of nitrocellulose are incubated with blocking buffer TBS-Tween with 5% not fat milk. The primary antibody is an anti-sarcomeric actin (Sigma). The secondary antibody is a fluorescence anti-mouse, combined with detection by the Odyssey system.

16. Imaging and quantification

In the order to quantify different morphology parameters of myotubes, the acquisition of immunofluorescence images is done with Zeiss EM S3/SyCoP3 Macro-apotome with software Zen for macroscope in the imaging plateform from Institute of Biology PARIS-SEINE.

Quantification of fusion index is defined by the number of nuclei in myotubes on total nuclei number in 5 fields per sample. We measure myotube diameter by the measure of myotube larger part. The counting of the nuclei number per myotube is done on fifty whole myotubes in the full sample image. All morphometric

analysis is done by Image J software.

17. Statistical analysis

The results were statistically analysed using TWO-WAY ANOVA to compare continuous variables between the 4 cell treatments examined. We also estimated multiple comparisons using the least significant difference test. The value of $p < 0.05$ was considered to indicate a statistically significant difference. Tukey HSD was used as a *post-hoc* test, whenever two ANOVA indicated interactions legitimating the use of this test. Simple comparisons were statistically analysed using ONE-WAY ANOVA. P values of < 0.05 were considered statistically significant.

REFERENCES

Affolter M, Montagne J, Walldorf U, Groppe J, Kloter U, LaRosa M, and Gehring W.J. (1994). The Drosophila SRF homolog is expressed in a subset of tracheal cells and maps within a genomic region required for tracheal development. Dev. Camb. Engl. 120, 743–753.

Andreyev HJ, Norman AR, Oates J, Cunningham D. (1998). Why do patients with weight loss have a worse outcome when undergoing chemotherapy for gastrointestinal malignancies? Eur J Cancer. 34(4):503-9.

Argilés JM, López-Soriano FJ, Toledo M, Betancourt A, Serpe R, Busquets S. (2011). The cachexia score (CASCO): a new tool for staging cachectic cancer patients. J Cachexia Sarcopenia Muscle. 2(2):87-93

Aversa Z, Costelli P, Muscaritoli M. (2017). Cancer-induced muscle wasting: latest findings in prevention and treatment. Ther Adv Med Oncol. 9(5):369-382.

Benny Klimek ME1, Aydogdu T, Link MJ, Pons M, Koniaris LG, Zimmers TA. (2010). Acute inhibition of myostatin-family proteins preserves skeletal muscle in mouse models of cancer cachexia. Biochem Biophys Res Commun. 391(3):1548-54.

Bilezikjian LM, Blount AL, Leal AM, Donaldson CJ, Fischer WH, Vale WW. (2004). Autocrine/paracrine regulation of pituitary function by Activin, inhibin and follistatin. Mol Cell Endocrinol. 225(1–2):29–36.

Bonetto A, Aydogdu T, Jin X, Zhang Z, Zhan R, Puzis L, Koniaris LG, Zimmers TA. (2012). JAK/STAT3 pathway inhibition blocks skeletal

muscle wasting downstream of IL-6 and in experimental cancer cachexia. Am J Physiol Endocrinol Metab. 1;303(3):E410-21.

Bonetto A, Penna F, Minero VG, Reffo P, Bonelli G, Baccino FM, Costelli P. (2009). Deacetylase inhibitors modulate the myostatin/follistatin axis without improving cachexia in tumor-bearing mice. Curr Cancer Drug Targets. 9(5):608-16.

Busquets S, Toledo M, Orpí M, Massa D, Porta M, Capdevila E, Padilla N, Frailis V, López-Soriano FJ, Han HQ, Argilés JM. (2012). Myostatin blockage using ActIIBR antagonism in mice bearing the Lewis lung carcinoma results in the improvement of muscle wasting and physical performance. J Cachexia Sarcopenia Muscle. 3(1):37-43.

Cash JN, Rejon CA, McPherron AC, Bernard DJ, Thompson TB. (2009). The structure of myostatin:follistatin 288: insights into receptor utilization and heparin binding. EMBO J. 2;28(17):2662-76.

