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THE ROLE OF MICAL2 IN PHYSIOLOGICAL AND PATHOLOGICAL MYOGENIC COMMITMENT

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Abbreviation list

α-SA:	Sarcomeric alpha actin
αSMA:	Alpha Smooth Muscle Actin
β-SG^{null}:	Sarcoglycan- β null
AP:	Alkaline Phosphatase
ARMS:	Alveolar rhabdomyosarcoma
BrdU:	Bromodeoxyuridine
BSA:	Bovine serum albumin
Cas9:	CRISPR associated protein 9
CH:	Calponin homology domain
cMyHC:	Cardiac myosin heavy chain
CRISPR:	Clustered regularly interspaced short palindromic repeat
CSA:	Cross-Sectional Area
CTX:	Cardiotoxin
DGC:	Dystrophin Glycoprotein Complex
DMD:	Duchenne Muscular Dystrophy
DMEM:	Dulbecco's Modified Eagle's Medium
EB:	Embryoid body
EGFR:	Epidermal growth factor-receptor

EMT:	Epithelial-mesenchymal transition
ESCs:	Embryonic stem cells
ERMS:	Embryonal rhabdomyosarcoma
esiRNA:	Endoribonuclease-prepared small interference RNA
F-actin:	Filamentous actin
FACS:	Fluorescence Activated Cell Sorter
FAD:	Flavin Adenine Dinucleotide
FBS:	Foetal Bovine Serum
FGF:	Fibroblast growth factor
FI:	Fusion index
G-actin:	Globular actin
GC:	Gastrocnemius
GFP:	Green Fluorescent Protein
GU:	Genitourinary tract
HGF:	Hepatocyte growth factor
hiPSCs:	Human induced pluripotent stem cells
HS:	Horse Serum
IF:	Immunofluorescence
LGMD:	Limb Girdle Muscular Dystrophies
LGMD2D:	Limb Girdle Muscular Dystrophy Type 2D

LGMD2E:	Limb Girdle Muscular Dystrophy Type 2E
LIF:	Leukemia inhibitory factor
MABs:	Mesoangioblasts
MAPK:	Mitogen-activated protein kinase
MDs:	Muscular Dystrophies
MET:	Mesenchymal-epithelial transition
MICALs	Molecules interacting with CasL (or Microtubule Associated Monooxygenase, Calponin And LIM Domain Containing)
MICAL1	Microtubule Associated Monooxygenase, Calponin And LIM Domain Containing-1
MICAL2	Microtubule Associated Monooxygenase, Calponin And LIM Domain Containing-2
MICAL3	Microtubule Associated Monooxygenase, Calponin And LIM Domain Containing-3
MO:	Flavoprotein monooxygenase domain
MRFs:	Muscle regulatory factors
MyHC:	Myosin Heavy Chain
MyoG:	Myogenin
NADPH:	Nicotinamide Adenosine Dinucleotide Phosphate

OCT4:	Octamer-binding transcription factor 4
PAX7:	Paired box protein 7
PBS:	Phosphate Buffer Saline
Pen/Strep:	Penicillin-Streptomycin
PHBH:	FAD-containing enzyme p-hydroxybenzoate hydroxylase
PI:	Propidium iodide
PMSF:	Phenylmethylsulfonyl Fluoride
PSCs:	Pluripotent stem cells
Q:	Quadriceps femoris
qRT-PCR:	quantitative Reverse Transcription Polymerase Chain Reaction
RBD:	Rab-binding domain
REDOX:	Oxidation-reduction reaction
RMS:	Rhabdomyosarcoma
ROS:	Reactive oxygen species
S1P:	Sphingosine 1-Phosphate
SCs:	Satellite cells
SD:	Standard Deviations
SEM:	Standard Error of the Mean
SGCA:	Alpha-Sarcoglycan

SGCB:	Beta-Sarcoglycan
SM-MHC:	Smooth muscle myosin heavy chain
TA:	Tibialis Anterior
TAZ:	Transcriptional coactivator with PDZ-binding motif
TBS:	Tris-buffered saline
TGFβ:	Transforming growth factor beta
TUB:	α-Tubulin
ULA:	Ultra low attachment
Utrn:	Utrophin
WB:	Western blot
YAP:	Yes-associated protein

English Summary

Muscle tissue represents 40% of human body mass and provides locomotion, posture support and breathing. Contractile myofibre units are mainly composed of two crucial components: thick myosin and thin actin (F-actin) filaments. F-Actin interacts with Microtubule Associated Monooxygenase, Calponin And LIM Domain Containing 2 (MICAL2), capable to make oxidation-reduction reactions by its FAD domain. Indeed, MICAL2 modifies actin subunits and promotes actin turnover by severing disaggregation and preventing repolymerisation.

An adequate supply of oxygen is essential during myogenesis and muscle fibres need to modify their oxygen demand from rest to cell contraction. The striking capability of MICAL2 to directly and mechanistically connect oxygen availability with F-actin depolymerisation, and hence cytoskeleton dynamics, was thought to be implicated into the process of myogenic differentiation. Therefore, we hypothesised that MICAL2 is involved in smooth, skeletal and cardiac muscle differentiations. Gaining this knowledge might help to understand the role of MICAL2 in muscle pathological conditions, including muscular dystrophies and rhabdomyosarcoma (RMS). Hence, unravelling MICAL2 involvement in muscle differentiation in physiological and pathological conditions was the main aim of this project.

The role of MICAL2 was deciphered firstly during differentiation to skeletal, smooth and cardiac muscle cells from myogenic progenitors. In this setting, MICAL2 was found more expressed in myogenic differentiated cells compared to their relative progenitors. Secondly, MICAL2 expression was assessed in dystrophic conditions where the pool of adult stem cells was exhausted. In this case, MICAL2 was more present when a degeneration/regeneration process occurred, localising in centrally-nucleated fibres, in both acute and chronic conditions. Loss and gain of function studies in C2C12 and satellite cells demonstrated that silencing or overexpressing MICAL2 had an impact on both proliferation and myogenic differentiation potential. Indeed, silencing MICAL2 resulted in an enhanced proliferation state of progenitor cells, with a consequent skeletal muscle differentiation impairment. While, on the contrary, overexpressing MICAL2 in

myogenic precursors differentiating towards skeletal muscle positively impacted on myotube formation compared to control cells. Moreover, RMS cell lines were explored for MICAL2 expression, showing an abundant presence of MICAL2 in these cancer cells. Loss of function experiments were performed to unveil the molecular impact of MICAL2, resulting in a slower and decreased proliferative stage. Further modulation of MICAL2 might reduce the tumorigenic capacity of RMS cells and might induce differentiation towards skeletal muscle.

In conclusion, our data indicate that MICAL2 is a novel regulator of myogenic differentiation, also outlining its multifaceted effects in determining the cellular response to the environment. In particular, in the pathophysiological context MICAL2 affects proliferation and cell migration, and controls muscle regeneration. Thus, we propose MICAL2 as potent modulator of skeletal myogenesis and perhaps crucial also for cardiac and smooth muscle progenitor cell fate.

Italian Summary

Il tessuto muscolare rappresenta il 40% della massa corporea umana e assicura la locomozione, il supporto posturale e la respirazione. Le unità contrattili delle miofibre sono composte principalmente da due componenti cruciali: filamenti spessi di miosina e filamenti sottili di actina (F-actina). La F-Actina interagisce con Microtubule Associated Monooxygenase, Calponin And LIM Domain Containing 2 (MICAL2), il quale è in grado di produrre reazioni di ossido riduzione tramite il dominio FAD. Infatti, MICAL2 modifica le subunità di actina causandone la depolimerizzazione e il relativo impedimento della ripolimerizzazione, promuovendo cosí il turnover dell'actina.

Un adeguato apporto di ossigeno è essenziale durante la miogenesi e, inoltre, le fibre muscolari devono modificare la domanda di ossigeno durante il passaggio da riposo a contrazione cellulare. La capacità sorprendente di MICAL2 di collegare direttamente e meccanicamente la disponibilità di ossigeno con la depolimerizzazione della F-actina, e quindi la dinamicità del citoscheletro, è stata ritenuta implicata nel processo di differenziamento miogenico. Pertanto, abbiamo ipotizzato che MICAL2 sia coinvolto nel differenziamento del muscolo liscio, scheletrico e cardiaco. Acquisire questa conoscenza potrebbe aiutare a comprendere il ruolo di MICAL2 nelle condizioni patologiche muscolari, incluse le distrofie muscolari e il rabdomiosarcoma (RMS). Quindi, la scoperta del coinvolgimento di MICAL2 nel differenziamento muscolare in condizioni fisiologiche e patologiche è il principale obiettivo di questo progetto.

Il ruolo di MICAL2 è stato decifrato in primo luogo durante il differenziamento delle cellule muscolari scheletriche, lisce e cardiache a partire dai progenitori miogenici. Durante questi processi, MICAL2 è stato trovato più espresso nelle cellule miogeniche differenziate rispetto ai loro relativi progenitori. In secondo luogo, l'espressione di MICAL2 è stata valutata in condizioni distrofiche, in cui la nicchia di cellule staminali adulte è esaurita. In questo caso, MICAL2 era maggiormente presente al verificarsi di un processo, sia acuto che cronico, di degenerazione/rigenerazione, presentando una localizzazione prettamente nucleare nelle fibre nucleate centralmente. Analisi di perdita o aumento della funzionalitá

hanno dimostrato, in C2C12 e nelle cellule satelliti, che il silenziamento o l'iperespressione di MICAL2 hanno un impatto sia sulla proliferazione che sul potenziale di differenziamento miogenico. Infatti, il silenziamento di MICAL2 ha comportato un aumento dello stato di proliferazione delle cellule progenitrici, con conseguente compromissione del differenziamento verso muscolo scheletrico. Mentre, al contrario, la iperespressione di MICAL2 nei precursori miogenici, indotti verso il differenziamento a muscolo scheletrico, ha avuto un impatto positivo sulla formazione di miotubi, rispetto alle cellule di controllo. Inoltre, diverse linee cellulari di RMS sono state esplorate per l'espressione di MICAL2, mostrando un'abbondante presenza di MICAL2 in queste cellule tumorali. Sono stati eseguiti, in seguito, esperimenti di perdita di funzione per svelare l'impatto molecolare di MICAL2 a livello tumorale, determinando uno stadio proliferativo più lento e diminuito. Un'ulteriore modulazione di MICAL2 potrebbe ridurre la capacità tumorigenica delle cellule di RMS e potrebbe indurre un'eventuale differenziamento verso il muscolo scheletrico.

In conclusione, i nostri dati indicano MICAL2 come un nuovo regolatore del differenziamento miogenico, delineando anche i suoi molteplici effetti nel determinare la risposta cellulare all'ambiente. In particolare, nel contesto pato-fisiologico, MICAL2 influenza la proliferazione e la migrazione cellulare e controlla la rigenerazione muscolare. Quindi, proponiamo MICAL2 come un potente modulatore della miogenesi scheletrica e forse cruciale anche per il destino delle cellule progenitrici del muscolo cardiaco e liscio.

Samenvatting

Spierweefsel omvat 40% van ons lichaamsgewicht en staat in voor onze beweging, houding en ademhaling. De contractiele eenheden van de spiervezels bestaan uit twee cruciale componenten: dikke myosine en dunne actine (F-actine) filamenten. F-actine kan interageren met Microtubule Associated Monooxygenase, Calponin And LIM Domain Containing 2 (MICAL2), een eiwit dat in staat is om oxidatie-reductie reacties te katalyseren via zijn FAD domein. MICAL2 kan, via deze interactie, actine subeenheden modificeren en de actine turnover promoten door disaggregatie te stimuleren en repolymerisatie tegen te gaan.

Voldoende zuurstoftoevoer is essentieel tijdens de myogenese vermits de spiervezels hun zuurstofverbruik aanpassen tijdens de transitie van rust naar contractie. Opvallend is dat MICAL2 het vermogen heeft om het zuurstofgebruik direct en mechanistisch te linken aan de F-actine depolymerisatie. Vandaar dat gedacht wordt dat de dynamiek van het cytoskelet van belang is tijdens de myogene differentiatie. Dit leidt tot onze hypothese dat MICAL2 een rol speelt in de differentiatie van zowel het hart, de skeletspier als de gladde spieren. Meer informatie hieromtrent kan ons helpen de rol van MICAL2 in pathologische condities, zoals musculaire dystrofie en rabdomyosarcoom (RMS), te ontrafelen. Vandaar zal het hoofddoel van mijn project bestaan uit het ontrafelen van de rol van MICAL2 tijdens de myogene differentiatie in zowel fysiologische als pathologische condities.

In eerste instantie werd de rol van MICAL2 onderzocht tijdens de differentiatie van myogene progenitoren naar skeletspier, hartspier en gladde spieren. Hier zagen we dat MICAL2 meer tot expressie komt in gedifferentieerde cellen dan in de progenitoren. Nadien werd de expressie van MICAL2 geanalyseerd in dystrofische condities, waarbij de stamcelpool uitgeput is. Hier werd vastgesteld dat MICAL2 meer aanwezig was in de centraal gelegen nuclei tijdens het proces van degeneratie en regeneratie in zowel acute als chronische condities.

Downregulatie of overexpressie van MICAL2 in C2C12s en satellietcellen had een impact op zowel de proliferatie als de differentiatie. Hierbij leidde downregulatie van MICAL2 tot een verhoogde proliferatie waardoor de spierdifferentiatie werd

gehinderd. Daartegenover had MICAL2 overexpressie een positieve impact op de vorming van myotubes tijdens de skeletspierdifferentiatie.

Verder werd in RMS lijnen een hoge MICAL2 expressie geobserveerd. Wanneer deze expressie naar beneden werd gebracht, vertraagde en daalde de deling van deze kankercellen. Verdere modulatie van MICAL2 zou dus kunnen leiden tot een verlaagde tumorigene capaciteit en een inductie van de differentiatie naar skeletspier.

In conclusie toont onze data aan dat MICAL2 een nieuwe regulator is van de myogenese en dus van belang is tijdens de differentiatie zowel skeletspier, hartspier als gladde spier. Verder werd zijn veelzijdigheid aangetoond om de correcte celrespons te bepalen bij verandering van de omgeving. Meer specifiek kan MICAL2 de proliferatie, migratie en spierregeneratie beïnvloeden in pathofysiologische omstandigheden.

1 Chapter One: Introduction

1.1 Myogenesis

Myogenesis is the process that ensures the generation of myoblasts to give rise to skeletal muscle tissue [1]. Myogenesis begins during embryogenesis, leading to the organisation of the different muscle patterns. [2]. It is known that myogenesis can be separated into two phases during development: an early embryonic and a later foetal phase (Figure I1) [3, 4]. Further studies demonstrated that the first phase results in the production of the primary myofibres, which derive from Pax3+ dermomyotomal progenitors in mice [5-7]. These primary myofibres were shown to express a specific set of proteins, such as the slow myosin heavy chain (MyHC) and myosin light chain 1 (MyLC1, Myl1) [8]. After formation of the myotome, the Pax3+ cells of the central dermomyotome were seen populating the myotome, providing the myogenic precursors involved in later phases of myogenesis [9-11]. Myogenesis progression is a rostral-to-caudal wave of maturation due to embryo elongation and sequential addition of new pairs of somites [12, 13]. In the trunk and limbs, the myogenic program was shown to be controlled by a core network of transcription factors, including Pax3 and a set of muscle regulatory factors (MRFs) consisting of Myf5, MyoD (Myod1), MRF4 (Myf6) and myogenin (MyoG) controlling the terminal differentiation of myoblasts into myocytes [12, 14-19]. Importantly, in 1987, Davis, Weintraub and Lassar described how a transfection of cDNA for a gene that they called Myogenic Differentiation 1 (MyoD1) could convert a small proportion of fibroblasts, and other differentiated cell types, to the skeletal muscle lineage [20]. This was the first proof that a single transcription factor could drive trans-differentiation, reprogramming the fate of a differentiated cell type. Myogenin (MyoG) was then identified in 1989 as a sequence that forced myoblasts to undergo myogenic differentiation, resulting in myoblasts exiting the cell cycle and fusing to form multinucleated myofibres [21].

During the course of postnatal muscle growth, individual fibres undergo hypertrophy through the addition of novel myofibrils [22, 23]. During secondary myogenesis, muscle growth is sustained mainly by cell fusion and the addition of myonuclei from proliferating Pax7+ progenitors [24]. Indeed, following the research of Keller [25] and Kelly [8], it was seen that, during the second phase of

myogenesis in mouse, a subset of the Pax3+ myogenic progenitors began to express Pax7 and downregulated Pax3. These Pax7+ myogenic precursors fused among themselves or to the primary fibres giving rise to secondary or foetal fibres [26, 27]. Van Horn and Crow saw that at this time, the fibres also expressed fast MyHC isoforms [28]. A subpopulation of the Pax7+ progenitors was seen to also form the pool of adult muscle stem cells – the satellite cells (SCs) [9, 11, 29].

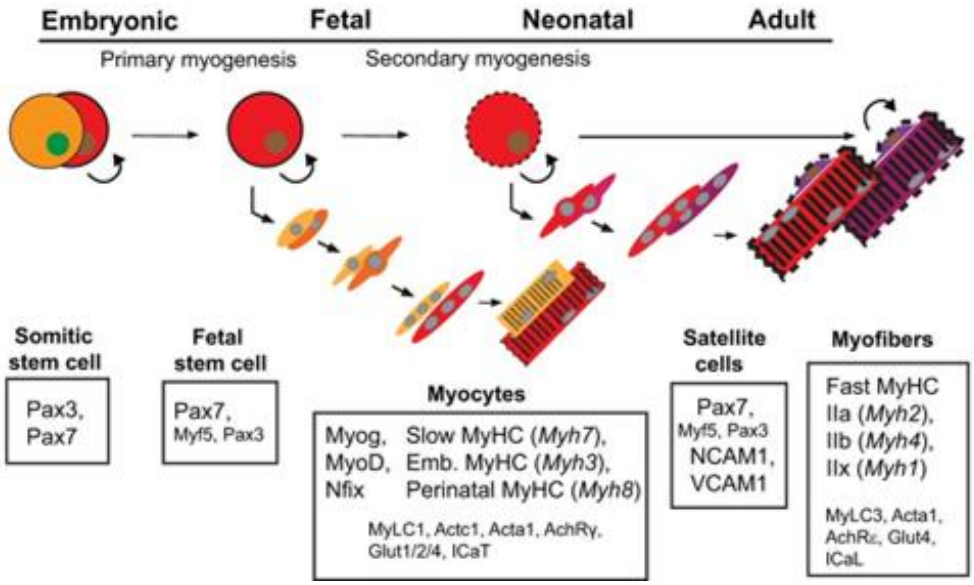

Figure I1. Stages of skeletal myogenesis from the embryo to the adult.

Adapted from Chal et al. [30]

1.2 Skeletal muscle

Muscle tissue represents 40% of human body mass and provides locomotion, posture support and breathing [31]. Skeletal muscle sections seen microscopically show a series of alternating light and dark bands perpendicular to the long axis. This pattern is known as striated muscle, when referring to skeletal muscle. Due to its elongated shape and the presence of multiple nuclei, a skeletal muscle cell is also referred to as a muscle fibre. Muscle fibres are formed during development by fusion of undifferentiated, mono-nucleated cells known as myoblasts into single, cylindrical, multi-nucleated cells [32]. Skeletal muscle differentiation is finalised around birth, but differentiated fibres continue to increase in size from infancy to adulthood. Adult skeletal muscle fibre diameters can range between 10 and 100 mm and their length may extend up to 20 cm. Spread throughout the length of muscle fibres, nuclei from the original myoblasts participate in regulation of gene expression and protein synthesis within their local domain, contributing to maintenance and function of these cells [33]. The striated pattern in skeletal muscle results from two types of filaments within the cytoplasm, known as thin (actin) and thick (myosin) filaments due to their relative thickness. One unit of thin and thick filaments is known as a sarcomere (from the Greek sarco, "muscle," and mer, "part") (Figure I2). The repetition of sarcomeres form myofibrils, which are the main part of the cytoplasm of a fibre. Thin filaments - which are about half the diameter of the thick filaments - are principally composed of actin protein. Actin filaments are formed by two twisted α helices that associate with the regulatory proteins tropomyosin and the troponins. Tropomyosin consist of two α-helical chains arranged as a coiled-coil rod. The primary function of tropomyosin and troponins is to regulate the interaction of actin filaments and thick filaments during force generation. The troponins are a complex of three proteins: troponin-C, troponin-I and troponin-T. Troponins are present at regular intervals along the entire length of the actin filament [34]. All together, these proteins play important roles in regulating contraction. An actin molecule is a globular protein composed of a monomer (globular-actin) that polymerizes with other actin monomers to form a polymer consisting of two intertwined, helical chains. Each actin molecule of these chains, contain a binding site for myosin. Thick filaments are composed

almost entirely of myosin. Myosin consists of two globular heads containing two heavy and four light chains and a long tail formed by the two convoluted heavy chains. The two globular heads form cross-bridges, extending out to the sides. These cross-bridges make contacts with the thin filament and exert force during muscle contraction. Each globular head contains two binding sites, one for binding to the thin filament and one for ATP [33, 35]. MyHC consist of different isoforms with different ATPase activities regulating the contraction characteristics of myofibres. Thus, the roles of muscle fibres differ: oxidative myofibres, containing MyHC isoforms with a lower ATPase activity and a stable source of ATP, operate skeletal support and endurance roles, while fast glycolytic fibres, composed of MyHC isoforms with high ATPase activity and ready access to ATP, give strength and speed for a short duration [36].