Cen, B, Selvaraj, A, Burgess, R.C, Hitzler, J.K, Ma, Z, Morris, S.W, and Prywes, R. (2003). Megakaryoblastic leukemia 1, a potent transcriptional coactivator for serum response factor (SRF), is required for serum induction of SRF target genes. Mol. Cell. Biol. *23*, 6597–6608.

Chacon-Cabrera A, Fermoselle C, Urtreger AJ, Mateu-Jimenez M, Diament MJ, de Kier Joffé ED, Sandri M, Barreiro E. (2014). Pharmacological strategies in lung cancer-induced cachexia: effects on muscle proteolysis, autophagy, structure, and weakness. J Cell Physiol. 229(11):1660-72.

Chai J, and Tarnawski AS. (2002). Serum response factor: discovery,biochemistry, biological roles and implications for tissue injury healing. J. Physiol. Pharmacol. Off. J. Pol. Physiol. Soc. *53*, 147–157.

Clarke BA, Drujan D, Willis MS, Murphy LO, Corpina RA, Burova E, Rakhilin SV, Stitt TN, Patterson C, Latres E, et al. (2007). The E3 Ligase MuRF1 degrades myosin heavy chain protein in dexamethasone-treated skeletal muscle. Cell Metab. *6*, 376–385.

Charvet C, Houbron C, Parlakian A, Giordani J, Lahoute C, Bertrand A, Sotiropoulos A, Renou L, Schmitt A, Melki J, et al. (2006). New role for serum response factor in postnatal skeletal muscle growth and regeneration via the interleukin 4 and insulin-like growth factor 1 pathways. Mol. Cell. Biol. *26*, 6664–6674.

Chen JL, Walton KL, Winbanks CE, Murphy KT, Thomson RE, Makanji Y, Qian H, Lynch GS, Harrison CA, and Gregorevic P. (2014). Elevated expression of Activins promotes muscle wasting and cachexia. FASEB J. Off. Publ. Fed. Am. Soc. Exp. Biol. 28, 1711–1723.

Creemers EE, Sutherland E, Cohen S, Nathan JA, Goldberg AL. (2015). Muscle wasting in disease: molecular mechanisms and promising therapies. Nat Rev Drug Discov. 14(1):58-74.

Cohen S, Brault JJ, Gygi SP, Glass DJ, Valenzuela DM, Gartner C, Latres E, and Goldberg AL. (2009). During muscle atrophy, thick, but not thin, filament components are degraded by MuRF1-dependent ubiquitylation. J. Cell Biol. *185*, 1083–1095.

Coletti D, Moresi V, Adamo S, Molinaro M, Sassoon D. (2005). Tumor necrosis factor-alpha gene transfer induces cachexia and inhibits muscle regeneration. Genesis. 43(3):120-8.

Collard L, Herledan G, Pincini A, Guerci A, Randrianarison-Huetz V, and Sotiropoulos A. (2014). Nuclear actin and myocardin-related transcription factors control disuse muscle atrophy through regulation of SRF activity. J. Cell Sci. *127*, 5157–5163.

Costelli P, Muscaritoli M, Bossola M, Penna F, Reffo P, Bonetto A, Busquets S, Bonelli G, Lopez-Soriano FJ, Doglietto GB, Argilés JM, Baccino FM, Rossi Fanelli F. (2006). IGF-1 is downregulated in experimental cancer cachexia. Am J Physiol Regul Integr Comp Physiol. 291(3):R674-83.

Damrauer Jeffrey S, Stadler Michael E, Acharyya Swarnali, Baldwin Albert S, Couch Marion E, Guttridge Denis C. (2008). Chemotherapy-induced muscle wasting: association with NF-κB and cancer cachexia. Basic Applied Myology 18 (5): 139-148.

Dopie J, Skarp KP, Rajakylä EK, Tanhuanpää K, and Vartiainen MK. (2012). Active maintenance of nuclear actin by importin 9 supports transcription. Proc. Natl. Acad. Sci. U. S. A. *109*, E544–552.