Muscle repair and regeneration complement developmental myogenesis, with myoblasts fusing together for de novo myotube formation, or fusing to damaged myofibres to replace lost myonuclei. Furthermore, skeletal muscle will maintain regeneration even after repeated injury, ensuring the generation of thousands of myoblasts on each occasion [37]. Therefore, if skeletal muscle fibres are damaged or destroyed after birth as a result of injury, or if myonuclei are required for homeostasis, hypertrophy or repair/regeneration, they undergo a repair process involving a population of resident undifferentiated stem cells known as satellite cells, embedded between the plasmalemma of the myofibre and the basal lamina [32, 38].

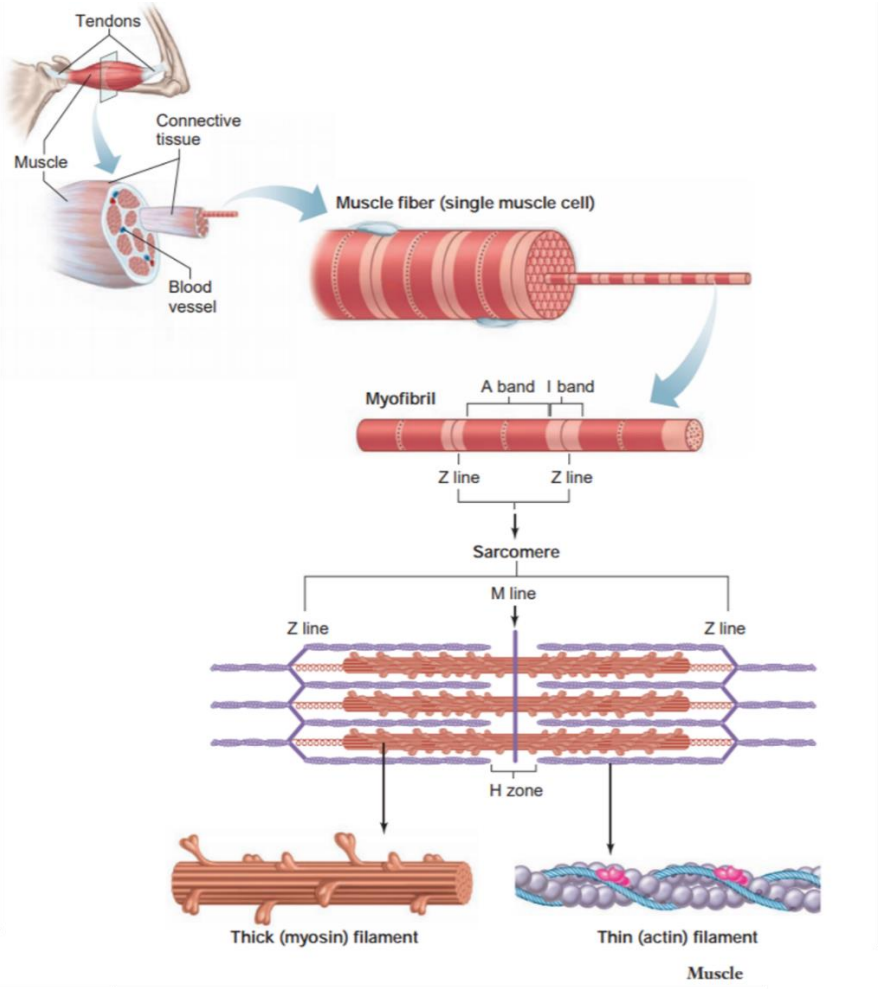

Figure I2: Structure of a skeletal muscle.

Adapted from Martini's human anatomy [35].

1.2.1 Satellite cells (SCs)

SCs are adult skeletal muscle stem cells that possess an extensive potential for regeneration after injury [39]. These cells were first identified in 1961 and were described as being located between the plasma membrane of muscle fibres and the surrounding basal membrane [38]. Satellite cells comprise 2% of total myonuclei and are normally quiescent, but in response to strain or injury, they become active and undergo mitotic proliferation. This process goes through asymmetric divisions providing a sufficient number of fusion-competent muscle progenitor cells that contribute to myofibre repair, but also uncommitted stem cells that remain in a quiescent state and serve to replenish the reservoir pool (Figure I3) [40]. This

process ensures that the regenerative demands due to muscle injuries can be met, throughout adulthood. Daughter cells involved in the repairing process then differentiate into myoblasts. These new myoblasts can either fuse together to form new fibres or fuse with stressed or damaged muscle fibres to reinforce and repair them [41]. Although satellite cells have considerable capacity to form new skeletal muscle fibres, this ability may not restore a severely damaged muscle to the original number of muscle fibres. Some compensations for a loss of muscle tissue also occur through a satellite cell-mediated increase in the size (hypertrophy) of the remaining muscle fibres [42]. Muscle hypertrophy also occurs in response to heavy exercise. Evidence suggests that this phenomenon occurs through a combination of hypertrophy of existing fibres, splitting of existing fibres, and satellite cell proliferation, differentiation, and fusion. Many hormones and growth factors are involved in regulating these processes, such as growth hormone, insulin-like growth factor, and testosterone [33].

A research study published by Michael Rudnicki et al. showed that satellite cells are positive for Pax7 expression and inactivation of Pax7 caused a severe depletion of these muscle stem cells, unequivocally linking satellite cells to the paired box transcription factor Pax7 [43]. Indeed, Pax7 expression was shown to be maintained in quiescent satellite cells in adult murine muscle [44] and in many other species, including human [45, 46]. Different studies targeting Pax7 to manipulate satellite cell genome [47-49] have shown the importance of these stem cells in muscle regeneration. Indeed, once satellite cells were ablated locally or systemically, muscle was unable to regenerate and did not recover this ability [50]. Therefore, muscle did not regenerate without satellite cells, and other potential myogenic stem cells did not compensate for their loss. Furthermore, as myonuclei do not express Pax7, they were immune from ablation in that study. The absence of measurable regeneration proved that, as expected, myonuclear de-differentiation does not occur to any significant degree under normal circumstances [50].

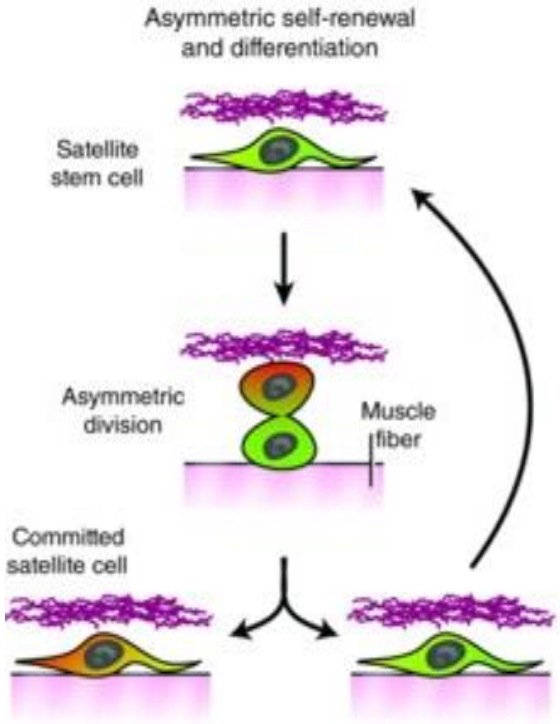

Figure I3: Asymmetric division of satellite cells.

Adapted from Dumont at al. [51]

1.3 Smooth muscle

Smooth muscle tissue can be found in the walls of blood vessels, around hollow organs such as the urinary bladder, at the base of hair follicles, and in layers around the circulatory, respiratory, digestive, and reproductive tracts [52]. Each smooth muscle cell is small and spindle-shaped, with a diameter between 2 and 10 μm, and a length ranging from 50 to 400 μm. Unlike skeletal muscle, they own a single oval nucleus which is centrally located (Figure I4). Differently from skeletal muscle fibres which are sometimes large enough to run the entire length of the muscles in which they are found, many individual smooth muscle cells are generally interconnected to form sheet-like layers of cells. Smooth muscle cells, as well as skeletal muscle cells, have thick and thin filaments, containing myosin and actin respectively. Tropomyosin is present in the thin filaments, but the regulatory protein

troponin is absent. Peculiarly, a protein called caldesmon is associated with the thin filaments and in some types of muscle, it may play a role in regulating contraction. The thin filaments are attached either to the plasma membrane or to the so-called dense bodies, which are cytoplasmic structures. Thick and thin filaments are oriented diagonally to the long axis of the cell. When the fibre shortens, the regions of the plasma membrane enlarge between the points where actin is anchored to the membrane. The thick and thin filaments do not have a regular alinement into sarcomeres and they are not organised into myofibrils, as in striated muscles. This pattern accounts for the absence of striations: it is the only non-striated muscle tissue. Contraction of smooth muscle cells normally occurs through the action of pacemaker cells. Although smooth muscle contractions may be triggered by neural activity, the nervous system regulating smooth muscle belongs to the autonomic nervous system division – rather than the somatic nervous system division. Thus, smooth muscle is not under direct voluntary control. Therefore, smooth muscle tissue is called non-striated involuntary muscle [35]. Smooth muscle, like skeletal muscle, uses cross-bridge movements between actin and myosin filaments to generate force, and calcium ions control cross-bridge activity. However, the organization of the contractile filaments and the process of excitation–contraction coupling are quite different in smooth muscle compared to skeletal muscle and even in respect to different smooth muscles [33]. Nonetheless, smooth muscle contraction occurs by a sliding-filament mechanism. Myosin content in smooth muscle is only about one-third compared to that in striated muscle. While actin content can be twice as great. However, in smooth muscle, force generation can vary to a broad range of muscle lengths compared to that of skeletal muscle. This highly adaptive property is necessary due to the constant changes in volume that hollow structures and organs surrounded by most smooth muscle cells undergo. As a consequence, smooth muscle cells can change in length in the walls of those organs having variable volume, to adapt to these changings. Contractile activity in smooth muscle occurs in response to Ca^{2+}-calmodulin interaction. This interaction stimulates phosphorylation of the light chain of myosin. A Ca^{2+} sensitisation of the contractile proteins follows the signal of the RhoA/Rho kinase pathway to inhibit the dephosphorylation of the light chain by myosin phosphatase, maintaining force

generation. While the opposite event of smooth muscle relaxation is seen to initiate by removal of Ca^{2+} from the cytosol and stimulation of myosin phosphatase [52]. Another difference with skeletal muscle fibres is that smooth muscle cells have the capacity to divide throughout the life of an individual, while skeletal muscle cells have limited ability to divide once they have differentiated. Hence, smooth muscle cells can divide, and smooth muscle tissue can regenerate in response to tissue injuries by paracrine factor stimulation [53].

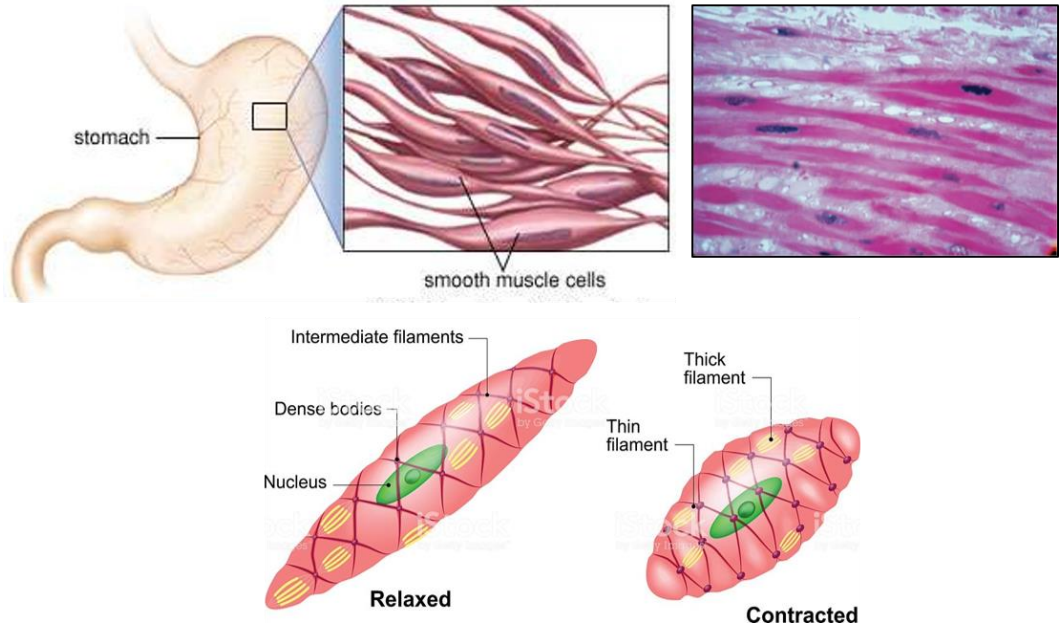

Figure I4. Non-striated cell organisation of smooth muscle.

Adapted from Martini et al. [35].

1.3.1 Mesoangioblasts (MABs)

Mesoangioblasts (MABs) are vessel-associated stem cells that can be isolated from the dorsal aorta during development or from post-natal cardiac and skeletal muscles, in different organisms, including murine, canine and human [54-57]. Their characterisation on murine organism showed them positive for several markers,

such as CD34, Sca1, CD140a, NG2 and Alkaline phosphatase (AP) [58]. A definition of MABs addresses them as a subset population of the skeletal muscle pericyte pool [56, 59]. Several cell lines originating from MABs showed stem cell characteristics by maintaining their potential to form mesodermal tissues, including blood vessels, bone, cartilage, and smooth, cardiac and skeletal muscle following transplantation [55]. Interestingly, as they were seen to differentiate to most mesodermal cell types including skeletal muscle, MABs were found to be a valid alternative for cell therapy of Muscular Dystrophies [55, 56]. Indeed, MABs were used in a phase I-II clinical trial [60].

Despite their skeletal muscle applications, MABs can differentiate towards smooth muscle as well [53, 55]. Different research articles showed expression of several members of the transforming growth factor beta (TGFβ) receptor family pathway, particularly Tgfbr2 and Tgfbr1, in MAB clones [61]. In this study, MABs readily responded to TGFβ1 by activating smooth muscle-specific gene transcription, such as Calponin 1 and Smooth Muscle Myosin Heavy Chain (SM-MHC) [61]. When Tgfbr2 was ablated, this differentiation pathway was partially inhibited [62], concluding that MABs preferred the smooth muscle differentiation pathway in culture [63]. In another study, Sphingosine 1-Phosphate (S1P) was reported to induce MAB differentiation towards smooth muscle [64]. Importantly, smooth muscle differentiation was driven by GATA6 up-regulation, together with that of LMCD1, as a novel mechanism of transcriptional regulation. Moreover, GATA6 played a role in the mechanism by which TGFβ triggered smooth muscle differentiation of MABs and it was reported that S1P biosynthesis was required for promoting biological action of cytokines [64]. This contribution added knowledge of the molecular mechanisms driving differentiation of these progenitors cells and disclosed SphK/S1P axis as a novel target to stimulate MAB differentiation towards smooth muscle [64]. Lastly, Msx2 and Necdin combined activities were shown to be required for smooth muscle differentiation in MABs. In particular, necdin was shown to promote cell cycle exit and cell survival by preventing p53-mediated apoptosis. Msx2 played roles in cell cycle control showing a proapoptotic effect in vivo, particularly in osteogenesis. Importantly, when both genes - alone or in combination - were silenced in MABs there was a reduction of αSMA, SM22,

calponin, and SM-MHC expression, underlying their importance in smooth muscle differentiation [65].

In this context, many other markers may be evaluated and addressed as smooth muscle promoting or inhibiting proteins in MABs differentiation.

1.4 Cardiac muscle

The heart is a muscular organ divided into right and left halves, each consisting of an atrium and a ventricle - also referred to as chambers. The heart is enclosed in the pericardium (a protective fibrous sac) located in the chest. A fibrous layer, called epicardium, also surrounds the heart. The narrow space between the pericardium and the epicardium is filled with a watery fluid that serves as a lubricant as the heart moves within the sac. The wall of the heart is called myocardium and is composed mainly of cardiac muscle cells [33]. A typical cardiac muscle cell - known as cardiomyocyte - is smaller than a skeletal muscle fibre, and has one centrally placed nucleus. Cardiac muscle sections seen microscopically - like skeletal muscle - appear as a series of alternating light and dark bands perpendicular to the long axis. This pattern is known, as already said for skeletal muscle, as striated muscle (Figure I5). However, despite the similarity with skeletal muscle fibres, cardiac muscle cells do not rely on neural activity to start a contraction. Instead, specialised cardiac muscle cells, called pacemaker cells, sustain a regular rate of contraction. Although the nervous system can influence the rate of pacemaker activity, it does not provide voluntary control over individual cardiac muscle cells. For these properties, cardiac muscle is called striated involuntary muscle.

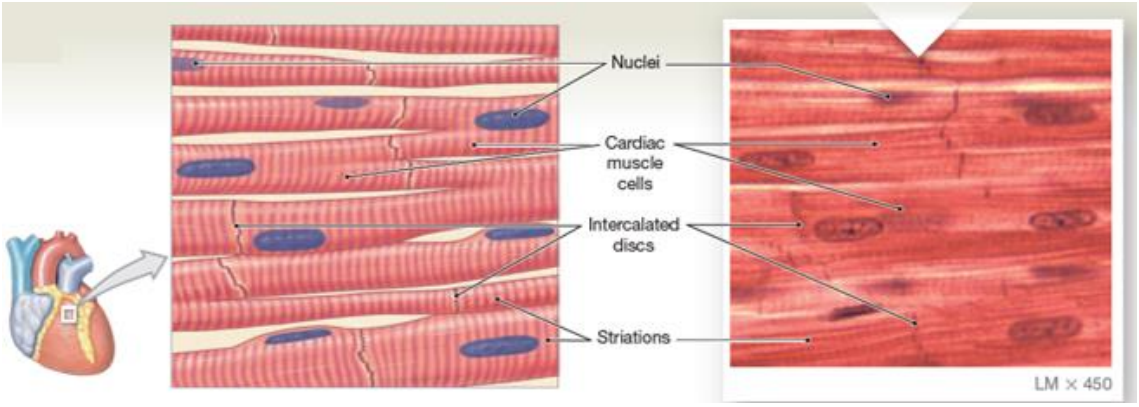

Figure I5. Striated organisation of cardiac muscle cells.

Adapted from Martini et al. [35].

Cardiac muscle cells form connections with one another; these extensive connections occur at specialized regions known as intercalated discs. Therefore, cardiac muscle tissue consists of a branching network of interconnected muscle cells. The anchoring junctions help convey contraction forces, and gap junctions at the intercalated discs help coordination of individual cardiac muscle cell activities [35]. The cardiac muscle cells of the myocardium are organised in layers that are tightly bound together and completely surround the blood-filled chambers. When the walls of a chamber contract, the cardiomyocytes of that area come together and exert pressure on the blood they enclose. Unlike skeletal muscle cells, which can be rested for prolonged periods and only a fraction of which are activated in a given muscle during most contractions, cardiomyocytes contract with every beat of the heart. Thus, cardiac muscle is an electrically excitable tissue that converts chemical energy stored in the bonds of ATP into force generation. Action potentials propagate along cell membranes, Ca^{2+} enters the cytosol, and force-generating cross-bridge cycling is activated [33]. Approximately 1% of cardiac cells do not function in contraction but have specialised properties that are essential for normal heart excitation. These cells constitute a network known as the conducting system of the heart and are electrically in contact with the cardiac muscle cells through gap junctions. The conducting system gives rise to the heartbeat and helps spread an action potential rapidly throughout the heart [66]. Remarkably, despite this

enormous workload, the human heart has a limited ability to replace its muscle cells. Indeed, like skeletal muscle fibres, cardiac muscle cells are incapable of dividing, and because this tissue lacks satellite cells, cardiac muscle tissue damaged by injury or disease cannot regenerate. As suggested by Bergmann and colleagues, only about 1% of heart muscle cells are replaced per year [67]. A genetic pulse-chase study in mice provides indication of adult mammalian cardiac self-repair following infarction [68]. A tamoxifen-dependent, cardiomyocyte-restricted Cre was transiently activated in healthy adult mice, causing a short-lived pulse of recombination that stably activated the Cre-dependent reporter in more than 80% of cardiac muscle cells. In the absence of injury, the reporter gene's expression was maintained over time, indicating the absence of detectable cardiac muscle cell replacement during normal aging. However, three months after induction of ischemic injury, the reporter was found to be diluted, suggesting that the cardiomyocytes surrounding the injury arose de novo from formerly undifferentiated cells, either intrinsic or extrinsic to the heart [68]. There was little evidence of replication of pre-existing myocytes, indicating that natural cardiac regeneration in the mouse occurs primarily through stem or progenitor cell contribution, rather than by cell cycle re-entry of differentiated cardiomyocytes. These evidences remark the importance of pluripotent stem cells in the cardiac field, although the clinical use of these cells has been prevented by crucial limitations, like potential tumorigenic and immunogenic issues, genetic instability and ethical issues [69]. However, research on stem cells differentiating towards cardiomyocyte-like cells, has shown to be important for cell and disease modelling, for example [70]. Moreover, new insight on the use of these cells might overcome the aforementioned problems and pave the way for new clinical applications.

1.4.1 Murine embryonic stem cells (mESCs)

Murine embryonic stem cells (mESCs) were isolated for the first time in 1981 by Evans and Kaufman [71]. These cells are pluripotent stem cells (PSCs) holding the potential to generate lineages of the three embryonic germ layers – endoderm,

mesoderm and ectoderm (Figure I6) [72]. Despite their great potential, PSCs ethical issues and immunological concerns, as well as safety matter when considering their use in cell therapy [69]. Indeed, PSCs can give rise to teratomas when differentiation is not fully accomplished. Nevertheless, the pluripotent potential, together with the great renewal capacity owned by PSCs, brought them to the spotlight for several cell-based therapy approaches and for development of diverse cell types and disease modelling in culture [73, 74]. Among these properties, cardiac progenitor cells and cardiomyocyte-like cells were differentiated in culture, allowing research to shed light on the cardiac field [75]. In spite of skeletal muscle differentiation from ESCs, which initially encountered difficulties in achieving good results, cardiac differentiation in culture became efficient and reproducible both in murine and human ESC differentiation thanks to the increasing knowledge on heart development in mouse embryos [76-78]. Various strategies were used to improve cardiac differentiation from ESCs in culture and different approaches were pursued to follow maturation and to study specific proteins, compounds or chemical factors that can have an impact on cardiac maturation from pluripotency culture [79]. For instance, Lee and colleagues showed how ouabain - a cardiac glycoside used to treat congestive heart failure and supraventricular arrhythmias due to re-entry mechanisms - facilitates cardiac differentiation of mouse embryonic stem cells through ERK1/2 pathway, increasing the knowledge of the differentiation processes of cultured ESCs [80]. This example might be followed to study other biological or chemical interactions involved in cardiac differentiation, as well as in skeletal muscle.

1.4.2 Human induced pluripotent stem cells (hiPSCs)

The ethical and legislative issues which arose due to the use of human ESCs derived from the inner cell mass of pre-implanted blastocysts, were overcome in 2006. In that year, Takahashi et al. generated induced pluripotent stem cells (iPSCs), by reprogramming mouse somatic cells to give them pluripotency features (Figure I6) [81]. Pluripotency was achieved by the introduction of a specific set of transcription

factors via retroviral transduction. Specifically, this transcription factor combination consists of Klf4, Sox2, Oct3/4 and c-Myc [81]. Similarly to ESCs, iPSCs exhibit ESC-specific markers, they are comparable in morphology and behaviour and generate teratomas when subcutaneously transplanted into nude mice [81]. In 2007, the same team generated iPSCs from human dermal fibroblasts, using the same cocktail of transcription factors - KLF4, SOX2, OCT3/4 and c-MYC [82]. Moreover, another cocktail was soon after identified, inducing pluripotency via SOX2, OCT3/4, NANOG and LIN28 [83]. Human iPSCs were similar to human ESCs in morphology, proliferation, surface antigens, gene expression, epigenetic status of pluripotent cell-specific genes, and telomerase activity. Furthermore, these cultured cells could widely differentiate into cell types of the three germ layers and could form teratomas [82, 84]. Many cell types have been generated by differentiating hiPSCs, benefitting fields dealing with regenerative medicine, disease modelling, drug discovery and toxicology [85-87]. The cardiogenic potential of the iPSCs was studied in both iPSCs derived from mice [88] and human [89]. Human iPSCs might be as well useful for understanding biological or chemical interactions involved in cardiac differentiation. In this regard, gene editing might be pursued to improve needed genetic therapies. It is shown in a recently published paper how gene editing nowadays induces double-strand breaks in the DNA and how techniques have evolved over the years [90]. Particularly, it was first described gene editing by zinc finger nuclease [91, 92], then TALENs came into the scene [93, 94], to finally pass the lead to the bacterial clustered regularly interspaced short palindromic repeat (CRISPR)-Cas9 system [95, 96]. These technologies can correct mutation in genetic disorders, or can mirror diseases in hiPSCs by inducing the same mutations of certain pathologies [97]. Moreover, if any amelioration would be seen in modulating biological or chemical agents in these cells, future applications might also embrace this way.

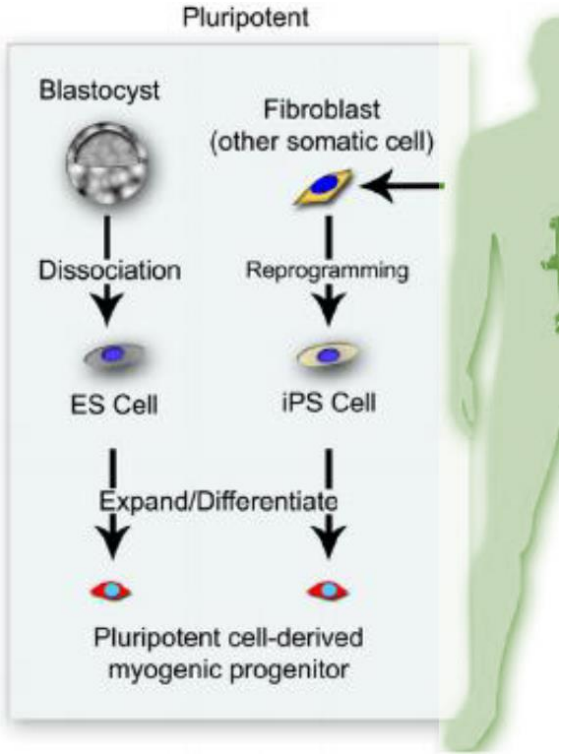

Figure I6. Stem cell sources for myogenic progenitor derivation.

Adapted from Hosoyama et al. [98]

1.5 MICALs family

Molecules interacting with CasL (or Microtubule Associated Monooxygenase, Calponin And LIM Domain Containing - MICALs) came into play for the first time in 2002 [99]. Two independent papers were published the same year. The first reported human MICAL1 characterisation in a screening study for CasL-interacting proteins [100], and the second study found the only drosophila Mical while screening for proteins binding Plexin A (a semaphorin receptor) to its cytoplasmic domain [101]. Additional research conducted between 2002 and 2006 identified two more MICAL genes in vertebrate, MICAL2 and MICAL3 respectively [100, 102-104]. Moreover, one MICAL-like gene was found in Drosophila and two other MICAL-like genes were described in mice and humans – MICAL-L1 and MICAL-L2 [101, 105-107]. In vertebrates, MICAL family members show ubiquitous

expression. MICALs were seen in neurons and glial cells during development and in the adult nervous system, in muscle, heart, fibroblasts, kidney, liver, gastrointestinal tract, bone marrow, testis, lung and spleen [108]. In the intracellular compartment, MICALs localisation is both cytoplasmic and nuclear. In fact, it was shown that MICAL isoforms localise also in association with plasma membrane and with compartments rich of actin, i.e. in the nucleus they are associated with nuclear actin [108].

Each protein member of the MICAL family is composed of multiple-domains (Figure I7). From the N-terminal, MICALs have a flavoprotein monooxygenase (MO) domain [109-111], a calponin homology (CH) domain typical of actin-binding proteins, a LIM domain (for 'Lin-11, Isl-1 and Mec-3') and a Rab-binding domain (RBD) with motifs forming a coiled-coil domain [112, 113] that is present only in MICAL1 and MICAL3. The MICALs MO domain is essential for its actin-depolymerising activity and it is structurally associated to NADPH-dependent MO domain of FAD-containing enzyme p-hydroxybenzoate hydroxylase (PHBH) [110, 111, 114]. The CH domain is an actin-binding domain involved in non-muscle cell motility, muscle contractility and protein signals [115]. CH domain is related to MO domain function since it makes NADPH oxidation faster in the presence of F-actin [109]. The LIM domain is composed of cysteine-rich zinc-finger structures, functioning as an anchor for transcription factors, kinases and cytoskeletal proteins. Therefore, it is involved in cytoskeleton architecture, cell adhesion, cell motility and signal transduction [116]. The RDB domain consists of a prolin-reach region (interacting with SH3 domain of Cas family), a glutamic acid-repeat region, and a coiled-coil motif. RDB domain, when present, may auto-inhibit the activity of MO domain [117].

MICALs make oxidation-reduction (redox) reactions on filamentous-actin (F-actin), by binding FAD and using NADPH and O_2 to depolymerise F-actin [117-120]. Several studies were published to explain how F-acting gets depolymerised by MICALs. A possible explanation is relative to MICALs reactive oxygen species (ROS) production capacity [110, 112, 121, 122]. Indeed, ROS can modify methionine, tryptophan and cysteine residues of actin, affecting both

polymerisation and depolymerisation [110, 123, 124]. Therefore, F-actin depolymerisation may happen through diffusible H_2O_2 (as a ROS product) that was found increased when F-actin and MICALs were bound together [120]. In addition, F-actin was seen to be a direct substrate of MICALs causing the oxidation of two actin methionine residues (M44 and M47) into methionine sulfoxide, showing that a direct contact between MICALs and F-actin is compulsory for depolymerisation to happen [118, 125]. Intriguingly, methionine sulfoxide reductases - SelR in Drosophila and MsrB proteins in mammals – were seen to revert actin oxidation mediated by MICALs. As a result, F-actin disassembly was blocked *in vitro* [126, 127]. This result was interesting in combination with the results reported in another paper that observed the two methionine residues increased in aged muscle actin due to ROS production [128] and it was assumed that ROS might cause oxidative damage disrupting F-acting. Therefore, Msr proteins were hypothesised to counteract the deleterious cell damage that was possibly caused by MICALs ROS production, although it was not directly linked to MICALs [127].

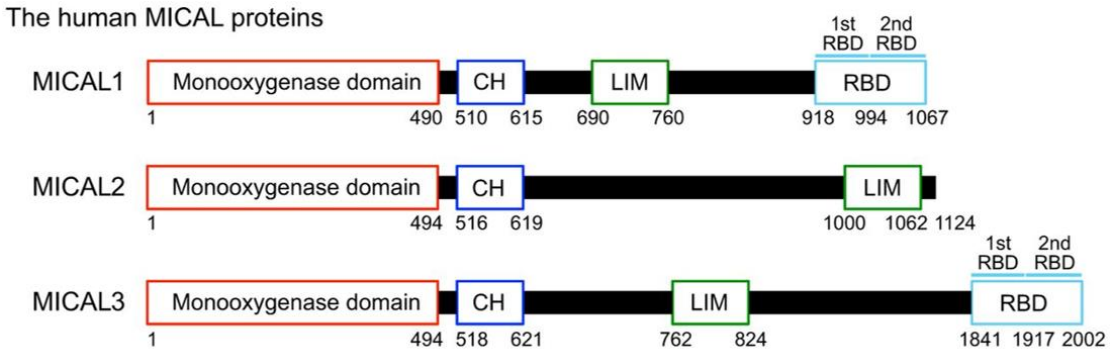

Figure I7. The human MICAL protein domain organisation.

Adapted from Fremont et al. [99]

After Terman showed in the first Drosophila study that Mical had a role in plexin-mediated axonal repulsion in vivo [101], MICALs were seen promoting F-acting disassembly during repulsive axon guidance by direct binding and oxidation of F-actin [101, 118]. Interestingly, MICALs were seen involved in many other functions within different cell types, all depending on dynamic actin cytoskeleton remodelling (Figure 8). Most of the studies reported MICALs in neuronal cell biology [101, 117, 129], but also MICALs involvement was seen in immunity [127], cell viability [122, 130, 131], bristle development [118], cell shape [132, 133], membrane trafficking [116, 134], nuclear actin regulation [119], skeletal muscle morphology and function [126, 135], cardiovascular integrity [136] and lately also cancer (particularly for MICAL2) [137, 138] (Figure I8).

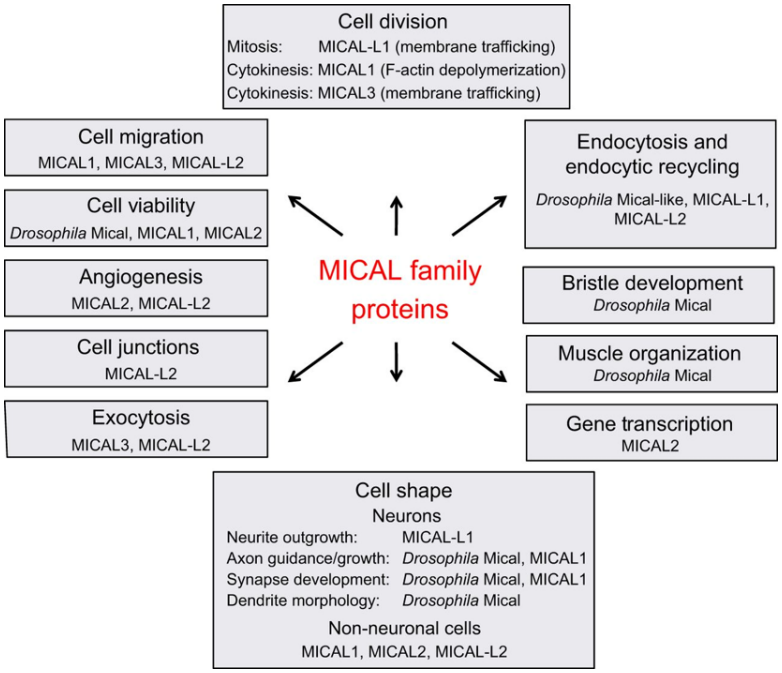

Figure I8. MICALs protein functions.

Adapted from Fremont et al. [99]

However, skeletal muscle morphology function was only shown in Drosophila Mical [126, 135], thus its implication and function is not clear in vertebrates and

there is no proof in the literature of which component of the MICAL family is responsible for this regulation. However, it seems that MICALs are indirectly involved in cardiovascular integrity by regulating semaphoring 3a expression. Indeed, semaphorin 3a overexpression led to a reduction of post-myocardial infarction arrhythmia [136]. Nevertheless, evidences of a direct role of MICALs on cardiac and smooth muscle is lacking.

1.5.1 MICAL2

Among the MICALs, MICAL2 emerged as the one involved in angiogenesis, cell viability, gene transcription [99] and interestingly it was found upregulated in a genome-wide profiling study in which MICAL2 was among a set of ten functionally linked genes involved in the disease evolution of mdx-mice, a mouse model of Duchenne muscular dystrophy (DMD) [139]. Despite mdx-mice carry the analogous genetic defect as DMD patients, their phenotype is milder than DMD patients [140]. It is well established that utrophin (Utrn) reappearing in mdx mice (and not in DMD patients) is one of the responsible genes contributing to the reduction of muscle fibre necrosis [141-143]. However, other factor must play a role, since utrophin alone is not sufficient to explain all the differences shown in comparison to DMD [140]. Therefore, the rationale of Marotta and colleagues in their study was to find out genetic differences in mdx skeletal muscle during the evolution of the disease, thus to find candidate genes that might be responsible for overcoming the lack of dystrophin [139]. The genome-wide analysis unveiled significant changes in some genes only during mdx disease evolution. Through a high confidence functional network analysis, two principal nodes emerged as possible pathways involved. The first comprise Dmd-Utrn-Myo10, as node including the two main genes involved in the disease and Adamts5-Thbs1-Spon1-Postn formed the second node, which was functionally associated with the first. These two nodes were functionally linked to each other by 10 genes, among which Mical2 was listed (Figure I9). Discussing the results, it was assumed that all these genes were candidate genes that might be implicated in the decreased muscle

necrosis occurring in mdx mice, referring to them as potential therapeutic target for DMD [139].

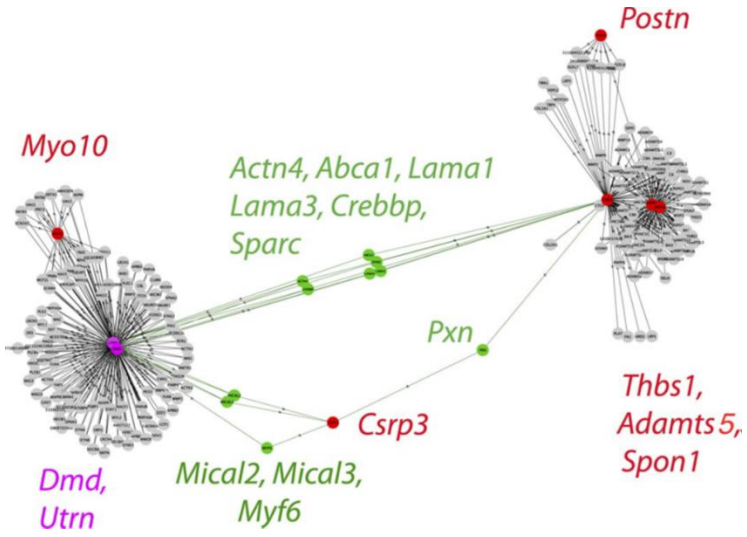

Figure I9. Functional gene network map of induced genes in skeletal muscle of mdx mice based on a probabilistic prediction.

Adapted from Marotta et al. [139]

In addition, MICAL2 was recently linked with cancer progression and metastasis of several cancer types [137, 138, 144]. The first study that found MICAL2 increased in cancer was in 2015, by Mariotti et al [137], in which the authors screened different histotypes of human gastric cancer (GC), renal carcinoma and the non-small cell lung cancer (NSCLC), finding MICAL2 more expressed in highly aggressive primary cancer and in metastatic emboli [137]. MICAL2 cancer involvement was also found in prostate cancer proliferation due to NADPH oxidation mediation [145]. Moreover, MICAL2 was associated to bladder cancer proliferation through a correlation with MKI67 [146]. Lastly, breast cancer was also associated to MICAL2 causing an increase in the expression of epidermal growth factor receptor (EGFR) [138].

In the first study, primary cancer cells in a state of epithelial to mesenchymal transition (EMT), prone to migrate from the site of origin, showed that MICAL2 was highly present. On the contrary, MICAL2 was decreased when mesenchymal to epithelial transition (MET) occurred after cell homing at the metastatic site [137]. Hence, the knowledge of MICAL2 oxygenation capacity on M44 and M47 F-actin residues, that promotes actin depolymerisation and cytoskeleton rearrangement, combined with the knowledge of F-actin disassembly and cytoskeleton dynamics conferring EMT capacity to epithelial cells, allowed the suggestion that MICAL2 was involved in this process [118, 145].

In breast cancer, MICAL2 was found linked with increased EGFR protein content, causing a boost in cell migration. In a loss and gain of function setting, it was proved that when MICAL2 was silenced, EGFR was destabilised and cell migration was consequently inhibited [138]. While, when overexpressing MICAL2, EGFR was found more stably expressed - due to a delay in degradation - and cell migration was enhanced. The way MICAL2 stabilised EGFR was due to an increased expression of Rac1, which is known to prevent EGFR degradation. Furthermore, the mechanism of the enhanced cell migration was found in the main downstream signalling cascades of EGF/EGFR involved in cell migration, P38/HSP27 and P38/MMP9 signalling, which positively correlated with MICAL2 overexpression. Moreover, these results were confirmed in human breast cancer samples, leading to the conclusion that MICAL2 regulated cell migration in breast cancer and it was associated with worse breast cancer prognosis [138].

In summary, MICAL2 redox regulation on F-actin is a crucial mechanism in many cell types, but it appears to be fundamental in muscle cells. Indeed, MICAL2 has a potential role in muscle disorders, being appointed as a candidate potential therapeutic target gene involved in DMD. Moreover, the strong correlation between MICAL2 and several cancers allows the speculation of an involvement of MICAL2 in the known muscle cancer, rhabdomyosarcoma. However, very little is known so far about MICAL2 in muscle physiological formation and pathological degeneration.

1.6 Muscle disorders: homeostasis regulation loss

Muscle homeostasis is a dynamic equilibrium that in muscle is obtained since the developmental stage. Indeed, muscle formation occurs in a specific set of protein synthesis and progenitor activation towards their differentiated stage [31, 147]. During adulthood, a steady state is reached in muscle tissue in which cellular turnover decreases and cellular plasticity is the result of a balanced anabolism/catabolism interplay. Nevertheless, the steady state may be disrupted upon injuries or excessive physical activities, causing damage and necrosis of several muscle cells. This condition triggers regeneration. Skeletal muscle regeneration is the repair process that takes place after a muscle damage or degeneration. Muscle regeneration after injury seems to follow the pattern of muscle development during embryogenesis. Initially, inflammation occurs and quiescent satellite cells become active and start proliferating, differentiating and fusing originating de novo fibres or repairing injured ones [148]. In this setting, Pax7+ SCs become active and give rise to an asymmetrical division with SCs that lose Pax7 expression, start expressing MyoD, Myf5 and myogenin to support myogenesis and muscle repair, or Pax7+ SCs not expressing myogenic markers that can repopulate the stem cell niche [149, 150]. Moreover, muscle repair is sustained also by secretion of several factors, like transforming growth factor-βs (TGF-βs), fibroblast growth factors (FGFs), hepatocyte growth factor (HGF) and others [151]. However, injuries and extreme physical activities are not the only cause of muscle degeneration/regeneration and loss of homeostasis. Chronic degeneration /regeneration processes may evolve due to a set of diseases termed muscular dystrophies (MDs) [152].

In addition, muscle progenitors can lose their capacity to exit the cell cycle and they do not undergo differentiation. These cells remain in a proliferation stage originating a cancer disease named rhabdomyosarcoma (RMS) [153].