Fleige A, Alberti S, Gröbe L, Frischmann U, Geffers R, Müller W, Nordheim A, and Schippers A. (2007). Serum response factor contributes selectively to lymphocyte development. J. Biol. Chem. *282*, 24320–24328.

Gallot YS, Durieux AC, Castells J, Desgeorges MM, Vernus B, Plantureux L, Rémond D, Jahnke VE, Lefai E, Dardevet D, Nemoz G, Schaeffer L, Bonnieu A, Freyssenet DG. (2014). Myostatin gene inactivation prevents skeletal muscle wasting in cancer. Cancer Res. 15;74(24):7344-56.

Gauthier-Rouviere C, Vandromme M, Tuil D, Lautredou N, Morris M, Soulez M, Kahn A, Fernandez A, and Lamb N. (1996). Expression

and activity of serum response factor is required for expression of the muscle-determining factor MyoD in both dividing and differentiating mouse C2C12 myoblasts. Mol. Biol. Cell *7*, 719–729.

Geneste O, Copeland JW and Treisman R. (2002). LIM kinase and Diaphanous cooperate to regulate serum response factor and actin dynamics. J. Cell Biol. *157*, 831–838.

Guerci A, Lahoute C, Hébrard S, Collard L, Graindorge D, Favier M,Cagnard N, Batonnet-Pichon S, Précigout G, Garcia L, et al. (2012). SRF-Dependent Paracrine Signals Produced by Myofibers Control Satellite Cell-Mediated Skeletal Muscle Hypertrophy. Cell Metab. *15*, 25–37.

Guttridge DC, Mayo MW, Madrid LV, Wang CY, Baldwin AS Jr. (2000). NF-kappaB-induced loss of MyoD messenger RNA: possible role in muscle decay and cachexia. Science. 29;289(5488):2363-6.
Hakkinen A, Pakarinen A, Hannonen P, Kautiainen H, Nyman K, Kraemer WJ, Hakkinen K (2005). Effects of prolonged combined strength and endurance training on physical fitness, body composition and serum hormones in women with rheumatoid arthritis and in healthy controls. Clin Exp Rheumatol 2005; 23(4):505-512.) .

Han HQ, Zhou X, Mitch WE, Goldberg AL. Myostatin/Activin pathway antagonism: molecular basis and therapeutic potential. Int J Biochem Cell Biol. 45(10):2333-47.

Hansen JS, Rutti S, Arous C, et al, (2016). Circulating follistatin is liver-derived and regulated by the glucagon-to-insulin ratio. J Clin Endocrinol Metab . 101(2):550–560.

He WA, Berardi E, Cardillo VM, Acharyya S, Aulino P, Thomas-Ahner J, Wang J, Bloomston M, Muscarella P, Nau P, Shah N, Butchbach

ME, Ladner K, Adamo S, Rudnicki MA, Keller C, Coletti D, Montanaro F, Guttridge DC. (2013). NF-κB-mediated Pax7 dysregulation in the muscle microenvironment promotes cancer cachexia. J Clin Invest. 123(11):4821-35.

Hirano H, and Matsuura Y. (2011). Sensing actin dynamics: structural basis for Gactin-sensitive nuclear import of MAL. Biochem. Biophys. Res. Commun. 414, 373–378.

Holick CN1, Newcomb PA, Trentham-Dietz A, Titus-Ernstoff L, Bersch AJ, Stampfer MJ, Baron JA, Egan KM, Willett WC. (2008). Physical activity and survival after diagnosis of invasive breast cancer. Cancer Epidemiol Biomarkers Prev. 17(2):379-86.

Holmes MD, Chen WY, Feskanich D, Kroenke CH, Colditz GA. (2005). Physical activity and survival after breast cancer diagnosis. 25;293(20):2479-86.

Irwin ML, Smith AW, McTiernan A, Ballard-Barbash R, Cronin K, Gilliland FD, Baumgartner RN, Baumgartner KB, Bernstein L. (2008). Physical activity and survival after diagnosis of invasive breast cancer.J Clin Oncol. 26(24):3958-64.

Johansen FE, and Prywes R. (1993). Identification of transcriptional activation and inhibitory domains in serum response factor (SRF) by using GAL4-SRF constructs. Mol. Cell. Biol. *13*, 4640–4647.

Koegel H, von Tobel L, Schäfer M, Alberti S, Kremmer E, Mauch C, Hohl D, Wang XJ, Beer HD, Bloch W, et al. (2009). Loss of serum response factor in keratinocytes results in hyperproliferative skin disease in mice. J. Clin. Invest. *119*,899–910.

Kuwahara K, Barrientos T, Pipes GCT, Li S, and Olson EN. (2005). Muscle specific signaling mechanism that links actin dynamics to serum response factor. Mol. Cell. Biol. *25*, 3173–3181.

L'honore A, Lamb N.J, Vandromme M, Turowski P, Carnac G, and Fernandez A. (2003). MyoD distal regulatory region contains an SRF binding CArG element required for MyoD expression in skeletal myoblasts and during muscle regeneration. Mol. Biol. Cell *14*, 2151–2162.

Lepper C, Partridge TA, and Fan CM. (2011). An absolute requirement for Pax7-positive satellite cells in acute injury-induced skeletal muscle regeneration. Dev. Camb. Engl. *138*, 3639–3646.

Li J, Zhu X, Chen M, Cheng L, Zhou D, Lu MM, Du K, Epstein JA, and Parmacek MS. (2005). Myocardin-related transcription factor B is required in cardiac neural crest for smooth muscle differentiation and cardiovascular development. Proc. Natl. Acad. Sci. U. S. A. *102*, 8916–8921.

Meyerhardt JA, Giovannucci EL, Holmes MD, Chan AT, Chan JA, Colditz GA, Fuchs CS. (2006). Influence of pre- and postdiagnosis physical activity on mortality in breast cancer survivors: the health, eating, activity, and lifestyle study. J Clin Oncol. 24(22):3527-34.

Minty A, and Kedes L. (1986). Upstream regions of the human cardiac actin gene that modulate its transcription in muscle cells: presence of an evolutionarily conserved repeated motif. Mol. Cell. Biol. *6*, 2125–2136.

Murphy M.M, Lawson JA, Mathew S.J, Hutcheson D.A, and Kardon G. (2011). Satellite cells, connective tissue fibroblasts and their

interactions are crucial for muscle regeneration. Dev. Camb. Engl. *138*, 3625–3637.

Ladner KJ, Caligiuri MA, Guttridge DC. (2002). Tumor necrosis factor-regulated biphasic activation of NF-kappa B is required for cytokine-induced loss of skeletal muscle gene products. J Biol Chem. 2003 Jan 24;278(4):2294-303.

Langen RC, Schols AM, Kelders MC, van der Velden JL, Wouters EF, Janssen-Heininger YM. (2006). Muscle wasting and impaired muscle regeneration in a murine model of chronic pulmonary inflammation. Am J Respir Cell Mol Biol. 35(6):689-96.

Langley B, Thomas M, Bishop A, Sharma M, Gilmour S, Kambadur R. (2002). Myostatin inhibits myoblast differentiation by down-regulating MyoD expression. J Biol Chem. 20;277(51):49831-40.

Lee SJ, McPherron AC. (2001). Regulation of myostatin activity and muscle growth. Proc Natl Acad SciUSA. 98(16):9306–9311

Le Grand F, Jones AE, Seale V, Scimè A, and Rudnicki MA. (2009). Wnt7a activates the planar cell polarity pathway to drive the symmetric expansion of satellite stem cells. Cell Stem Cell 4, 535–547.

Livak KJ, Schmittgen TD. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 25(4):402-8.

Lundquist MR, Storaska AJ, Liu TC, Larsen SD, Evans T, Neubig RR, and Jaffrey SR. (2014). Redox modification of nuclear actin by MICAL-2 regulates SRF signaling. Cell *156*, 563–576.