1.6.1 Muscular dystrophies

Muscular dystrophies (MDs) are inherited neuromuscular disorders that primarily affect striated muscle, mainly characterised by progressive muscle wasting and weakness, leading to physical disability and a shortened life expectancy [154]. In 1852, MDs were described for the first time by Meryon reporting a series of clinical cases in young boys [155]. Since that first description, many studies and huge knowledge were added to these diseases. More than 30 subtypes are considered as MDs, including congenital forms, and they are classified in different groups according to the distribution of predominant muscle weakness. Duchenne muscular dystrophy (DMD) is the most common one and together with Becker and Emery-Dreyfuss (XR) MDs, belong to the X-linked inherited diseases. Other MDs are limb-girdle muscular dystrophies (LGMDs), a heterogeneous group of several dystrophies affecting mainly upper limbs and characterised by a specific mutation in different genes of the dystrophin glycoprotein complex (DGC), particularly affecting dystrophin or one of the sarcoglycan proteins (Figure I10). MDs are also distal, facioscapulo-humeral and oculopharyngeal. In almost all cases, the genetic cause is known and this allows a precise diagnosis, even before birth [156]. The onset of clinical manifestations varies. MDs symptoms may occur from birth or childhood, but may also rise during adulthood. Congenital MD symptoms are generally evident at birth or in the first months of life. While, DMD and some of the LGMDs may manifest in early or late childhood or also during adolescence, when normal ambulation is already reached. Other LGMDs and milder MDs do not have clinical manifestation until adulthood [152]. Not only skeletal muscle is affected in MDs, in fact, respiratory muscles, cardiac muscles and smooth muscles can also be affected in some MD forms, in particular, DMD and some of the LGMDs present these complications. These complications eventually lead to respiratory failure and cardiac decompensation, causing premature death [157].

The most common dystrophy form - the X-linked DMD - has an incidence of 1:3600-1:6000 male births worldwide [158]. It is caused by mutations in the dystrophin gene on the X chromosome [159] and the clinical signs usually present at four years of age [160]. The disease progression is very quick and usually patients

necessitate a wheelchair at the age of 10. Moreover, by the same age, most DMD patients develop severe cardiomyopathy [161], characterised by congestive heart failure that together with respiratory complications, leads to death by the age of 30 [162, 163]. In healthy muscle, dystrophin is located on the intracellular surface of the sarcolemma along the length of myofibres [164]. It is bound with dystroglycan, sarcoglycan, and neuronal nitric oxide synthase present on plasma membrane of myofibres, to form the DGC. The essential function of dystrophin in the muscle is to stabilise the fibres during contractions by binding to F-actin with the N-terminal domain and to β-dystroglycan with the C-terminal domain, to serve as a bridge and anchor for proteins [165, 166]. In DMD, what leads to a severe muscle wasting, respiratory and cardiac failure and death before the age of 30 [167], is dystrophin mutations [168]. Indeed, the loss of dystrophin disrupts the DGC complex, causing membrane instability with increased susceptibility to injury, and fibre necrosis. In addition, in DMD patients, the chronic degeneration/regeneration process compromise the myofibre regenerative ability causing satellite cells exhaustion [166] and replacement of muscle with fibroadipose tissue [165].

LGMDs onset occur after birth with progressive weakness and muscle atrophy predominantly affecting hips, shoulders, and proximal extremity muscles. As a group, the LGMDs have a minimum prevalence of approximately 1:20000 [169]. This form is characterised by high heterogeneity and shows different patterns of inheritance [170]. Two severe forms of LGMD are LGMD type 2D (LGMD-2D), with a mutation in the α-sarcoglycan (SGCA), and the type 2E (LGMD-2E), which has a β-sarcoglycan (SGCB) gene mutation [171, 172].

Different animal models can recapitulate MDs, and are widely used for studying the disease and finding new therapies. The most used animal model for DMD is the mdx murine model, which lacks of the dystrophin protein in the muscle, exhibits necrosis, centrally located nuclei and the muscle degeneration characteristic of DMD [161]. LGMD-2D is recapitulate in the sarcoglycan-α-null murine model [57]. While, LGMD-2E is mirrored in the sarcoglycan-β-null mouse model, developing both muscular dystrophy and cardiomyopathy with progressive and severe wastage of both muscle types [173].

There is no available cure for DMD yet. The current strategies are based on pharmacological treatment, which aims at the pathological effects, such as inflammation and decrease in muscle mass, rather than the actual cause. Cortisone therapy is the common treatment, together with prevention and management of complications [174]. However, research is evolving to develop possible therapies targeting the cause of the disease.

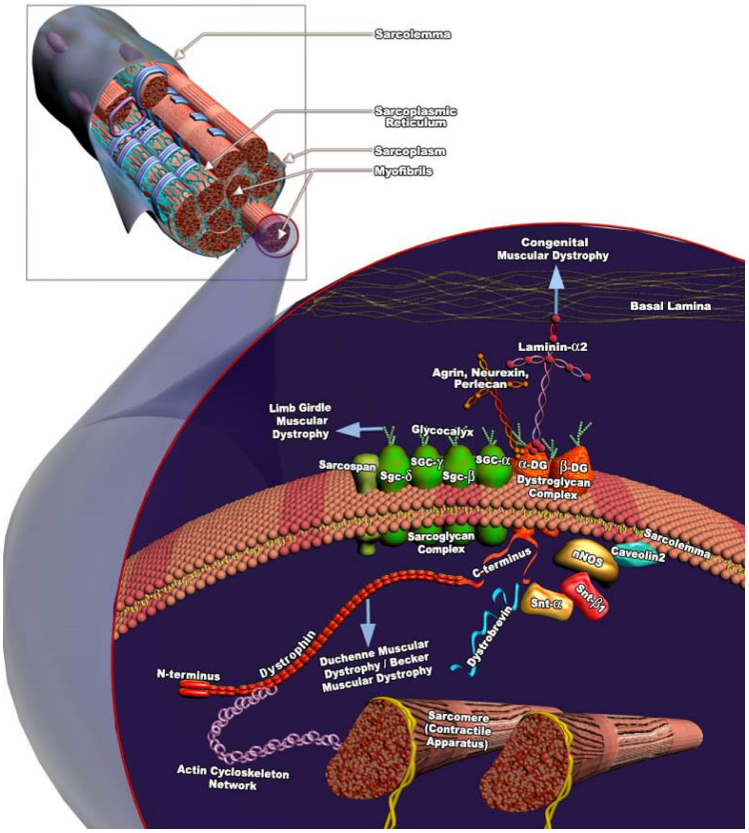

Figure I10. DGC complex and MDs related to its gene modifications.

Adapted from https://www.qiagen.com/br/shop/genes-and-pathways/pathway-details/?pwid=308

1.6.2 Rhabdomyosarcoma

Rhabdomyosarcoma (RMS) is the most common paediatric soft tissue sarcoma and is one of the 10 most common childhood malignancies. RMS incidence is approximately 6 cases per 1000000 population per year, which accounts for ~250 new cases in children each year, in the United States. Two major histologic subtypes of RMS are reported in the literature: embryonal RMS (ERMS), and alveolar RMS (ARMS). ERMS affects younger patients and manifests between 2 and 6 years, in which the typically developing sites are the head/neck and the genitourinary (GU) tract. ARMS is the most aggressive form of RMS, it develops in older patients between 10 and 18 years, and typically originates in trunk and extremity locations (Figure I11) [175]. In case of non-metastatic disease, 70% of patients have an event-free survival rate, while when metastases occur only 25-30% of patients survive in the long term [176]. The pathogenesis of RMS is not fully understood yet. However, what is known is that malignant myoblasts fail to exit the cell cycle and are restrained from fusing into syncytial muscle [177]. It was suggested that MET proto-oncogene, macrophage migration inhibitory factor (MIF), and P53 may play a role in oncogenic transformation and tumour progression [178-180]. Moreover, it was shown that the transcription factor FOXO can fuse with either the PAX3 or PAX7 transcription factor, at the genetic level. These fusion proteins were identified in patients with ARMS [181]. When these PAX/FOXO fusion proteins are together, the DNA binding domain of PAX is linked with the regulatory domain of FOXO. Consequently, PAX activity was found increased, causing de-differentiation and proliferation of myogenic cells [182]. PAX3-FOXO fusion accounts for 55% of the cases and it is more common than the PAX7-FOXO fusion seen only in 23% of the cases. Moreover, PAX3-FOXO fusion is associated with worse overall survival [183]. Histologically, ERMS cells display spindle-shaped or round morphology, resembling embryonic skeletal muscle cells with a heterogeneous genetic background [184]. While, ARMS cells are shown to be formed by aggregates of small round undifferentiated cells separated by dense hyalinised fibrous septa reminiscent of lung alveolar architecture, driven by the fusion protein PAX3-FOXO or PAX7-FOXO [185].

RMS is stratified in the TNM staging. TNM is a pre-treatment staging system determined by the site and size of the primary tumour, degree of tumour invasion, nodal status, and metastatic presence [186]. Based on risk stratification, current treatments for RMS include chemotherapy, radiation and surgery. Up to date, the gold-standard chemo-therapeutic combination consist of three chemo-therapeutic agents, which are vincristine, actinomycin D and cyclophos-phamide (called VAC regimen) [187]. However, this treatment has severe side effects in the long term, including infertility, second cancers and toxicity. Therefore, the late sequelae in adulthood due to the currently available treatments has pushed for the search of novel strategies targeting key mediators of the molecular machinery involved in the pathogenesis of RMS [187].

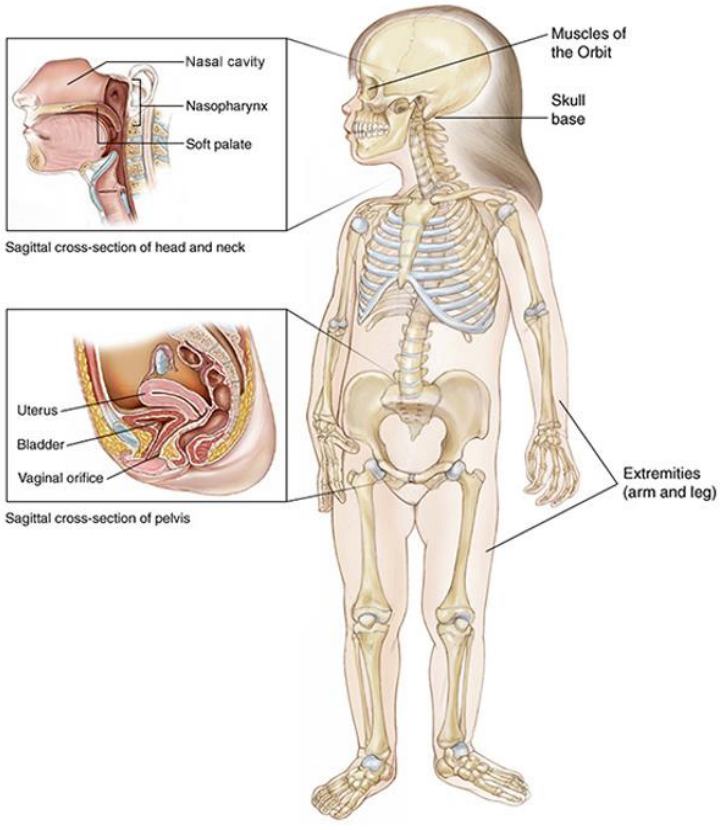

Figure I11. RMS most common sites of origin.

Adapted from https://www.cancer.net/cancer-types/rhabdomyosarcoma-childhood/view-all

1.7 Aims of the study

An adequate supply of oxygen is essential for the normal function of muscle cells, which need to modify their oxygen demand from rest to cell contraction. Although little is known about the smooth muscle mitochondrial respiration, oxidative organs (including the heart) have a high oxygen demand [33]. The striking capability of MICAL2 to directly and mechanistically connect oxygen availability with F-actin depolymerisation, and hence cytoskeleton dynamics, was thought to be implicated into the process of myogenic differentiation. Therefore, we hypothesised that modulations of MICAL2 had an impact on muscle progenitor commitment and that the link of MICAL2 with tumour progression could be found also in rhabdomyosarcoma (RMS) cell lines. Gaining this knowledge might help the regeneration when a chronic condition occurs or to better understand why rhabdomyosarcoma cells are unable to exit cell cycle. Hence, unravelling MICAL2 involvement in muscle differentiation in physiological and pathological conditions was the main aim of this project. The role of MICAL2 was deciphered firstly during differentiation to skeletal muscle cells from myogenic progenitors by gain and loss of function experiments. Secondly, MICAL2 expression was assessed in dystrophic conditions where the pool of adult stem cells was exhausted. Moreover, rhabdomyosarcoma cell lines were explored for MICAL2 expression and loss of function experiments were performed to unveil the molecular impact of MICAL2. Finally, MICAL2 expression was monitored during cardiac and smooth muscle differentiation. To achieve these goals, the research was articulated in different objectives, listed below.

i) Assess the role of MICAL2 involvement in skeletal, smooth and cardiac muscle commitments.

ii) Characterise MICAL2 expression in muscular disorders: DMD, LGMD2E murine models

iii) Modulation of MICAL2 during myogenesis: loss and gain of function studies and

iv) Characterisation and modulation of MICAL2 in RMS cell lines.

The novelty of this project is to establish a molecular link between MICAL2 and myogenesis, in physiological and dystrophic condition and in aberrant myogenic cell proliferation (RMS) that could be relevant for further in vivo studies.

2 Chapter two: Materials and Methods

2.1 Isolation, culture and skeletal muscle differentiation of murine satellite cells (mSCs)

Tibialis anterior (TA), *gastrocnemius* (GC) and *quadriceps femoris* (Q) of 4 to 6 weeks-old C57/Bl6 mice were minced to extract murine satellite cells (mSCs) after a muscle double enzymatic digestion consisting of a solution of 0,02% collagenase D (Sigma Aldrich) and 0,06% of pancreatin (Sigma Aldrich) dissolved in sterile PBS. After the first enzymatic digestion lasting for 1 hour, the supernatant was mixed to Fetal Bovine Serum (FBS) in order to block the reaction, while the remaining pieces were digested again with the same mix of pancreatin and collagenase for the following 30 minutes. After that, the mix of serum and cells was double filtered (70um and 40um). Cells were seeded and expanded in 9,4 cm^2 plates. Cells were grown in high glucose Dulbecco's Modified Eagle's Medium (D-MEM) supplemented with 20% FBS, 1% penicillin/streptomycin solution [100 units], 2 mmol/L glutamine, 1 mmol/L sodium pyruvate and 1% chicken embryo extract (all from Thermo Fisher Scientific, except otherwise specified). Cells were maintained at 37°C in a humidified atmosphere of 5% CO_2. Medium change was every two days. Differentiation to myotubes was induced by shifting 70% confluent cultures to DMEM supplemented with 2% horse serum, 1% penicillin/streptomycin solution [100 units] and 1 mmol/L sodium pyruvate. Most of the cells were fused to myotubes within 2 days.

2.2 Culture and skeletal muscle differentiation of C2C12-cells

Murine C2C12 skeletal myoblasts (ATCC, Manassas, VA, USA) were grown in high glucose DMEM supplemented with 10% FBS, 1% penicillin/streptomycin solution [100 units], 1 mmol/L sodium pyruvate. Cells were maintained at 37°C in a humidified atmosphere of 5% CO_2. Differentiation to myotubes was induced by shifting 80% confluent cultures to DMEM supplemented with 2% horse serum, 1% penicillin/streptomycin solution [100 units] and 1 mmol/L sodium pyruvate. The medium was changed every second day, and within 5 days most of the cells were fused to myotubes.

2.3 Culture and smooth muscle differentiation of murine mesoangioblasts (mMABs)

Murine MABs were previously isolated in the translational cardiomyology laboratory by *Giovannelli* et al. [188]. These cells were cultured and expanded on collagen-coated plates in high glucose Dulbecco's modified Eagle medium (D-MEM high glucose) culture medium supplemented with 20% FBS, 1% penicillin/streptomycin solution [100 units], 2 mmol/L glutamine, 1 mmol/L sodium pyruvate, 1% NEAA, 0.5% b-mercaptoethanol. When cells reached 80% to 85% confluence, they were split in a 1:4 ratio.

To induce smooth muscle differentiation, $5x10^3$ cells/ cm^2 were plated in collagen-coated plates, incubated at 37°C with maintenance medium. After 24 h, cells underwent media change with smooth muscle differentiation medium for 8 days. Smooth muscle differentiation was induced in mMABs by transforming growth factor-b (TGF-b) treatment added to D-MEM high glucose with 2% horse serum, 1% penicillin/streptomycin solution, 2 mmol/L glutamine, 1 mmol/L sodium pyruvate, and 10 ng/ml TGF-b (Peprotech, Oak Park, California). Medium change was every day.

2.4 Culture and cardiac differentiation of murine embryonic stem cells (mESCs)

Murine ESCs were derived from blastocyst of C57/Bl6 embryos following the methods published by Kohueiry at al. [189] and kindly given to our use by professor Koh's laboratory – Stem Cell Institute KU Leuven (SCIL), Leuven, Belgium. mESCs were maintained with feeder cells in Glasgow's Modified Eagle's Medium (G-MEM) supplemented with 7,5% FBS, 1% penicillin/streptomycin solution [100 units], 2 mmol/L glutamine, 1 mmol/L sodium pyruvate, 1% NEAA 1:500 β-mercaptoethanol and 1:10000 Leukemia inhibitory factor (LIF) was freshly added to every media change. Maintenance media change was every day. Prior to cardiac differentiation, two days adaptation on feeder-free gelatin-coated wells in serum maintenance media was done. Cardiac differentiation was induced by embryoid bodies (EBs) formation via hanging drop method, putting in one drop (20 µl/ drop)

~500 cells. Each drop was forming one EB. EBs were grown in cardiac differentiation medium for 48 hours in hanging drops. Drops were then collected - 100 EBs were put in a 9,4 cm^2 surface - in cardiac differentiation medium the second day, to be grown in suspension in ultra-low attachment (ULA) plates for 5 days. Cardiac differentiation medium consists of G-MEM supplemented with 20% FBS, 1% penicillin/streptomycin solution [100 units], 2 mmol/L glutamine, 1 mmol/L sodium pyruvate, 1% NEAA and 1:500 β-mercaptoethanol. Medium change was every two days. At day 5 of ULA plate culture, EBs were culture in adhesion in gelatin-coated plates (~100 EBs/ 9,4 cm^2 well). From day 9, the first beating areas appeared. Cells were kept in culture until day 11 of cardiac differentiation.

2.5 Culture and cardiac differentiation of human induced pluripotent stem cells (hiPSCs)

The hiPSCs used were an episomal hiPSC-line, viral-integration-free human induced pluripotent stem cell (iPSC) line generated using cord blood-derived CD34+ progenitors with seven episomally expressed factors (Oct4, Sox2, Klf4, Myc, Nanog, Lin28, and SV40 T; Gibco by Thermo Fisher Scientific). A second hiPSC-line has been generated in the translational cardiomyology laboratory - in KU Leuven, where the experiments were performed - from human fibroblasts reprogrammed via Sendai virus carrying Yamanaka factors, which have been shown to be critical for efficient generation of iPSCs (Invitrogen, CytoTune 2 iPSCs). To maintain iPSCs in culture, hiPSCs were cultured and maintained feeder-free (GeltrexTM LDEV-Free, hESC-Qualified, Reduced Growth Factor Basement Membrane Matrix) in complete serum-free mTeSR medium, consisting of basal medium and 5x supplement with recombinant human bFGF and TGF-β, at 37°C in a humidified atmosphere of 5% CO_2. Every 4 to 6 days of growth, cells were passaged non-enzymatically in colonies at a 1:12 split ratio, using EDTA (Thermo Fisher Scientific). Human iPSCs were differentiated into functional CMs, according to the ActA-induced EB-based cardiac differentiation protocol, described by *Duelen* et al. [190].

2.6 Culture of human RMS cell lines

Two human ERMS cell lines, namely embryonal RD (eRD) and RD18, and one human ARMS cell line, namely RH30 were generated and cultured in the laboratory of professor Fanzani [184] - at the University of Brescia, Italy – and kindly given to us. The three cell lines were grown in high glucose DMEM supplemented with 10% FBS, 1% penicillin/streptomycin solution [100 units], 1 mmol/L sodium pyruvate. Cells were maintained at 37°C in a humidified atmosphere of 5% CO_2. The medium was changed every second day, and cells were split when at ~80% confluence. To perform transient transfection experiments, cells were plated 24h prior to transfections at a 10000 cells / cm^2 density.

2.7 Transient transfection

Transient transfections were performed for both loss and gain of function assays in C2C12, mSCs and RH30 RMS cell line. When cells were ~50% confluent, transfections were done by using lipofectamine 2000 (Invitrogen). A solution made of Opti-MEM medium with 1:20 lipofectamine 2000 was incubated at room temperature (RT) for 5 minutes. In parallel, the same volume of Opti-MEM was incubated for 5 minutes with either 1:20 of Mical2-esiRNA (Mission esiRNA Sigma Aldrich) for silencing experiments, or with 1 µg of pMICAL2-eGFP-N2 overexpressing plasmid (Clontech, kindly given to our use by professor Debora Angeloni, Sant'Anna Institute, University of Pisa, Italy) for gain of function experiments. The Opti-MEM solution with either esiRNA or plasmid was put in the lipofectamine solution and incubated together for 20 minutes at RT. Meanwhile, culture medium was removed and cells were washed once with PBS. The transfection solution was added to the cells and left for 9 hours at 37°C in a humidified atmosphere of 5% CO_2. Maintenance medium was added after 9 hours to stop the transfection. After one night in maintenance medium, cells could be kept proliferating, undergo differentiations or be treated with bromodeoxyuridine (BrdU) for further flow cytometry analyses.

2.8 Flow cytometry analysis (FACS)

Flow cytometry analysis (FACS) was employed for monitoring the cell cycle, after BrdU incorporation. After a transfection to silence MICAL2 in C2C12, cells were kept proliferating for 24 h and 36 h time points. At each time point, 100000 cells were treated with 1:500 from 10 µM mouse anti-BrdU (BD Biosciences), which was incorporated in newly synthesized DNA of replicating cells. Treated cells were incubated for 1 hour at 37°C in a humidified atmosphere of 5% CO_2. Cells were then detached, counted and 1 x 10^5 cells were suspended in 100 µl of appropriate culture medium. After washing the cells once in 1% BSA/PBS and spinning at 500 x g for 15 minutes at RT, the pellet was resuspended in 200 µL of 1X PBS on ice. 5 mL of 70% ethanol was placed and cells were incubated on ice for 30 minutes to undergo fixation. The cells were then centrifuged 500 x g for 10 minutes at 10°C. Supernatant was carefully removed and pellets were loosen by vortexing. 1 mL of 2N HCl/Triton X-100 was added to the cells while maintaining a vortex. This step was followed by an incubation at RT for an additional 30 minutes, thus to denature DNA and produce single-stranded molecules. Cells were centrifuged at 500 x g for 10 minutes. The supernatant was aspirated and pellets were resuspended in 1 mL of 0.1 M $Na_2B_4O_7$ x 10 H_2O, pH 8.5, to neutralise the acid. Cells were centrifuged at 500 x g for 10 minutes. The supernatant was aspirated and pellets were resuspended in 1 mL of 0.5% Tween 20/1% BSA/PBS. For indirect immunostaining, 0.5 µg/ml of anti-BrdU (Sigma Aldrich) were added to the cells in a PBS-BSA 1%-tween 0.5% solution and incubated for 30 minutes at RT. After centrifuging for 5 minutes at 500 x g, supernatant was discarded and cells were resuspended in a PBS-BSA 1%-tween 0.5% solution with 16 µg/ml of 647 anti-mouse (Alexa Fluo) secondary antibody. They were then incubated for 30 minutes in the dark at RT. After washing the cells with PBS-BSA 1%-tween 0.5% and centrifuging them for 5 minutes at 500 x g, cells were incubated for 30 minutes in the dark at RT with 0.015 µg of propidium iodide (PI) suspended in PBS. After washing with PBS and centrifuging for 5 minutes at 500 x g, cells were resuspended in 300 µl of PBS, and filtered in FACS tubes. Lastly, cells were analysed and quantified by flow cytometry (BD FACS Canto I or II with HTS; BD Biosciences) and FlowJo Software (FlowJo LLC) was used for data interpretation.

2.9 Quantitative real-time PCR

For quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR) assays, RNA was isolated through Purelink® RNA mini kit (Thermo Fisher Scientific), and treated with Turbo™ DNA-free kit (Thermo Fisher Scientific) to purify RNA samples. 1 μg RNA was reverse-transcribed using Superscript III Reverse Transcriptase First-Strand Synthesis SuperMix (Thermo Fisher Scientific). After having cDNA from the reverse transcription, qRT-PCR was performed with the Platinum SYBR Green QPCR SuperMix-UDG (Thermo Fisher Scientific). The oligonucleotide primer sequences are listed in Table 1. A 10-fold dilution series ranging from 10^{-3} to 10^{-8} of 50 ng/μL human genomic DNA was used to evaluate the primer efficiency. Relative expression values were obtained by normalising Ct values of the tested genes to Ct values of the housekeeping genes *Gapdh, Hprt* and *Tbp* for murine samples and *GAPDH, HPRT,* and *RPL13a* were used as housekeeping genes of human samples.

Table 1. List of primers

Gene	Primer orientation	Primer sequences 5' > 3'
mMical2	Forward	AAAGAGAGGAGGAAGGAACATGG
	Reverse	GAACTGGAAGGCTGAAACGTA
mMyoD	Forward	GAGCAAAGTGAATGAGGCCTT
	Reverse	CACTGTAGTAGGCGGTGTCGT
mMyogenin	Forward	ATGGAGCTGTATGAGACATCCCC
	Reverse	CGACACAGACTTCCTCTTACAC
mMyhc	Forward	TTCATTAGTTTCCCAGCTCTCC

	Reverse	AGGCACTCTTGGCCTTTATC
mα-Sma	Forward	AGTCGCTGTCAGGAACCCTGAGACG
	Reverse	ATCTTTTCCATGTCGTCCCAGTTG
mCalponin	Forward	ACATCATTGGACTGCAGATG
	Reverse	CAAAGATCTGCCGCTTGGTG
mSm22	Forward	TCCAGTCCACAAACGACCAAGC
	Reverse	GAATTGAGCCACCTGTTCCATCTG
mSm-Myhc	Forward	TCAACGCCAACCGCAGGAAGCTG
	Reverse	TGCTAAGCAGTCTGCTGGGCT
mOct4	Forward	CCAGGCAGGAGCACGAGTGG
	Reverse	CCACGTCGGCCTGGGTGTAC
mNanog	Forward	GAGTGTGGGTCTTCCTGGTC
	Reverse	GAGGCAGGTCTTCAGAGGAA
mBrach	Forward	TGCTGCCTGTGAGTCATA
	Reverse	ACAAGAGGCTGTAGAACATG
mMixL1	Forward	ACCACCAGGCCTGACAACCT
	Reverse	TGGGTGCACACCATACCACA
mcMyhc	Forward	ATCTCTGACAACGCCTATC
	Reverse	GATAGGCGTTGTCAGAGAT
mTnnt3	Forward	GCGTTCTGAGGACTCGTTG

	Reverse	CGTGAAGCTGTCGGCATAAG
mGapdh	Forward	TGGTGAAGGTCGGTGTGAAC
	Reverse	GCTCCTGGAAGATGGTGATGG
mHprt	Forward	TGGATACAGGCCAGACTTTGTT
	Reverse	CAGATTCAACTTGCGCTCATC
mTbp	Forward	CAAACCCAGAATTGTTCTCCTT
	Reverse	ATGTGGTCTTCCTGAATCCCT
h*MICAL2*	Forward	CAACCCGTGTGTGTCTCATC
	Reverse	GTGGATGCCTGGACAAAGTT
h*OCT4*	Forward	CGAGCAATTTGCCAAGCTCCTGAA
	Reverse	GCCGCAGCTTACACATGTTCTTGA
h*NANOG*	Forward	TGGCCGAAGAATAGCAATGGTGTG
	Reverse	TTCCAGGTCTGGTTGCTCCACATT
h*BRACH*	Forward	ACCCAGTTCATAGCGGTGAC
	Reverse	AAGCTTTTGCAAATGGATTG
h*MIXL1*	Forward	CTGAGGAGCCATGACTGACA
	Reverse	TGGGAGTGTGGGCTTAAAAC
hc*MyHC*	Forward	GCCCTTTGACATTCGCACTG
	Reverse	CGGGACAAAATCTTGGCTTTGA
h*TNNT2*	Forward	ACAGAGCGGAAAAGTGGGAAG

	Reverse	TCGTTGATCCTGTTTCGGAGA
h*GAPDH*	Forward	TCAAGAAGGTGGTGAAGCAGG
	Reverse	ACCAGGAAATGAGCTTGACAAA
h*HPRT*	Forward	TGACACTGGCAAAACAATGCA
	Reverse	GGTCCTTTTCACCAGCAAGCT
h*RPL13a*	Forward	CCTGGAGGAGAAGAGGAAAGAGA
	Reverse	TGGAGGACCTCTGTGTATTTGTCAA

2.10 Cryosection preparation from murine models' tissue

All animal procedures were performed at the Translational cardiomyology laboratory according to the guidelines of the Animal Welfare Committee of KU Leuven (Ethical Committee approval number P161/2018.) and Belgian/European legislation. Mice were sacrificed by cervical dislocation, muscles were snap frozen in liquid nitrogen-cooled isopentane and kept at -80ºC for further analyses. The samples were then cut transversally in 7 µm sections using a cryostat machine (Leica, Wetzlar, Germany). Immunofluorescence analyses were performed to characterise MICAL2 in skeletal muscle of different murine models.

2.11 Immunofluorescence assay

For both *in vitro* cultures and tissue cryosections, cells were fixed with 4% paraformaldehyde for 15 minutes at RT and after three PBS washes, permeabilisation by 1% Bovine Serum Albumin (BSA) + 0.2% or 0.5% triton was done for 30-45 minutes at RT to increase permeability. Cells were then blocked for

30 minutes with 10% donkey serum at RT followed by overnight incubation at 4ºC with different primary antibodies at the indicated dilution listed in Table 2. Secondary antibody incubation was done by using the appropriate Alexa Fluor 488-, 594- and/or 647-conjugated secondary antibody (Thermo Fisher Scientific; 4 µg/mL). Nuclei were stained with Hoechst (33342, Thermo Fisher Scientific; 1:10000) for 1 minute. Analyses were assessed using an Eclipse Ti Microscope and NIS-Elements AR 4.11 Software (both from Nikon). Confocal images were obtained by using ZEISS LSM 800 with Airyscan microscope. Images were quantified and analysed using ImageJ software.

2.12 Western blotting

Western blotting (WB) analyses were performed on cell or tissue lysates using RIPA buffer (Sigma-Aldrich) supplemented with 10 mM Sodium Fluoride, 0.5 mM Sodium Orthovanadate, 1:100 Protease Inhibitor Cocktail, and 1 mM Phenylmethylsulfonyl Fluoride (PMSF). The same amount of protein samples (40 µg) have been heat-denatured in sample-loading buffer (50 mM Tris–HCl, pH 6.8, 100 mM DTT, 2% SDS, 0.1% bromophenol blue, 10% glycerol). SDS-polyacrylamide gel electrophoresis was used for resolving and then proteins were transferred to nitrocellulose membranes (Protran, Nitrocellulose membrane, Sigma-Aldrich). Membranes were blocked with Tris-buffered saline (TBS) containing 0.05% Tween and 5% skim milk powder (Sigma-Aldrich). This step has been followed by overnight incubation with different primary antibodies at the indicated dilution listed in Table 2. All secondary horseradish peroxidase (HRP)-conjugated antibodies (BioRad) were diluted 1:5000 in TBS-Tween and 2.5% skim milk powder. After incubation with SuperSignal Dura Chemiluminescence substrate (Thermo Scientific), the polypeptide bands were detected with GelDoc chemiluminescence detection system (BioRad). Quantitation was performed on gels loaded and blotted in parallel. Relative densitometry was obtained by normalising the protein band versus background and a housekeeping protein (Table 2) using the QuantityOne software (BioRad).

Table 2. List of primary antibodies for WB and IF assays

Primary antibody	WB dilution	IF dilution
rabbit anti-MICAL2 (OriGene TA331975)	1:500	-
rabbit anti-MICAL2 (SantaCruz)	-	1:50
mouse anti-MF20 (Developmental Studies Hybridoma Bank, DSHB)	1:3	1:20
mouse anti-α-SarcomericActinin (Abcam 9465)	-	1:100
mouse anti-Ki67 (BD Biosciences 556003)	-	1:300
rabbit anti-Laminin (Sigma 9393)	-	1:300
rabbit anti-Lamin A/C (Epitomics 2966-1)	-	1:600
rabbit anti-GFP (Invitrogen A11122)	1:1000	-
mouse anti-p-ERK (SantaCruz)	1:300	-
rabbit anti-Total-ERK (SantaCruz)	1:300	-
mouse anti-cMyHC (Abcam 50967)	1:500	1:100
goat anti-SOX2 (SantaCruz)	-	1:50
rabbit anti-α-SMA (Abcam ab15734)	1:800	-
mouse anti-α-SMA Cy3 conjugated (Sigma C6198)	-	1:200
mouse anti-α-Tubulin (Sigma T5168)	1:1000	-
Hoechst (33342, Thermo Fisher Scientific)	-	1:10000

2.13 Statistical analysis

Statistical analyses and graphs of the results were performed on GraphPad Prism 7.0 (GraphPad Software, San Diego, CA, USA). Two-tailed t test or one-way ANOVA were used to compare interrelated samples. While two-way ANOVA was used to compare multiple factors. Confidence intervals were fixed at 95% ($p < 0.05$), 99% ($p < 0.01$) and 99.9% ($p < 0.001$). Data is reported as mean ± standard error of the mean (SEM). The number of independent experiments and any other specific information regarding the statistical test used and significance of the differences are specified in the figure legends.

3 Chapter three: Results

3.1 MICAL2 characterisation in C2C12 and satellite cell myogenic differentiation.

MICAL2 plays an important role on actin turnover, which is needed in all the cell types, but in muscle cells it is very important for the functionality and since little is known about MICAL2 involvement in muscle cells, the first aim to be accomplished was to characterise MICAL2 in this compartment. To assess the role of MICAL2 during skeletal muscle differentiation, C2C12-cells and satellite cell (mSCs) isolated from C57/Bl6 mice were differentiated towards skeletal muscle. MICAL2 mRNA and protein expression was then tested in C2C12-cells using qRT-PCR, IF and WB during cell proliferation (day 0) and differentiation (day 5). The localisation of MICAL2 protein during myogenic differentiation was also confirmed in mSCs during proliferation and differentiation (day 2). Key transcription factors involved in myogenic differentiation, including MyoD and Myogenin, were also analysed by qRT-PCR. As expected MyoD was more expressed in proliferating myogenic cells compared to differentiated cells and the opposite was observed for Myogenin that acts later in the myogenic differentiation pathway (Figure 1a). Furthermore, the late myogenic marker myosin heavy chain (MyHC) [191] was also upregulated at the differentiated stage (Figure 1b). qRT-PCR data revealed that Mical2 expression significantly increased after myotubes formation (Figure 1a).

IF analysis showed MICAL2 localisation in both proliferating and differentiated C2C12 and mSCs. While MICAL2 localised in both cytoplasm and nuclei during proliferation (d0), MICAL2 showed a predominant nuclear localisation in myotubes (d5), in both cellular types (Figure 1b). WB analysis showed MICAL2 present in both proliferation (d0) and skeletal muscle differentiation (d5) condition. The protein band and its quantification showed MICAL2 increased in differentiated cells (d5) compared to proliferating cells (d0) (Figure 1c). In conclusion, these results showed a basal MICAL2 content in proliferation that increased in differentiation conditions, where MICAL2 is manly localised in nuclei (Figure 1b).

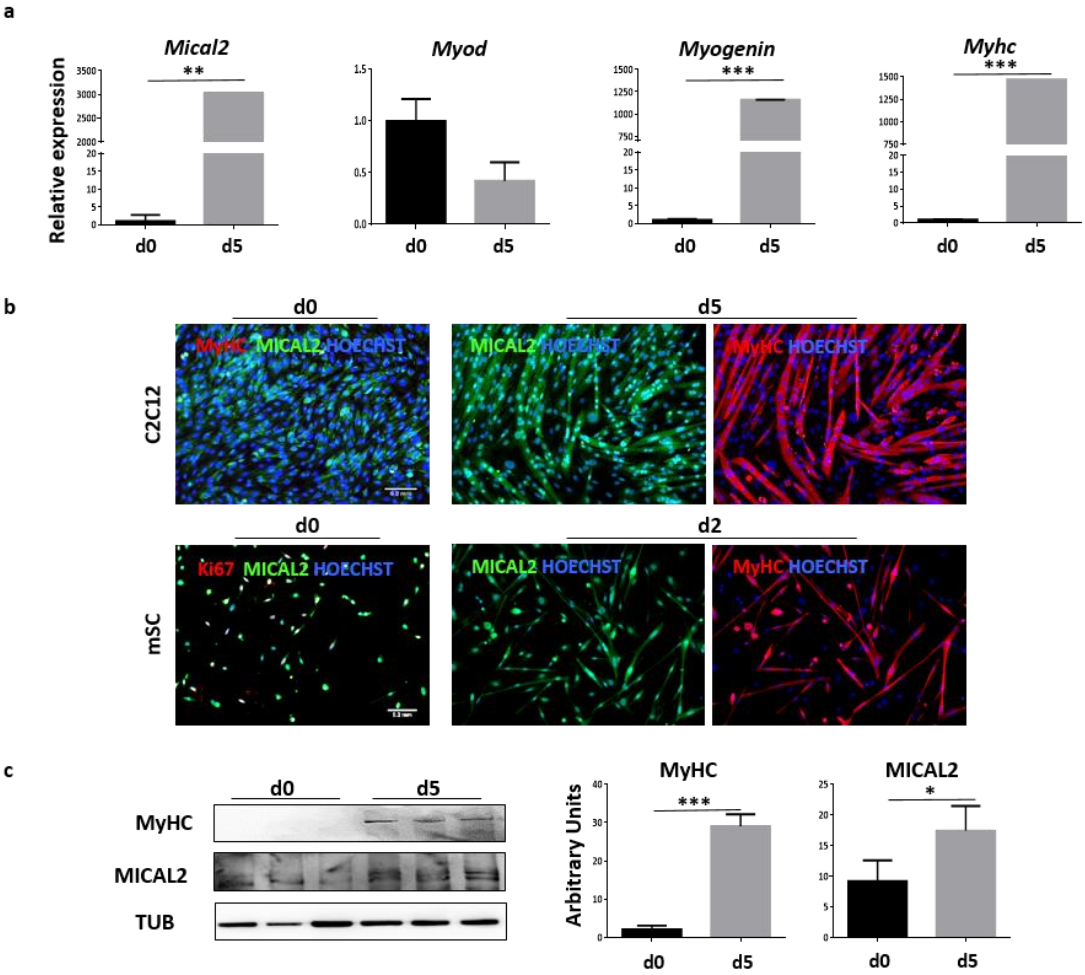

Figure 1. MICAL2 characterisation in proliferating C2C12 and mSC and in differentiated myotubes. a) qRT-PCR for the myogenic markers MyoD, Myogenin, MyHC and for MICAL2 at day0 and day5 of skeletal muscle differentiation. Values are expressed as relative expression, normalised on three different housekeeping genes (*Gapdh, Hprt and Tbp*). **b**) IF assay for MICAL2 (green) and MyHC (red) at day0 and day5 (C2C12) or day2 (mSC) of skeletal muscle differentiation. Nuclei were stained with HOECHST (blue). Scale bars indicate 200 µm. **c**) WB for MyHC and MICAL2 proteins on proliferating (d0) and differentiating (d5) C2C12-cells normalised by α-TUBULIN (TUB). The relative quantification is on the right. N=3. * = p<0,05; ** = p<0,01; *** = p<0,001by two tailed t-test.

3.2 MICAL2 characterisation during smooth and cardiac muscle differentiation of pluripotent stem cells.

Previous experiments have looked at skeletal muscle differentiation showing the importance of MICAL2 in this compartment. Here we test MICAL2 involvement in cardiac and smooth muscle fates of pluripotent stem cells (PSCs), this is important because: 1) PSCs have been shown to generate mesodermal progenitors allowing regeneration of the entire muscle pool [192]; 2) It is known that differentiation of mesodermal progenitors contributes to cardiac and skeletal muscle formation [193]; and 3) MICAL2 is also expressed in both cardiac and smooth muscles [194]. Therefore, MICAL2 was monitored by qRT-PCR, IF and WB during differentiation of murine and human PSCs toward mesodermal progenitors. Then qRT-PCR, IF and WB were performed on mesodermal progenitors subjected to smooth and cardiac muscle differentiation to monitor MICAL2 mRNA and protein.

3.3 MICAL2 characterization during smooth muscle differentiation from progenitors.

To assess the role of MICAL2 along smooth muscle differentiation, MICAL2 was investigated in murine mesoangioblasts (mMABs) - vessel-associated muscle-derived adult stem cells - for mRNA expression and protein content. All the experiments here consider day 0 as proliferating mMABs, day 2 and day 5 as progenitor cells and day 8 as complete smooth muscle differentiation. Gene expression of key transcription factors involved in smooth muscle differentiation, including *α-Smooth Muscle Actin* (*α-Sma*), *Sm22*, *Calponin* and *smooth-muscle MyHC* (*sm-MyHC*), confirmed an efficient differentiation by qRT-PCR (Figure 2a) and are in line with literature [195, 196]. qRT-PCR on differentiating smooth muscle cells revealed a higher *Mical2* expression after smooth muscle cell formation, with a slight increase along differentiation (d2 and d5) (Figure 2a). MICAL2 protein was checked by IF and WB, together with other structural proteins such as α-SMA. IF on proliferating and differentiated mMABs showed MICAL2 localisation in both circumstances, as well as the localisation of the other smooth

muscle-differentiation markers (Figure 2b). Indeed, MICAL2 was lower expressed during proliferation (d0), compared to the nuclear localisation seen in smooth muscle cells at day 8 of differentiation (Figure 2b). The same trend has kept for the protein band shown by the WB and the relative quantification, showing MICAL2 increased in differentiated cells (Figure 2c). These results show that MICAL2 is highly expressed in nuclei of differentiated smooth muscle cells relative to its progenitor, indicating the importance of its nuclear role.