Luo Y, Yoneda J, Ohmori H, et al, (2014). Cancer usurps skeletal muscle as an energy repository. *Cancer Research*. 74(1):330–340.

Guizoni D.M, Oliveira Jr S. A, Paula F. M., Lima A. R. R, Bonomo C, Damatto R. L, Cezar M. D. M, Pagan L. U, Zornoff L. A. M, Okoshi K. and Okoshi M. P, (2013). Influence of late exercise training on myostatin and follistatin expression in soleus muscle of rats with chronic heart failure. The FASEB J. 27(1):1085-8.

Medjkane S, Perez-Sanchez C, Gaggioli C, Sahai E, Treisman R. (2009). Myocardin-related transcription factors and SRF are required for cytoskeletal dynamics and experimental metastasis. Nat Cell Biol11(3):257-68.

Miano JM, Long X and Fujiwara K. (2007). Serum response factor: master egulator of the actin cytoskeleton and contractile apparatus. Am. J. Physiol. Cell Physiol. *292*, C70–81.

Miller SC, Ito H, Blau HM, Torti FM. (1988). Tumor necrosis factor inhibits human myogenesis *in vitro*. Mol Cell Biol. 8(6):2295-301.

Miralles F, Posern G, Zaromytidou, A.-I, and Treisman, R. (2003). Actin dynamics control SRF activity by regulation of its coactivator MAL. Cell *113*, 329–342.

Mitchell PO, Pavlath GK. (2004). Skeletal muscle atrophy leads to loss and dysfunction of muscle precursor cells. Am J Physiol Cell Physiol. 287(6):C1753-62.

Modak C, Chai J, (2010). Serum response factor: Look into the gut. World J Gastroenterol. 14; 16(18): 2195–2201.

Mokalled MH, Johnson AN, Creemers EE and Olson EN. (2012). MASTR directs MyoD-dependent satellite cell differentiation during skeletal muscle regeneration. Genes Dev. *26*, 190–202.

Moresi V, Pristerà A, Scicchitano BM, Molinaro M, Teodori L, Sassoon D, Adamo S, Coletti D. (2008). Tumor necrosis factor-alpha inhibition of skeletal muscle regeneration is mediated by a caspase-dependent stem cell response. Stem Cells. 26(4):997-1008.

Munoz RM, Han H, Tegeler T, Petritis K, Von Hoff DD, Hoffman SA. (2013). Isolation and characterization of muscle fatigue substance with anti-tumor activities. J Cancer. 9;4(4):343-9

Murphy KT, Chee A, Gleeson BG, Naim T, Swiderski K, Koopman R, Lynch GS. (2011). Antibody-directed myostatin inhibition enhances muscle mass and function in tumor-bearing mice. Am J Physiol Regul Integr Comp Physiol. 301(3):R716-26.

Olson EN and Nordheim A. (2010). Linking actin dynamics and gene transcription to drive cellular motile functions. Nat. Rev. Mol. Cell Biol. *11*, 353–365.

Padilha CS, Borges FH, Costa Mendes da Silva LE, Frajacomo FTT, Jordao AA, Duarte JA, Cecchini R, Guarnier FA, Deminice R. (2017). Resistance exercise attenuates skeletal muscle oxidative stress, systemic pro-inflammatory state, and cachexia in Walker-256 tumor-bearing rats. Appl Physiol Nutr Metab. 42(9):916-923.

Pawlowski R, Rajakyla EK, Vartiainen MK and Treisman R. (2010). An actin regulated importin alpha/beta-dependent extended bipartite NLS directs nuclear import of MRTF-A. EMBO J 29, 3448-3458.

Pelosi M, De Rossi M, Barberi L, Musarò A. (2014). IL-6 impairs myogenic differentiation by downmodulation of p90RSK/eEF2 and mTOR/p70S6K axes, without affecting AKT activity. Biomed Res Int. 2014;2014:206026.

Penna F, Bonetto A, Muscaritoli M, Costamagna D, Minero VG, Bonelli G, Rossi Fanelli F, Baccino FM, Costelli P. (2010). Muscle atrophy in experimental cancer cachexia: is the IGF-1 signaling pathway involved? Int J Cancer. 1;127(7):1706-17.

Peterson JM, Bakkar N, and Guttridge DC. (2011). NF-□B signaling in skeletal muscle health and disease. Curr. Top. Dev. Biol. *96*, 85–119.

Pigna E, Berardi E, Aulino P, Rizzuto E, Zampieri S, Carraro U, Kern H, Merigliano S, Gruppo M, Mericskay M, Li Z, Rocchi M, Barone R, Macaluso F, Di Felice V, Adamo S, Coletti D, Moresi V. (2016). Aerobic Exercise and Pharmacological Treatments Counteract Cachexia by Modulating Autophagy in Colon Cancer. Sci Rep. 31;6:26991.

Pipes GCT, Creemers EE and Olson EN. (2006). The myocardin family of transcriptional coactivators: versatile regulators of cell growth, migration, and myogenesis. Genes Dev. *20*, 1545–1556.

Polge C, Heng AE, Jarzaguet M, Ventadour S, Claustre A, Combaret L, Béchet D, Matondo M, Uttenweiler-Joseph S, Monsarrat B, et al. (2011). Muscle actin is polyubiquitinylated *in vitro* and *in vivo* and targeted for breakdown by the E3 ligase MuRF1. FASEB J. Off. Publ. Fed. Am. Soc. Exp. Biol. *25*, 3790–3802.

Pollard TD, and Borisy GG. (2003). Cellular motility driven by assembly and disassembly of actin filaments. Cell *112*, 453–465.

Posern G, Miralles F, Guettler S, and Treisman R. (2004). Mutant actins that stabilise Factin use distinct mechanisms to activate the SRF coactivator MAL. EMBO J. 23, 3973– 3983.

Posern G, and Treisman R. (2006). Actin together: serum response factor, its cofactors and the link to signal transduction. Trends Cell Biol. 16, 588–596.

Rashidlamir A, Attarzadeh Hosseini SR, Hejazi K, Motevalli Anberani SM . (2016). The effect of eight weeks resistance and aerobic training on myostatin and follistatin expression in cardiac muscle of rats. J Cardiovasc Thorac Res. 8(4):164-169.

Roan E, Waters CM, Teng B, Ghosh M, Schwingshackl A. (2014). The 2-pore domain potassium channel TREK-1 regulates stretch-induced detachment of alveolar epithelial cells. PLoS One. 19;9(2):e89429.

Romanello V, Sandri M. (2015). Mitochondrial quality control and muscle mass maintenance. *Frontiers in Physiology*. 2015;6:p. 422.

Rommel C, Bodine SC, Clarke BA, Rossman R, Nunez L, Stitt TN, Yancopoulos GD, Glass DJ. (2001). Mediation of IGF-1-induced skeletal myotube hypertrophy by PI(3)K/Akt/mTOR and PI(3)K/Akt/GSK3 pathways. Nat Cell Biol. 3(11):1009-13.

Ruiz-Ortega M, Lorenzo O, Rupérez M, König S, Wittig B, Egido J. (2000). Angiotensin II activates nuclear transcription factor kappaB through AT(1) and AT(2) in vascular smooth muscle cells: molecular mechanisms. Circ Res. 23;86(12):1266-72.

Sadeh M, Czyewski K, Stern LZ. (1985). Chronic myopathy induced by repeated bupivacaine injections J Neurol Sci. 67(2):229-38.

Sakuma K, Akiho M, Nakashima H, Akima H, and Yasuhara M. (2008). Age related reductions in expression of serum response factor and myocardin-related transcription factor A in mouse skeletal muscles. Biochim. Biophys. Acta *1782*, 453–461.

Sanders PM, Russell ST, Tisdale MJ. (2005). Angiotensin II directly induces muscle protein catabolism through the ubiquitin-proteasome proteolytic pathway and may play a role in cancer cachexia. Br J Cancer. 22;93(4):425-34.