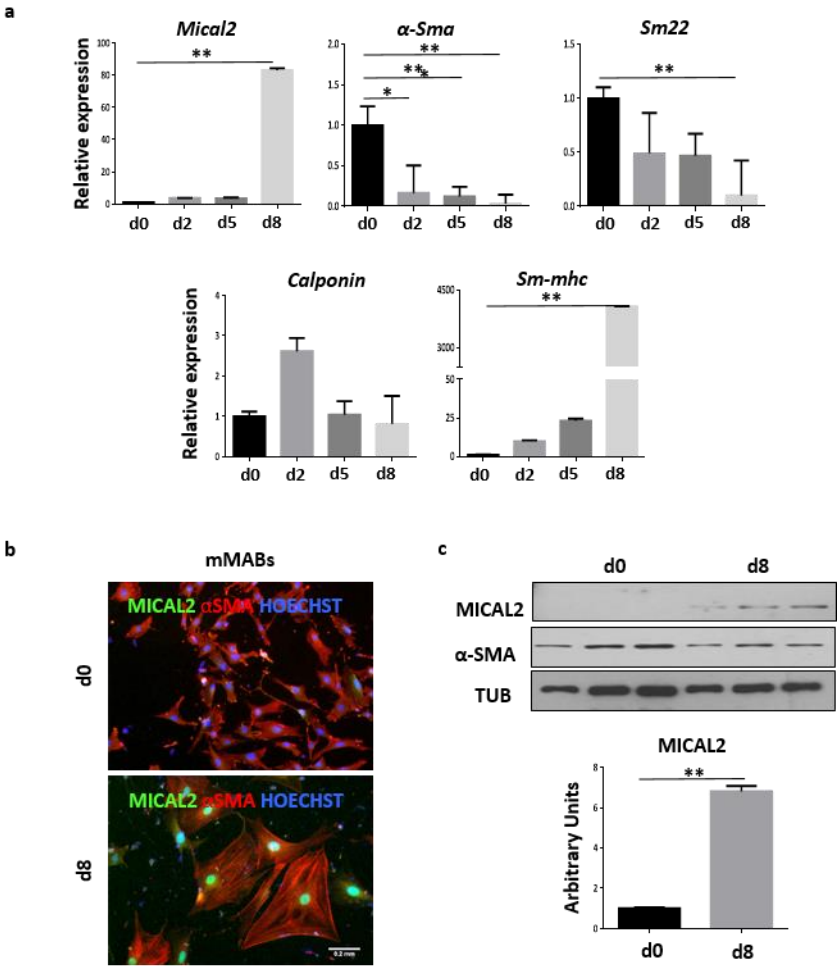

Figure 2. MICAL2 characterization in mMABs smooth muscle differentiation. a) qRT-PCR for mesodermal progenitor markers α-SMA, SM22, Calponin, smooth muscle marker SM-MyHC, and for MICAL2 at day 0, day 2, day 5 and day 8 of smooth muscle differentiation. Values are expressed as relative expression, normalised on three different housekeeping genes (*Gapdh, Hprt and Tbp*). **b**) IF assay for α-Smooth Muscle Actin (α-SMA; red) and MICAL2 (green). Nuclei were stained with HOECHST (blue). Scale bars indicate 200 µm. **c**) WB and relative quantification for MICAL2 and α-SMA, on proliferating (d0) and differentiating (d8) mMABs normalised on α-TUBULIN (TUB). N= 3. * = p<0,05; ** = p<0,01; *** = p<0,001 one way ANOVA test and two tailed t-test.

3.4 MICAL2 characterization during cardiac differentiation from murine embryonic stem cells (mESCs).

mESCs were differentiated into beating cardiomyocyte-like cells using a differentiation protocol starting from embryoid body (EB) aggregation (day 0) and generating cardiomyocyte-like cells at day 11. mESCs used here are derived from the blastocyst of C57/Bl6 embryos and have been screened for mRNA expression and protein content.

Gene expression of key transcription factors from pluripotency until cardiomyogenic differentiation, including Nanog, Oct4, Brachyury, MixL1, cardiac-MyHC (cMyHC) and TnnT3, were analysed by qRT-PCR. The gene expression revealed a higher *Mical2* expression in mature cardiomyocyte-like cells (Figure 3a). MICAL2 protein localisation and content were checked by IF and WB, together with SOX2 pluripotency marker and Sarcomeric α-Actin (α-SA) structural proteins. IF clarified MICAL2 sub-cellular localisation in both proliferating and differentiated mESCs, together with the other cardiomyogenic-differentiation markers. Indeed, MICAL2 was poorly present in the pluripotency stage - compared to differentiated cells - and was localised in both the cytoplasm and nucleus during proliferation (d0). While the signal had a predominant nuclear localisation in cardiomyocyte-like cells (d11), seen by confocal images (Figure 3b). The same trend has kept for the protein band shown by WB and its quantification (Figure 3c). These results have shown a basal MICAL2 content in proliferation and a significant increase in cardiac differentiation conditions, migrating from cytoplasm to nuclei in mESCs, providing further evidence that MICAL2 has a role in cardiac differentiation.

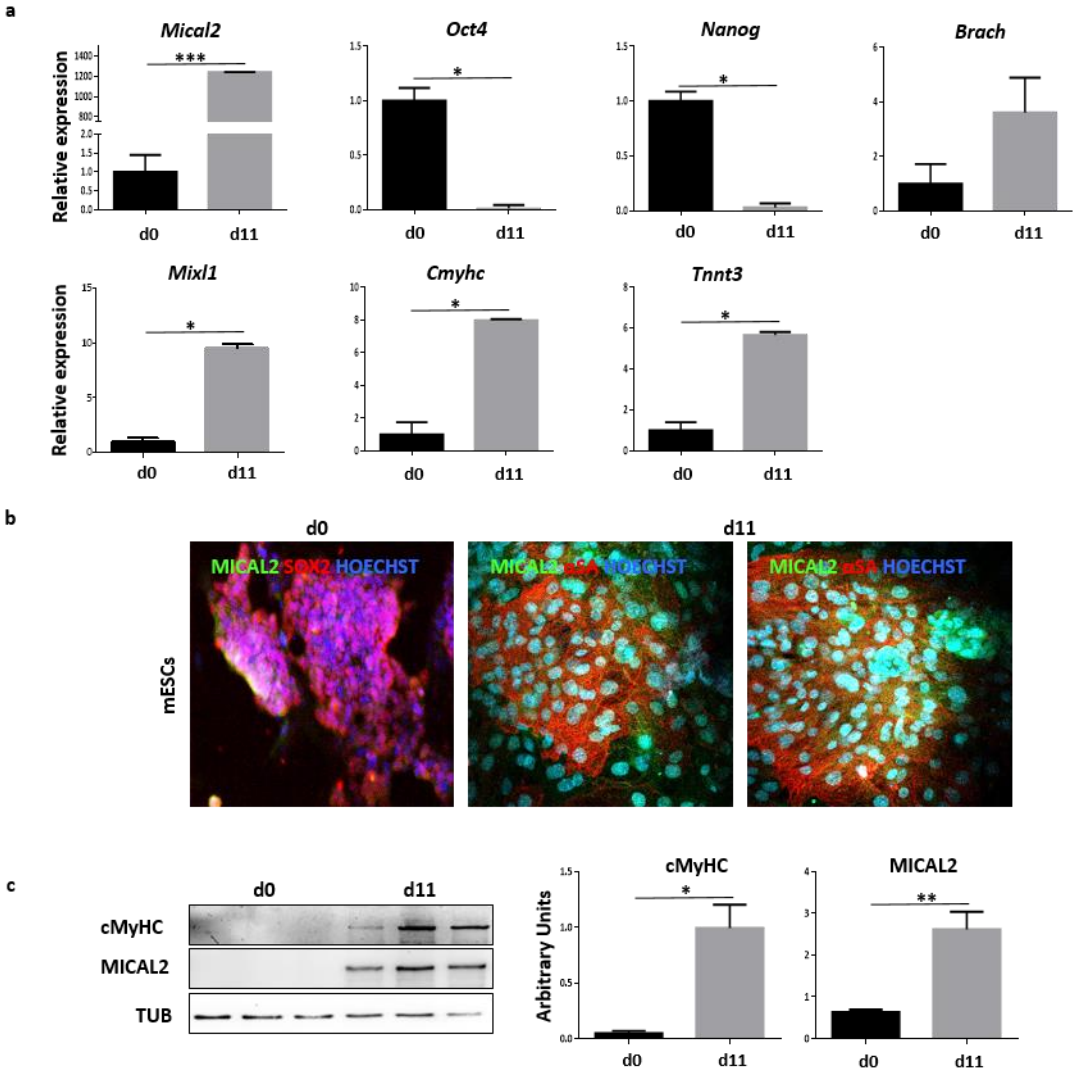

Figure 3. MICAL2 characterization in murine embryonic stem cells (mESCs) differentiated to cardiomyocyte-like cells. a) qRT-PCR for pluripotency markers Oct4, Nanog, mesoderm and progenitor markers Brach, MixL1, cardiac differentiation markers cMyHC and TnnT3, and for MICAL2 in proliferating mESCs (day 0) and in beating cardiomyocyte-like cells (day 11). Values are expressed as relative expression, normalised on three different housekeeping genes (*Gapdh, Hprt and Tbp*). **b**) IF assay in mESCs at d0 for SOX2 (red) and MICAL2 (green) and d11 of cardiac differentiation for α-Sarcomeric Actinin (αSA, red) and MICAL2 (green). Nuclei were stained with HOECHST (blue). Scale bars indicate 200 µm. **c**) WB and relative quantification for c-MyHC and MICAL2 on proliferating (d0) and differentiating (d11) mESCs normalised on α-TUBULIN (TUB). N=3. * = p<0,05; ** = p<0,01 by two tailed t-test.

3.5 MICAL2 characterization during cardiac differentiation from human induced pluripotent stem cells (hiPSCs).

In order to confirm the relevance of MICAL2 in cardiac commitment we monitored its expression in hiPSCs subjected to cardiac differentiation. We used here an episomal (viral-integration-free) hiPSC-line generated using cord blood-derived CD34+ progenitors transfected with seven factors (Oct4, Sox2, Klf4, Myc, Nanog, Lin28, and SV40 T; Gibco by Thermo Fisher Scientific). A second hiPSC-line, generated in house at the translational cardiomyology laboratory - KU Leuven from human fibroblasts reprogrammed via Sendai virus carrying Yamanaka factors [197], was also used. Importantly, the differentiation protocol for human cells has been set up in the laboratory [190]. All the experiments on the hiPSCs specify day 0 as pluripotency stage, day 2 as the mesodermal stage, day 5 as the progenitor stage and day 11 for differentiated cardiac-like cells, reaching beating cardiomyocyte-like cell formation. To follow step by step cardiac differentiation, gene expression of key human transcription factors from pluripotency up until cardiomyogenic differentiation, including *Nanog, Oct4, Brachyury, MixL1, cardiac-MyHC (cMyHC) and TnnT2*, were analysed by qRT-PCR. Here, gene expression showed a gradual increase in *Mical2* expression along the mesodermal, progenitor and cardiomyocyte-like cell stages during differentiation in culture (Figure 4a). MICAL2 protein was checked by IF and WB, together with other structural proteins such as Sarcomeric α-Actin (α-SA), proving cardiac differentiation. IF clarified MICAL2 localisation in differentiated hiPSCs, together with the other cardiomyogenic-differentiation markers. In cardiomyocyte-like cells (d11) MICAL2 signal had a predominant nuclear localisation (Figure 4b). The same trend kept for the protein band shown by WB and the relative quantification. Indeed, MICAL2 had a basal protein level in pluripotency stage (d0) that was kept along mesoderm (d2) and progenitor (d5). While the protein band for MICAL2 in cardiomyocyte-like cells was very intense at day 11 of differentiation, where also cMyHC was significantly present (Figure 4c). Drawing on these results, it can be assumed that MICAL2 has a basal expression from pluripotency to progenitor stage, whereas at later time points of cardiac differentiation MICAL2 expression

significantly increases in human cells, confirming what was seen for murine cardiac differentiation (Figure 4).

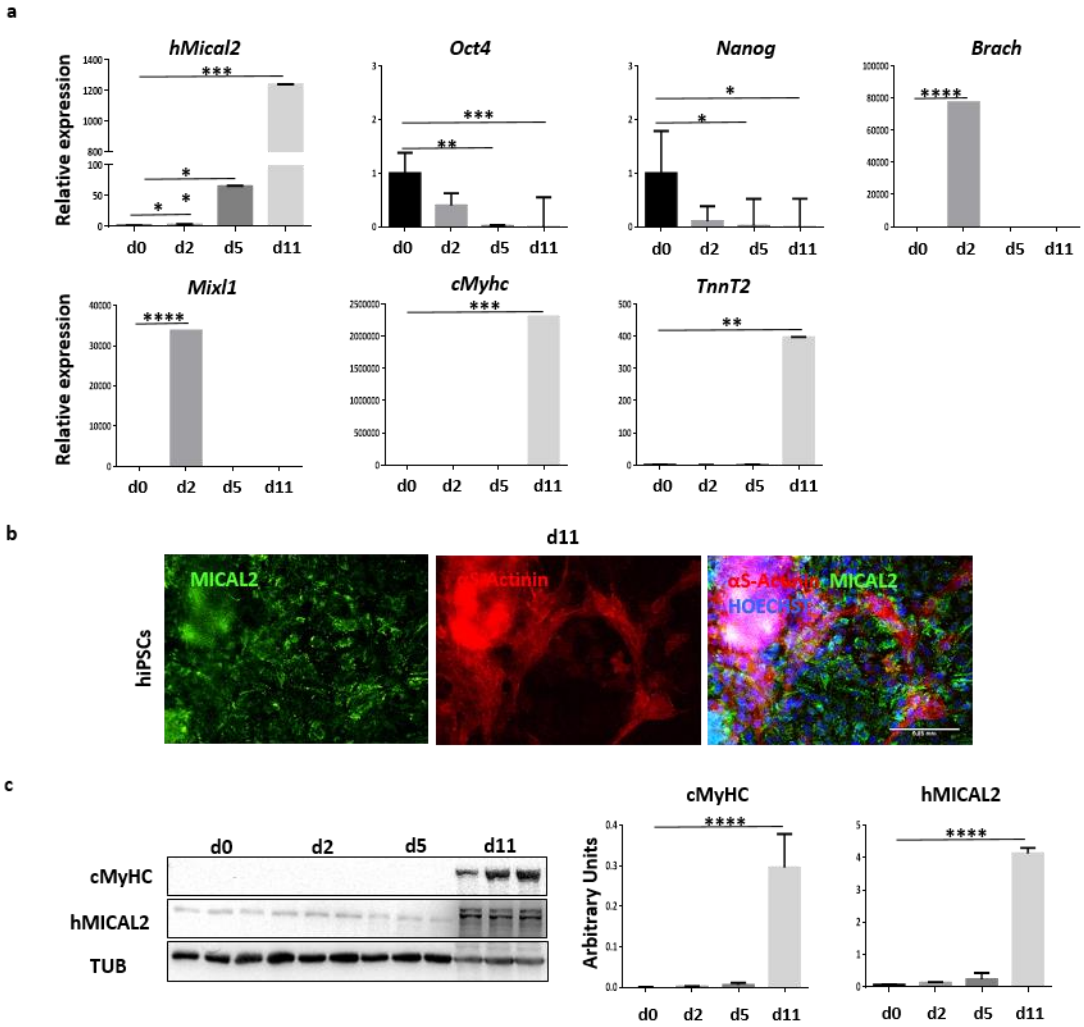

Figure 4. MICAL2 characterization in human induced pluripotent stem cells (hiPSCs) differentiated into cardiomyocyte-like cells. a) qRT-PCR analysis for pluripotency markers Oct4, Nanog, mesoderm and progenitor markers Brach, MixL1, cardiac differentiation markers cMyHC and TnnT3, and for hMical2 in proliferating hiPSCs (day 0), mesodermal cells (day 2), progenitor cells (day 5) and in beating cardiomyocyte-like cells (day 11). Values are expressed as relative expression, normalised on three different housekeeping genes (h*Gapdh, hHprt and hRpl13a*). **b**) IF assay in hiPSCs at d11 of cardiac differentiation for α-Sarcomeric Actinin (αS-Actinin, red) and MICAL2 (green). Nuclei were stained with HOECHST (blue). Scale bars indicate 200 µm. **c**) WB and relative quantification for c-MyHC and hMICAL2 in proliferating hiPSCs (day 0), mesodermal cells (day 2), progenitor cells (day 5) and in beating cardiomyocyte-like cells (day 11) and normalised on α-TUBULIN (TUB). N=3. * = p<0,05; ** = p<0,01; *** = p<0,001; **** = p<0,0001 by one-way ANOVA test.

3.6 MICAL2 impact in muscle disorders.

Having gained knowledge on MICAL2 upon proliferation and skeletal muscle differentiation in normal conditions and after its modulation, it was important to proceed further with characterising MICAL2 in muscle dysregulated scenarios, to understand whether MICAL2 is differentially expressed compared to the healthy counterpart. Therefore, further analyses have been conducted on constant degeneration/regeneration processes going on in dystrophies – in particular Duchenne muscular dystrophy (DMD) and limb girdle muscular dystrophy type 2e (LGMD-2E).

3.6.1 MICAL2 characterisation in dystrophic muscles.

Sarcoglycan- β null (β-SG^{null}, animal model with C57/Bl6 background resembling LGMD type 2E) mice develop both muscular dystrophy and cardiomyopathy with progressive and severe wastage of both muscle types [198]. SCID/MDX mice exhibit necrosis, centrally located nuclei and the muscle degeneration characteristic of DMD and may be used as a dystrophic model to evaluate skeletal muscle regeneration [199]. Thus, to characterise the presence of MICAL2 during chronic degeneration/regeneration processes these two murine models - β-SG^{null} and SCID/MDX - have been chosen. Moreover, cardiotoxin (Ctx) injections were performed to induce acute regeneration, for comparison studies to the chronic counterpart. All animal procedures were performed according to the guidelines of the Animal Welfare Committee of KU Leuven (Ethical Committee approval number P161/2018.) and Belgian/European legislation. The number of animals was calculated by a sample size calculator to reach statistical significance and determined that five mice per group were enough to reach statistical power. Mice were sacrificed by cervical dislocation. Freshly isolated TA, GC, Q and heart muscles from dystrophic β-SG^{null}, SCID/MDX mice have been tested for MICAL2 protein, and compared with all the same muscles of healthy C57/Bl6 mice to assess MICAL2 expression following acute and chronic degeneration/regeneration. Cryosections of TA underwent IF staining for MICAL2 and myogenic markers, including laminin for skeletal muscle. IF experiments on muscle sections showed increased MICAL2 content in all acute and chronic degeneration/regeneration

situations - C57 ctx, β-SG^{null} and SCID/MDX muscles - compared to C57/Bl6 control muscles. This happened particularly in centrally-nucleated fibres, where F-actin is constantly recruited in the regeneration process (Figure 5a). WB was then used to investigate the presence of MICAL2 in both healthy and pathologic mice, confirming that MICAL2 was more abundant in pathologic muscles (Figure 5b). In conclusion, acute and chronic muscle regeneration recruited more MICAL2 in regenerating nuclei, compared to healthy control muscles.

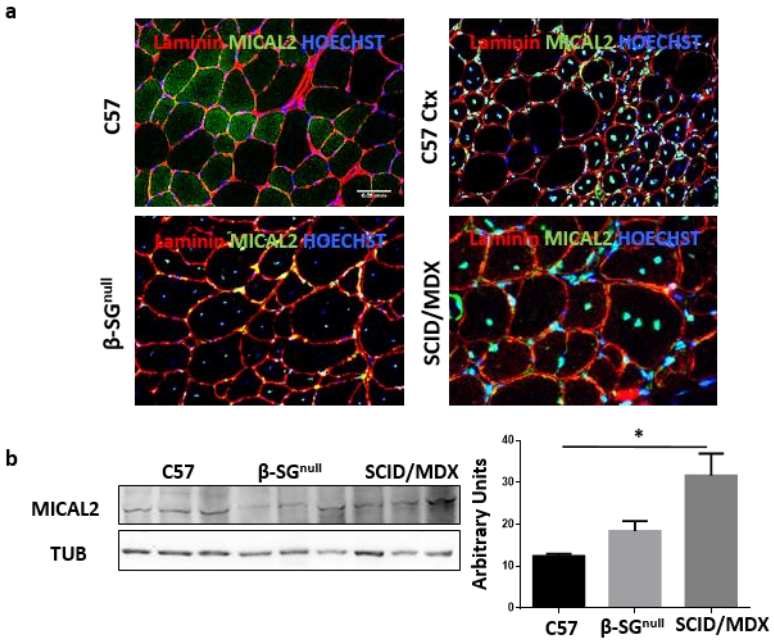

Figure 5. MICAL2 characterization in muscle fibres. a) Upper panels show IF for Laminin (red) and MICAL2 (green) on *tibialis anterior* (TA) cross-sections of control C57/Bl6 (C57), acute regeneration of C57/Bl6 after cardiotoxin injection (C57 ctx), chronic regeneration of β-Sg^{null} and SCID/MDX dystrophic models. Nuclei were stained with HOECHST (blue). Magnification 20x. Scale bar indicates 500 µm. **b**) WB and relative quantification for MICAL2 on *gastrocnemius* of control C57/Bl6 (C57) and chronic regeneration of β-Sg^{null} and SCID/MDX dystrophic models normalised on α-TUBULIN (TUB). N=3. * = p<0,05 by one-way ANOVA test.