Schröter H, Mueller CG, Meese K and Nordheim A. (1990). Synergism in ternary complex formation between the dimeric glycoprotein p67SRF, polypeptide p62TCF and the c-fos serum response element. EMBO J. *9*, 1123–1130.

Schwarzkopf M, Coletti D, Sassoon D, Marazzi G. (2006). Muscle cachexia is regulated by a p53-PW1/Peg3-dependent pathway. Genes Dev. 2006 Dec 15;20(24):3440-52.

Sepulveda PV, Lamon S, Hagg A, Thomson RE, Winbanks CE, Qian H, Bruce CR, Russell AP, Gregorevic P. (2015). Evaluation of follistatin as a therapeutic in models of skeletal muscle atrophy associated with denervation and tenotomy. Sci Rep. 11;5:17535.

Sotiropoulos A, Gineitis D, Copeland J, and Treisman R. (1999). Signal-regulated activation of serum response factor is mediated by changes in actin dynamics. Cell *98*, 159–169.

Soulez M, Rouviere CG, Chafey P, Hentze, D, Vandromme M, Lautredou N, Lamb N, Kahn A, and Tuil D. (1996). Growth and

differentiation of C2 myogenic cells are dependent on serum response factor. Mol. Cell. Biol. *16*, 6065–6074.

Sun Y, Boyd K, Xu W, Ma J, Jackson CW, Fu A, Shillingford JM, Robinson GW, Hennighausen L, Hitzler JK et al. (2006). Acute myeloid leukemiaassociated Mkl1 (MRTF-A) is a key regulator of mammary gland function. Mol. Cell. Biol. *26*, 5809–5826.

Tedesco FS, Moyle LA, Perdiguero E. (2017). Muscle Interstitial Cells: A Brief Field Guide to Non-satellite Cell Populations in Skeletal Muscle. Methods Mol Biol. 1556:129-147.

Tran TH, Shi X, Zaia J and Ai X. (2012). Heparan Sulfate 6-O-endosulfatases (Sulfs) Coordinate the Wnt Signaling Pathways to Regulate Myoblast Fusion during Skeletal Muscle Regeneration. J. Biol. Chem. 287, 32651–32664.

Treisman R. (1987). Identification and purification of a polypeptide that binds to the c-fos serum response element. EMBO J. *6*, 2711–2717.

Trendelenburg AU, Meyer A, Jacobi C, Feige JN, Glass DJ. (2012). TAK-1/p38/nNFκB signaling inhibits myoblast differentiation by increasing levels of Activin A. Skelet Muscle. 7;2(1):3.

Van Gammeren D, Damrauer JS, Jackman RW, Kandarian SC. (2009). The IkappaB kinases IKKalpha and IKKbeta are necessary and sufficient for skeletal muscle atrophy. FASEB J. 23(2):362-70.

Vartiainen MK, Guettler S, Larijani B and Treisman R. (2007). Nuclear actin regulates dynamic subcellular localization and activity of the SRF cofactor MAL. Science *316*, 1749–1752.

Wallace MA, Della Gatta PA, Ahmad Mir B, Kowalski GM, Kloehn J, McConville MJ, Russell AP, and Lamon S. (2016). Overexpression of Striated Muscle Activator of Rho Signaling (STARS) Increases C2C12 Skeletal Muscle Cell Differentiation. Front. Physiol. 7, 7.

Wang Z, Wang DZ, Pipes, GCT, and Olson EN. (2003). Myocardin is a master regulator of smooth muscle gene expression. Proc. Natl. Acad. Sci. U. S. A. *100*,7129–7134.

White JP, Baltgalvis KA, Puppa MJ, Sato S, Baynes JW, Carson JA. (2011). Muscle oxidative capacity during IL-6-dependent cancer cachexia. Am J Physiol Regul Integr Comp Physiol. 300(2):R201-11.

Winbanks CE, Weeks KL, Thomson RE, Sepulveda PV, Beyer C, Qian H, Chen JL, Allen JM, Lancaster GI, Febbraio MA, Harrison CA, McMullen JR, Chamberlain JS, Gregorevic P. (2012). Follistatin-mediated skeletal muscle hypertrophy is regulated by Smad3 and mTOR independently of myostatin. J Cell Biol. 25;197(7):997-1008.