3.7 Modulation of MICAL2 during myogenesis: loss and gain of function studies.

After having characterised MICAL2 in skeletal muscle differentiation, the scientific question to be addressed was whether modulations of MICAL2 can influence the

myogenic program. In order to understand how silencing or upregulation of MICAL2 might affect the proliferation and/or the differentiation fate and thus reveal its relative importance for myotube differentiation, perturbation studies were performed in C2C12 and mSCs.

3.7.1 Effects of MICAL2 loss of function in C2C12 and mSCs under proliferative conditions.

MICAL2-knockdown in C2C12 cells and mSCs was achieved by administrating Mical2-esiRNA to cells in culture, through a lipofectamine transient transfection. Mical2-esiRNA is an endo-ribonuclease prepared small interference RNA that targets Mical2 mRNA sequence, triggering its silencing. All the transfections were compared to lipofectamine treated cells, to control for effects due to lipofectamine itself. At first, the silencing efficiency of the Mical2-esiRNA was tested to understand how long it could last and if that was long enough to proceed with further experiments. C2C12 were transfected for 9 hours and then taken for investigations at three different time points (24h, 48h and 72h). Gene expression and protein amount – measured by qRT-PCR and WB, respectively – showed a 95% reduction of Mical2 mRNA and 70% reduction of its protein after 24h compared to lipofectamine-treated control cells (Figure 6a). The same trend kept for the second time point, 48h after transfection in both mRNA and protein. However, 72h after transfection, Mical2 mRNA and protein expression was only reduced by ~60% and 50%, respectively, relative to lipofectamine-treated control cells (Figure 6a). These results demonstrated that Mical2-esiRNA efficiently reduced the expression of Mical2 for up to 72h, confirming that it was a suitable technique for future experiments. In order to test whether MICAL2 had an impact on proliferation, the first circumstance to be evaluated was the cell cycle. To quantify cellular proliferation, BrdU incorporation in C2C12-cells was analysed by flow cytometry on C2C12-cells 24h and 36h after transfection. This experiment showed that there was a higher number of cells in the S phase of MICAL2 silenced cells compared to lipofectamine treated cells, meaning that cells lacking MICAL2 were more prone to proliferation. In particular, at the 24h time point, BrdU analysis

showed that 15% of control cells were in S phase compared to 22% in MICAL2 silenced cells. The second time point at 36h after transfection confirmed this result with ~12% of control cells in the S phase versus ~21% of silenced cells in the same proliferation phase (Figure 6b). The increased proliferation was also observed by immunostaining for the nuclear proliferation marker Ki67 in C2C12 and mSCs under proliferation condition. Indeed, in both MICAL2-silenced cell types more nuclei were counted compared to controls. Moreover, the proliferation quantification - expressed as ratio of Ki67+ nuclei and total number of nuclei – was significantly higher for MICAL2 silenced cells compared to lipofectamine treated cells (Figure 6c). To test which pathways were responsible for the increase in proliferation, phosphorylated ERK (P-ERK) - Mitogen-Activated Protein Kinase (MAPK) [200] - was evaluated since it is known to regulate muscle growth and function. The results showed that protein ratio between P-ERK and the total ERK was higher following MICAL2 silencing and gradually decreased over time in cells recovering from silencing (Figure 6d). Taken together these results proved that silencing MICAL2 led to a higher proliferation rate compared to controls.

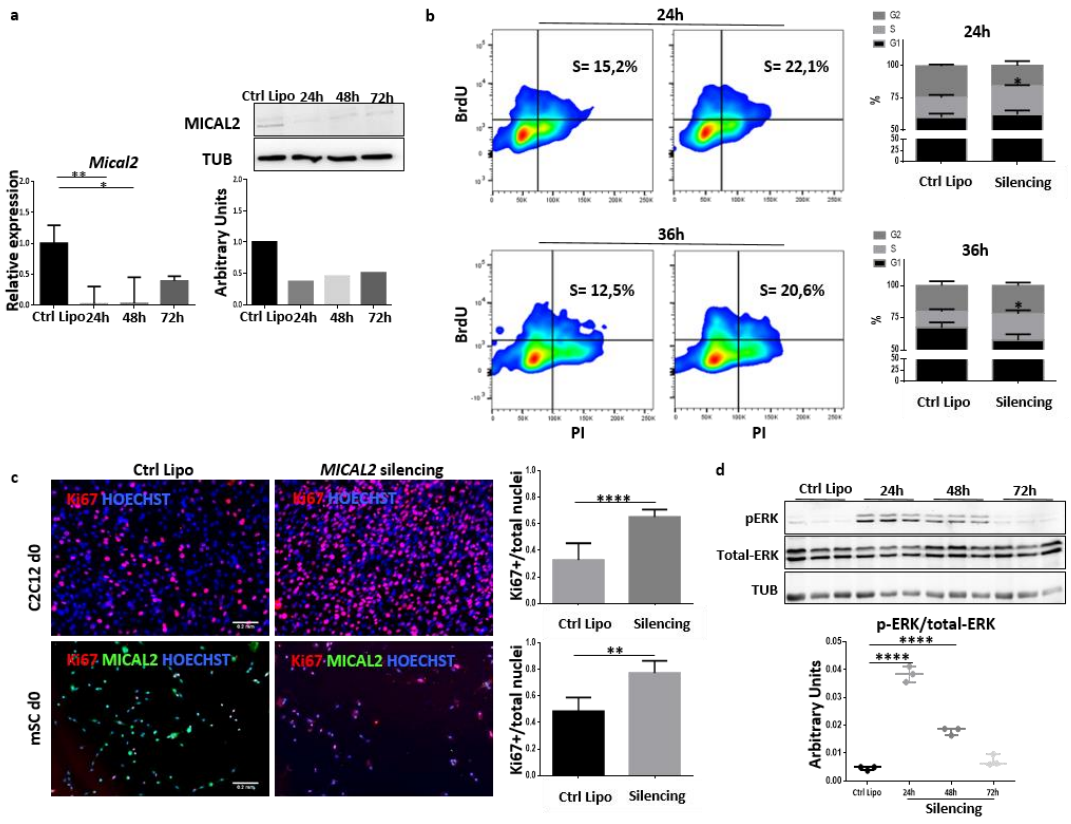

Figure 6. Effect of MICAL2 silencing on proliferating C2C12 and mSCs. a) qRT-PCR for Mical2 gene expression (left panel), showing the efficiency of MICALesiRNA silencing at 24h, 48h and 72h time points, compared to lipofectamine treated control cells (Ctrl Lipo). Values are expressed as relative expression, normalised on three different housekeeping genes (Ga*pdh, Hprt and Tbp*). WB and relative quantification for MICALesiRNA silencing at 24h, 48h and 72h time points (right panel), compared to lipofectamine treated cells as control (Ctrl Lipo), normalised by α-TUBULIN (TUB) content. **b**) Flow cytometry analysis for BrdU incorporation in proliferating C2C12-cells at 24h and 36h following lipofectamine treatment, for control cells (Ctrl Lipo) and MICAL2esiRNA treatment for silenced cells (MICAL2silencing). N=3. **c**) IF on proliferating C2C12 (upper panels) for the nuclear marker Ki67 (red) after 24h of lipofectamine treatment, for control cells (Ctrl Lipo) and MICAL2esiRNA treatment for silenced cells (MICAL2silencing). Nuclei were stained with HOECHST (blue). N=5. IF on proliferating mSCs (lower panels) for the nuclear marker Ki67 (red) and MICAL2 (green) after 24h of lipofectamine treatment, for control cells (Ctrl Lipo) and MICAL2esiRNA treatment for silenced cells (MICAL2silencing). Nuclei were stained with HOECHST (blue). N=5. Scale bars indicate 200 μm. Quantification of proliferation is expressed as ratio of Ki67+ nuclei and total number of nuclei. **d**) WB on proliferating C2C12 after 24h, 48h and 72h of MICAL2esiRNA compared to lipofectamine treated samples (Ctrl Lipo) for pERK and Total-ERK normalised on α-TUBULIN (TUB) and relative ratio below. N=3. * = p<0,05; ** = p<0,01; **** = p<0,0001 by one-way ANOVA.

3.7.2 Effects of MICAL2 loss of function in C2C12 and mSCs subjected to myogenic differentiation.

After having assessed that MICAL2 silencing had an effect on proliferation, we elucidated whether this knockdown also had an impact on myotube formation. C2C12 and mSCs were first transfected with Mical2-esiRNA at the same conditions as previously described in materials and methods section 2.7. Cells were then placed in skeletal muscle differentiation culture medium, for five days and two days, for C2C12 and mSCs, respectively. Intriguingly, MICAL2 silencing had an impact on the differentiation of cells towards a skeletal muscle phenotype, preventing cells from fusing and forming myotubes (Figure 7). In addition, IF analysis revealed that MICAL2 silenced cells do not express MyHC protein and do not form fused myotubes in culture compared to lipofectamine treated cells, which are positive for MyHC and accomplish skeletal muscle differentiation *in vitro*. This experiment has been repeated five times and fusion index (FI) was calculated as ratio of two or more MyHC+ nuclei within myocytes versus the total number of

nuclei. FI showed a significant difference between MICAL2 silenced cells and lipofectamine treated cells, with very high numbers for the former and almost null for the latter (Figure 7a-b; lower panels). Moreover, mSCs differentiated towards skeletal muscle were checked for Ki67 proliferation marker via IF, and relative ratio between Ki67+ nuclei and total number of nuclei calculated, this showed that more silenced cells were Ki67+ compared to lipofectamine treated cells (Figure 7b; upper panels). Additionally, MyHC protein was analysed via WB and quantified corroborating the aforementioned result in which control lipofectamine treated cells have more abundant MyHC content compared to MICAL2 silenced cells (Figure 7c).

In conclusion, MICAL2 loss of function led to an impairment of skeletal muscle differentiation and the exposure to MICAL2 esiRNA treatment resulted in a detrimental effect on C2C12 and mSCs myogenic differentiation compared to controls.

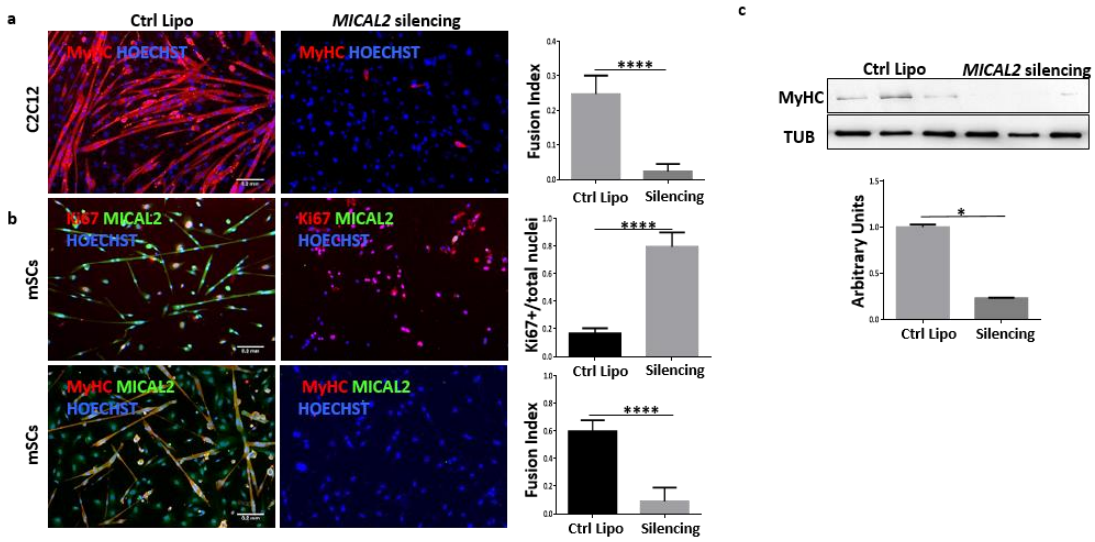

Figure 7. Effect of MICAL2 silencing on differentiated C2C12 and mSCs. a) Upper panels indicate IF for MyHC (red) in control lipofectamine treated (Ctrl Lipo, panels on the left) and Mical2-esiRNA treated (MICAL2 silencing, panels on the right) C2C12 after five days of skeletal muscle differentiation. Nuclei were stained with HOECHST (blue). N=5. Fusion index (FI) quantification is expressed as ratio of nuclei into MyHC+ myotubes and total number of nuclei. **b**) Upper panels indicate IF for Ki67 (red) and MICAL2 (green) in control lipofectamine treated (Ctrl Lipo, panels on the left) and Mical2-esiRNA treated (MICAL2 silencing, panels on the right) mSCs after two days of skeletal muscle differentiation. Quantification of proliferation is expressed as ratio

of Ki67+ nuclei and total number of nuclei. N=5. The lower panels show IF for MyHC (red) and MICAL2 (green) in control lipofectamine treated (Ctrl Lipo) and Mical2-esiRNA treated (MICAL2 silencing) mSCs after two days of skeletal muscle differentiation. Nuclei were stained with HOECHST (blue). N=5. Fusion index (FI) quantification is expressed as ratio of nuclei in MyHC+ myotubes and total number of nuclei. All the scale bars indicate 200 µm. **c**) WB and relative quantification in control lipofectamine treated (Ctrl Lipo) and Mical2-esiRNA treated (MICAL2 silencing) C2C12 after five days of skeletal muscle differentiation for MyHC normalised on α-TUBULIN (TUB). N=3. * = p<0,05; ** = p<0,001; **** = p<0,0001 by two tailed t-test.

3.7.3 MICAL2 gain of function studies in C2C12 cells subjected to myogenic differentiation.

To proceed further, the ensuing question was to assess whether MICAL2 overexpression was able to affect myogenic differentiation. In order to get overexpressing cells - C2C12 at first - the strategy was to start with a lipofectamine transient transfection using pMICAL2-eGFP-N2 overexpressing plasmid, kindly given by professor Debora Angeloni (Sant'Anna Institute –University of Pisa, Pisa - Italy) [137]. As already described for the silencing experiments, all the transfections have been compared to lipofectamine treated cells, to avoid any effect due to lipofectamine itself. Furthermore, the overexpression efficiency has been tested to understand how long it lasts and if that was long enough to proceed with further experiments. C2C12-cells were transfected for 9 hours and then taken for investigations after three different time points (24h, 48h and 72h). Gene expression and protein amount – measured by qRT-PCR and WB, respectively – showed a very significant *Mical2* mRNA overexpression after 24h (~100% increased relative expression). The protein at the same time point increased three-fold compared to lipofectamine-treated control cells. This was shown by a higher band of MICAL2 in the WB, with a molecular weight (MW) of ~140kD, since MICAL2 (~110kD) was fused with eGFP (~27kD) in the plasmid construct, as confirmed by a positive protein band for eGFP at ~140kD MW. However, for the second time point at 48h after transfection, *Mical2* mRNA was already back to normal expression and protein content was only slightly higher than controls. At the third time point, 72h after transfection both mRNA and protein were back to normal (Figure 8a). Hence, C2C12 cells were transfected and a few hours after stopping the transfections, cells

were placed in skeletal muscle differentiation culture medium, for five days. Analysing MyHC protein content by IF unveiled thicker myotubes in MICAL2 overexpressing cells compared to lipofectamine treated cells, as well untreated cells (Figure 8b, upper panels). MICAL2 protein was not visually different at the IF image among MICAL2-overexpressing and control cells at d5 of the differentiation protocol, due to the transient effect of overexpression lasting less than five days (Figure 8b; lower panels). Lastly, WB and relative quantification supported the positive result, shown as thicker MyHC protein band in MICAL2-overexpressing C2C12 compared to lipofectamine treated control cells (Figure 8c). In conclusion, MICAL2 gain of function analyses have enlightened a positive effect upon skeletal muscle differentiation, showing more massive myotube formation with more MyHC+ myotubes.

In addition, due to these promising results, in collaboration with professor Rik Gijsbers (Molecular Virology and Gene Therapy - Department of Pharmaceutical and Pharmacological Sciences, KU Leuven) Mical2-cDNA was inserted in a Malony Lentiviral Vector (MLV)-EF1a (EF1a -based on pCH_EF1a_HER2-CO_IRES_Bsd; BB: pSRS11-SF-adapter cl.12), and used to infect several cell-types to stably overexpress MICAL2. The efficiency of this viral construct has been tested in mESCs and the differentiation potential of the overexpressing cell lines is currently under investigation for both skeletal and cardiac potential (data not shown).

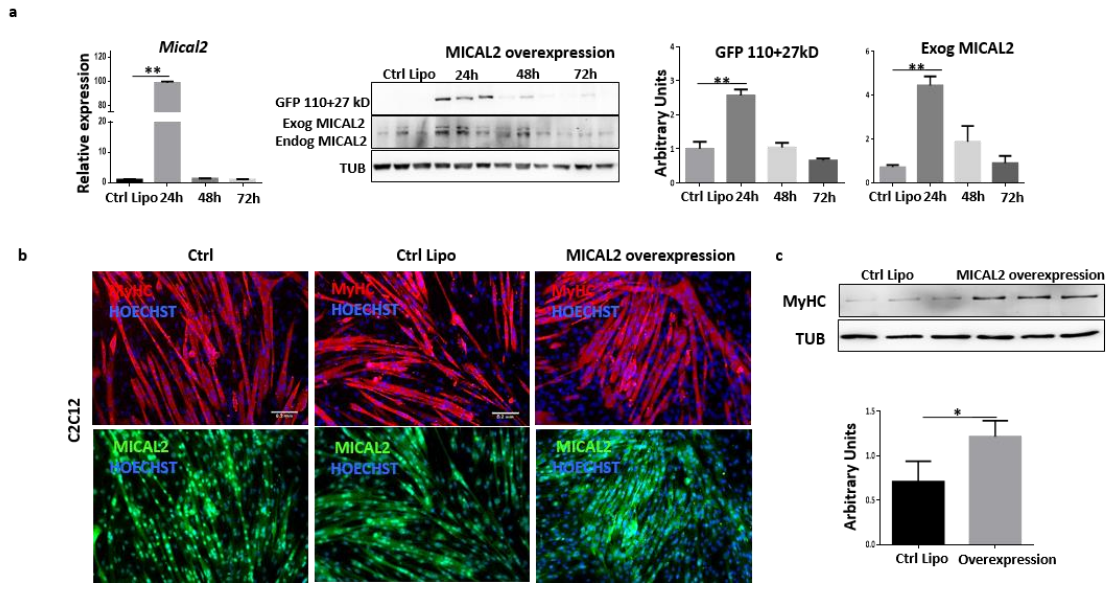

Figure 8. MICAL2 overexpression effects in C2C12-cells. a) On the left panel, qRT-PCR for Mical2 gene expression showing the efficiency of Mical2 overexpressing plasmid at 24h, 48h and 72h time points, compared to lipofectamine treated cells as control (Ctrl Lipo). Values are expressed as relative expression, normalised on three different housekeeping genes (*Gapdh, Hprt and Tbp*). On the right panel, WB and relative quantification of exogenous MICAL2-eGFP-fused protein (~140kD) and eGFP protein at 140kD in MICAL2 overexpressing C2C12 at 24h, 48h and 72h time points, compared to lipofectamine treated cells as control (Ctrl Lipo), normalised by α-TUBULIN (TUB). N=3. **b**) IF for MyHC (red, upper) and MICAL2 (green, lower) in control (Ctrl), control lipofectamine treated (Ctrl Lipo) and MICAL2 overexpressing C2C12 after five days of skeletal muscle differentiation. Nuclei were stained with HOECHST (blue). N=5. Scale bar indicates 200 µm. **c**) WB and relative quantification in control lipofectamine treated (Ctrl Lipo) and MICAL2 overexpressing C2C12 after five days of skeletal muscle differentiation for MyHC normalised on α-TUBULIN (TUB). N=3. * = p<0,05; ** = p<0,01; **** = p<0,0001 by one-way ANOVA test.

3.8 MICAL2 characterization in muscle disorders.

Moreover, another dysregulated muscle differentiation scenario to link MICAL2 with has concerned malignant myoblasts not able to exit the cell cycle and restrained from fusing into syncytial muscle, causing RMS [177].

3.8.1 MICAL2 characterisation in rhabdomyosarcoma.

To further understand the role of MICAL2 in muscle disorders, RMS was investigated. Since eRMS (RD18 and eRD) were already characterised and known to be cells owning spindle-shaped or round morphology and resembling embryonic skeletal muscle cells with a heterogeneous genetic background [184] they were chosen in this study as model of embryonal RMS. Moreover, another well-established cell line (RH30) resembling an aggressive and invasive form of aRMS [185] was used in the experimental setting, in order to test both embryonal and alveolar RMS forms. The three aforementioned cell lines were screened via IF and WB for the presence of MICAL2. Not surprisingly and similarly to other cancer types [130, 137, 138, 146] MICAL2 was very highly expressed in these cancer cells. Indeed, a representative picture of immune-stained RH30 cells depict bright MICAL2 in both cytoplasm and nuclei, co-localising with Ki67 marker (Figure 9a, upper and lower panels). MICAL2 protein was also observed via WB, and was highly expressed in RD18 and RH30, compared to control cells (Figure 9b). The conclusion driven by this characterisation showed MICAL2 highly expressed in cancer cells, suggesting an involvement in cancer progression.