Yang SH, Shore P, Willingham N Lakey JH, and Sharrocks AD. (1999). The mechanism of phosphorylation-inducible activation of the ETS-domain transcription factor Elk-1. EMBO J. *18*, 5666–5674.

Zammit PS, Heslop L, Hudon V, Rosenblatt JD, Tajbakhsh S, Buckingham ME, Beauchamp JR, Partridge TA. (2002). Kinetics of myoblast proliferation show that resident satellite cells are competent to fully regenerate skeletal muscle fibers. Exp Cell Res 281:39–49

Zammit PS, Golding JP, Nagata Y, Hudon V, Partridge TA, and Beauchamp JR. (2004). Muscle satellite cells adopt divergent fates: a mechanism for self-renewal? J. Cell Biol. 166, 347–357.

Zheng B, Han M, Bernier M, and Wen J. (2009). Nuclear actin and actin-binding proteins in the regulation of transcription and gene expression. FEBS J. 276, 2669–2685.

Zhou X, Wang JL, Lu J, Song Y, Kwak KS, Jiao Q, Rosenfeld R, Chen Q, Boone T, Simonet WS, Lacey DL, Goldberg AL, Han HQ. (2010). Reversal of cancer cachexia and muscle wasting by ActIIBR antagonism leads to prolonged survival. Cell. 20;142(4):531-43.

Zhu J, Li Y, Lu A, Gharaibeh B, Ma J, Kobayashi T, Quintero Andres J. and Huard J. (2011). Follistatin Improves Skeletal Muscle Healing after Injury and Disease through an Interaction with Muscle Regeneration, Angiogenesis, and Fibrosis Am J Pathol. 179(2): 915–930.

LIST OF PUBLICATIONS

1- Baccam Alexandra, Ramella Martina, Boccafoschi Francesca, Mericskay Mathias, Li Zhenlin, Coletti Dario.

Molecular pathways involved in the crosstalk between cytokines and mechanical cues in cancer cachexia. J Cachexia Sarcopenia Muscle. 2016 Sep; 7(4): 501–502. Published online 2016 Sep 6. doi: 10.1002/jcsm.12141

2- Baccam Alexandra, Hassani Medhi, Sviercoich-Benoni Alexandra, Adamo Sergio, Moresi Viviana and Coletti Dario

Basking in their Niche: Stem Cells with Myogenic Potential as a Target to Combat Cachexia. Curr Updates Stem Cell ResTher. [march 2017) 1: 1.1

POSTER / COMUNICAZIONI A CONGRESSO

1- Baccam Alexandra, Ramella Martina, Boccafoschi Francesca, Mericskay Mathias, Li Zhenlin, Coletti Dario.

Molecular pathways involved in the crosstalk between cytokines and mechanical cues in cancer cachexia. 8th International Conference on Cachexia, Sarcopenia and Muscle Wasting, 2015 J Cachexia Sarcopenia Muscle. 2016 Sep; 7(4): 501–502, Paris, FRANCE

2- Baccam Alexandra, Ramella Martina, Boccafoschi Francesca, Mericskay Mathias, Li Zhenlin, Coletti Dario.

Molecular pathways involved in the crosstalk between cytokines and mechanical cues in cancer cachexia. 1rd day of muscle exercice, 2017 june, Paris, FRANCE

3- Baccam Alexandra, Hassani Medhi, Benoni Alexandra, Ramella Martina, Boccafoschi Francesca, Parlakian Ara, Li Zhenlin, Xue Zhigang, Adamo Sergio, Coletti Dario

Mechanisms involved in the cross-talk between humoral and mechanical cues underlying muscle wasting in cachexia. 10th International Conference on Cachexia, Sarcopenia and Muscle Wasting, 2017 J Cachexia Sarcopenia Muscle. 201 nov; doi: 10.1002/jcsm.12255, Roma, ITALY