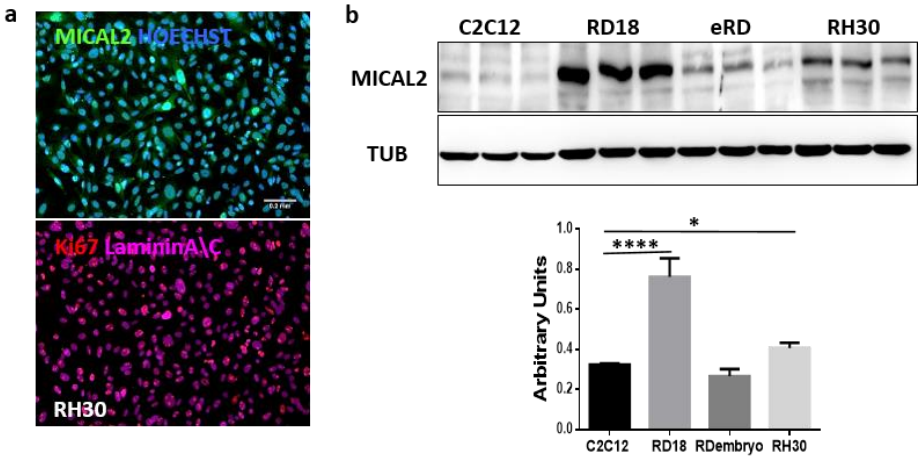

Figure 9. MICAL2 characterization on hRMS cells a) Representative IF assay on proliferating RH30 rhabdomyosarcoma cell line for MICAL2 (green; upper panel), Ki67 (red) and Laminin A/C (cyan; lower panel). Nuclei were stained with HOECHST (blue). Scale bar indicate 200 µm. **b**) WB and relative quantification for MICAL2 on proliferating controls C2C12-cells (C2C12) and three hRMS cell-lines (RD18, eRD and RH30) and α-TUBULIN (TUB) was used for normalisation. N=3. * = p<0,5; **** = p<0,0001 by one-way ANOVA test.

3.8.2 Modulation of MICAL2 during cancer progression: loss of function studies.

Since the alveolar form has been well described as the most aggressive type of RMS, with higher chances of metastasis [175], MICAL2 modulation was performed on the RH30 cell line. Thus, in order to understand whether and how the modulation of MICAL2 might influence the myogenic program, RH30 perturbation studies were performed to investigate any effect caused by MICAL2 on proliferation and/or differentiation during the progression of this cancer. RH30 cells underwent a MICAL2-knockdown by the administration of Mical2-esiRNA to culture plates, through a lipofectamine transient transfection. All the transfections were compared to lipofectamine treated cells. At first, the silencing efficiency was tested to understand how long it could last and if this time was long enough to proceed with further experiments. RH30 cells were transfected for 9 hours and then taken for investigation at three different time points (24h, 48h and 72h). Gene expression and protein amount – measured by qRT-PCR and WB, respectively – demonstrated a 95% reduction of *Mical2* mRNA after 24h and the protein at the same time point decreased by ~80% compared to lipofectamine-treated control cells. The same trend kept for the second time point, 48h after transfection for mRNA expression, while MICAL2 protein silencing was ~75%. 72h after the transfection, Mical2 mRNA remained unchanged (~85-90% silencing), whereas protein levels rose to ~50% of the baseline (Figure 10a). Once the efficiency was set and it was good enough to proceed to experiments, the first circumstance to be examined was the proliferation rate of these cancer cells. Intriguingly, under proliferative conditions, MICAL2-silencing led to a lower number of cells compared to controls. This is shown by IF staining for Ki67, a proliferation marker, after culturing MICAL2-silenced RH30 cells and lipofectamine treated control cells for 72h under proliferation condition (Figure 10b). In order to explain this result, ERK pathway was evaluated as proliferation screening, since ERK activation (P-ERK) was shown to inhibit myotube formation, repressing skeletal myogenesis [201]. Thus, to evaluate the MICAL2-silencing effects on this pathway, WB analysis and relative ratio between P-ERK and total-ERK were performed, showing that MICAL2 knockdown is associated with decreased P-ERK content, compared

to lipofectamine treated control cells over time. The same time points performed for the efficiency test were used for this experiment, proving that P-ERK decreased with the same trend as MICAL2 (Figure 10c). In conclusion, these results suggested that silencing MICAL2 affects the proliferation capacity of aRMS cells, causing these cells to proliferate slower.

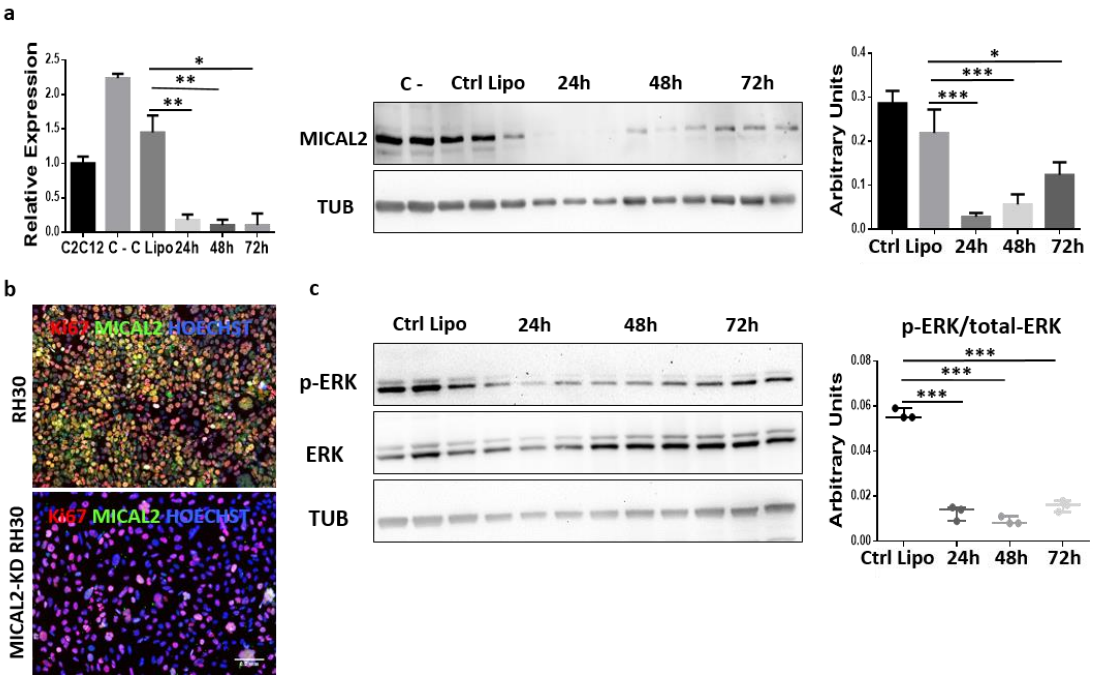

Figure 10. MICAL2 silencing effect on RH30 cells. a) From left to right, qRT-PCR, WB and relative quantification in RH30 control cells (C-), lipofectamine treated RH30 (Ctrl Lipo) and for MICAL2esiRNA treated cells after 24h, 48h and 72h of silencing for MICAL2 mRNA and protein content, respectively. Values of qRT-PCR are expressed as relative expression, normalised on three different housekeeping genes (G*apdh, Hprt and Rpl13a*). WB normalised on α-TUBULIN (TUB). N=3. **b**) IF assay in lipofectamine treated control (RH30) and MICAL2esiRNA treated RH30 (MICAL2-KD RH30) for Ki67 (red) and MICAL2 (green) after 72h of proliferation. Nuclei were stained with HOECHST (blue). Scale bar indicates 200 µm. **c**) WB on proliferating RH30 after 24h, 48h and 72h of MICAL2esiRNA compared to lipofectamine treated samples (Ctrl Lipo) for pERK and Total-ERK normalised on α-TUBULIN (TUB) and relative ratio on the right. N=3. * = p<0,05; ** = p<0,01; *** = p<0,001 by one-way ANOVA test.

4 Chapter four: Discussion

Despite the importance of actin remodelling in myogenic differentiation little is known regarding nuclear monooxygenase that promotes depolymerization of F-actin. This is mainly due to the activity of MICAL2 that mediates oxidation of methionine in order to depolymerise actin filament in reversible manner. In 2009, the group of Marotta identified MICAL2 as one of top 10 genes implicated in the remodelling of mdx muscles in a genome-wide screening. Thus, to fill the gap between in silico analysis and speculative hypothesis we decided to investigate the role of MICAL2 in myogenic differentiation using cell and transgenic animal models. Taken together, our results demonstrate for the first time that the expression of the redox enzyme MICAL2 converges in skeletal, smooth and cardiac muscle differentiated cells. Indeed, we show that MICAL2 increases its expression upon muscle differentiation from adult progenitor cells (mSCs, C2C12-cell line and mMABs), or pluripotent cells (mESCs and hiPSCs). Thus, MICAL2 can be included among myogenic markers commonly expressed in skeletal, smooth and cardiac muscles that regulate muscle progenitor fates.

It was proven, for instance, that serum response factor (SRF) is a transcription factor common to the three muscle lineages [202] and that based on its interactions it can either modulate skeletal, smooth and cardiac muscle commitments and functions [203-206]. Not surprisingly, SRF is finely regulated by MICAL2 [119]. Indeed, when MICAL2 depolymerises F-actin it causes G-actin export from nuclei, determining myocardin-related transcription factor-A (MRTF-A) accumulation in the nucleus. Therefore in this way, SRF can bind MRTF-A promoting the activation several genes [119] in smooth and cardiac muscle regulation [207-209]. A direct or indirect effect of MICAL2 regulation on SRF/MRTF-A axis might be further investigated to elucidate whether modulations of MICAL2 have an impact on SRF/MRTF-A myogenic gene activation.

On the contrary, specific genes are peculiar of one muscle linage and their activation only leads to one muscle commitment. While MyoD and MyoG are crucial only for skeletal muscle commitments [20], GATA4, TBX5 and NKX2.5 regulate cardiac differentiation [210, 211]. TGF-β, instead, can activate several

signalling pathways such as RhoA, Notch and SRF/myocardin leading to smooth muscle cell fate determination and maturation [212]. However, it is shown that regulating one of the aforementioned cross-talks could induce lineage promiscuity in myogenic progenitors. An example is the expression of miR669a and miR669q, which is known for negatively regulating MyoD activation [213]. In LGMD-2E, cardiac progenitors undergo aberrant skeletal muscle differentiation and this was found to be due to the absence of miR669a and miR669q in those cells, causing a switching from cardiac to skeletal muscle commitment [213]. Since MICAL2 regulation is important for both striated muscles, it could be interesting to investigate its interaction with MyoD and miR669 family and its role in this aberrant phenotype.

MICAL2 is localised in the nucleus where it acts as redox enzyme. It is known that MICAL1 and MICAL3 present a coil-coil domain able to auto inhibit their cellular activities when they are in the cytoplasm [99]. However, such domain is not present in MICAL2 and its auto-inhibition mechanism is not yet known, although it is not active in the cytoplasm [99]. We showed here that MICAL2 localises in the cytoplasm of myogenic progenitors (mSCs and C2C12-cells) and it moves into nuclei when the cells differentiate toward skeletal muscle. The nuclear localisation is also shown in smooth muscle differentiated mMABs and in cardiomyocyte-like cells originated from PSCs. In addition, MICAL2 is poorly expressed in the cytoplasm of healthy muscle fibres, while in acute and chronic degeneration/regeneration conditions it is highly expressed in the regenerating nuclei. Thus, MICAL2 is a protein shuttle shifting between cytoplasm and nucleus to maintain cellular homeostasis. A famous shuttle between cytoplasm and nucleus, which works in a similar manner, is YAP-TAZ system. YAP and TAZ are localised in the cytoplasm of cells experiencing low levels of mechanical signalling, such as in rounded cells attached to a soft ECM, or to a small adhesive area [214]. While, YAP and TAZ are nuclear in cells under high mechanical signalling, such as cells cultured on rigid substrates, or undergoing deformation and cytoskeletal tension [215]. Similarly to MICAL2, YAP/TAZ nuclear localisation occurs when cardiomyocyte progenitor cells differentiate towards cardiac-like cells [216]. Moreover, YAP/TAZ shuttling is activated in response to dynamic modifications

and seems to be required for cardiac progenitor cell motility on stiff surfaces [216]. Indeed, cell migration is another important effect that might involve MICAL2 action as documented in previous studies [137, 138], and epithelial to mesenchymal transition (EMT) studies fully support this hypothesis [137]. MICAL2 was addressed as a regulator of EMT and the reverse process mesenchymal to epithelial transition (MET) in several cancer types. Particularly, high expression of MICAL2 correlates with EMT and thus with cell migration, whereas MET occurs when MICAL2 is turned off, or decreased in cancer cells [99, 137, 138]. It is also likely that MICAL2 could have an impact in non-tumour cell migration since depolymerisation/re-polymerisation cycles are required for cell rolling. This could be relevant also for SCs, C2C12-cells [217], MABs and mesodermal progenitors [218, 219] that are known to have migration capacity. Further investigation using scratch and invasion assay are necessary to understand whether MICAL2 is involved in cell migration of non-tumour cells.

Nevertheless, what we found in physiological conditions regarding MICAL2 expression is not in line with the literature about cancers. Indeed, high MICAL2 correlates with high proliferation in several tumours [99, 137, 138]. While in our findings, MICAL2 is higher expressed in differentiated myogenic cells – in all the three lineages - compared to their progenitors. Moreover, overexpressing MICAL2 caused a better skeletal muscle differentiation. To shed light on this difference, we investigated MICAL2 expression in tumour cells belonging to the myogenic line, rhabdomyosarcoma (RMS) cells. RMS cells are, indeed, myogenic aberrant cells incapable to exit the cell cycle and finalise their myogenic differentiation, even if the myogenic signature is on [175, 186]. Not very surprisingly, RMS cells showed a sustained MICAL2 expression. Moreover, as for other cancer cells reported in literature, silencing MICAL2 resulted in a decreased proliferation as shown by less activation of the ERK pathway. This apparent discrepancy needs deeper investigation as well the reason that makes MICAL2 highly expressed in muscular dystrophies. However, if this high expression occurs for the same reason is not clear yet. What is clear is that in the two muscle disorders myogenic cell progenitors act very differently. While in the cancer environment there is a constant proliferation, in the dystrophic situation there is a constant

regeneration going on, in which myogenic cell progenitors try to reconstitute the myogenic pool [156, 186]. Thus, MICAL2 could be an important therapeutic target in both pathological conditions. Therefore, pathway analyses might unveil its role during the progression of these pathological conditions compared to their physiologic counterpart.

In conclusion, we provide evidence that MICAL2 is a crucial modulator for myogenic differentiation in all the three muscle types. Translocation of MICAL2 to the nucleus is likely required to provide redox modification of nuclear actin and for its positive interaction with myogenic transcription factors. This regulatory switch is necessary in skeletal myogenic progenitors to elevate the expression of genes such as myogenin and MyHC, and to stimulate myogenic differentiation. However, the pathological nuclear localisation indicates that MICAL2 is also necessary to sustain proliferation of skeletal muscle progenitors, as highlighted by our perturbation studies. Thus, MICAL2 is a novel regulator of skeletal myogenic differentiation and a possible therapeutic target for muscle disorders.

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March 1, 2019

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Dear Dr. Giarratana,

Thank you for the submission of your manuscript entitled "The role of MICAL2 in physiological and pathological myogenic commitment" to Journal of Cell Biology in the Article format. It was received on 2019-03-01, and the manuscript reference number is 201902169. Please use this number on all correspondence about the manuscript that you send to the JCB editorial staff.

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With best wishes,

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Academic Institution: University of CATANIA- Department of Public health and legal medicine - (Italy)

PhD Student: Double Degree Programme

From 01/11/2015 to present: PhD program of Medical Biotechnologies. Academic Institution (Main Institution): University “G. D'Annunzio" of CHIETI-PESCARA - (Italy)

From 25/07/2016 to present PhD program of Biomedical Sciences. Academic Institution (Partner Institution): KU Leuven, 3000 Leuven (Belgium)

Acquired skills

- HPLC-MS/MS and Gas Chromatography/Mass Spectrometry
- Animal handling: FELASA-B Certification (achieved 2016/11/22)
- Cell culture
- Nucleic acid extraction
- PCR, qRT-PCR
- Immunohistochemistry, Immunofluorescence
- Western blot
- Histological techniques (paraffin embedding, slides preparation and staining)
- CRISPR/Cas9 technology

Publications

- Toralf Fosen J, Høiseth G, Sempio C , GIARRATANA N, Enger A, Mørland J, Morini L (2018). Hair EtG: Reduced segment levels accompanying hair growth. Drug testing and analysis, ISSN: 1942-7603, doi: 10.1002/dta.2474. IF= 3.469

- Ceccarelli G, Presta R, Lupi S M, GIARRATANA N, Bloise N, Benedetti L, Cusella De Angelis G M, Rodriguez y Baena R (2017). Evaluation of Poly(Lactic-co-glycolic) Acid Alone or in Combination with Hydroxyapatite on Human-Periosteal Cells Bone Differentiation and in Sinus Lift Treatment. MOLECULES, ISSN: 1420-3049, doi: 10.3390/molecules22122109 I.F.= 1.288

- Toralf Fosen J, Morini L, Sempio C, GIARRATANA N, Enger A, Mørland J, Høiseth G (2017). Ethyl Glucuronide Elimination Kinetics in Fingernails and Comparison to Levels in Hair. ALCOHOL AND ALCOHOLISM, vol. Volume 52, p. 580-586, ISSN: 1464-3502, doi: https://doi.org/10.1093/alcalc/agx035 I.F.= 2.724

- Busardò FP, Zaami S, Baglio G, Indorato F, Montana A, GIARRATANA N, Kyriakou C, Marinelli E, Romano G. (2015). Assessment of the stability of exogenous gamma hydroxybutyric acid (GHB) in stored blood and urine specimens. EUROPEAN REVIEW FOR MEDICAL AND PHARMACOLOGICAL SCIENCES, p. 4187-4194, ISSN: 1128-3602 I.F.= 1.778

Oral communications:

- GIARRATANA N, Costamagna D, Duelen R, Fulle S, Sampaolesi M. MICAL2 affects myogenic differentiation. IX meeting Stem Cell Research Italy (SCR Italy), 21-23/06/2018 University of Milan, Milan, Italy.
- GIARRATANA N, Costamagna D, Duelen R, Fulle S, Sampaolesi M. Impact of MICAL2 on physiological and pathological myogenic commitment. 2nd YOUNG SCIENTIST WORKSHOP: FROM BASIC SCIENCE TO CLINICAL APPLICATION, 19-21/06/2018, University of Pavia, Pavia, Italy.
- GIARRATANA N, Costamagna D, Duelen R, Fulle S, Sampaolesi M. Role of MICAL2 in myogenesis. IIM, XXIV meeting, Assisi, Italy 12-15/10/2017.
- GIARRATANA N, Tamburro O, Benedetti L, Angeloni D, Cusella De Angelis MG, Ceccarelli G, Sampaolesi M. The role of MICAL2 in myogenic differentiation. In: IJAE Italian Journal of Anatomy and Embryology. ITALIAN JOURNAL OF ANATOMY AND EMBRYOLOGY, vol. 121, FIRENZE: Paolo Romagnoli, ISSN: 1122-6714, Roma, Italy 15-17/09/2016
- Ceccarelli G, Benedetti L, Presta R, Alloni M, GIARRATANA N, Balli M, Rodriguez Y Baena R, Rizzo S, Cusella De Angelis MG. Study of the effects of different biomaterials on osteogenic differentiation of oral-periosteal cells. In: IJAE Italian Journal of Anatomy and Emryology. ITALIAN JOURNAL OF ANATOMY AND EMBRYOLOGY, vol.

121, FIRENZE: Paolo Romagnoli, ISSN: 1122-6714, Roma, Italy 15-17/09/2016

- GIARRATANA N, Morini L, Groppi A. Ricerca dell'etg nelle unghie con finalità d'impiego nell'ambito del rilascio/rinnovo patente. Giornata studio della sezione giovani GTFI - attuali problematiche e recenti innovazioni normative in ambito tossicologico-forense. Mestre-Venezia, Italy 28/04/2015

Posters:

- GIARRATANA N, Duelen R, Fulle S, Costamagna D, Sampaolesi M. MICAL2 effects on physiological and pathological myogenic fate. Departmental day 2019. KU Leuven, Leuven, Belgium. 28/02/2019.

- GIARRATANA N, Duelen R, Fulle S, Costamagna D, Sampaolesi M. MICAL2 effects on physiological and pathological myogenic fate. 5th annual meeting of Belgian society for stem cell research (BeSSCR). Leuven, Belgium. 26/10/2018.

- GIARRATANA N, Duelen R, Fulle S, Costamagna D, Sampaolesi M. Role of MICAL2 in myogenesis. External Advisory Board (EAB) Meeting Stem Cell Institute (SCIL). Leuven, Belgium. 10/01/2018.

- Busardò F, Barbera N, Indorato F, GIARRATANA N, Romano G. Assessment of the stability of exogenous Gamma Hydroxybutyrric Acid (GHB) in stored blood and urine specimens. 51st annual meeting of The International Association of Forensic Toxicology (TIAFT). MADEIRA – Portugal. 02-06/09/2013

Awards:

- Prof. Paolo Bianco award for the best oral presentation to GIARRATANA N. MICAL2 affects myogenic differentiation. IX meeting Stem Cell Research Italy (SCR Italy), 21-23/06/2018 University of Milan, Milan, Italy.

Conflict of interest

The author declares no conflict of interest.

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