Effect of uniaxial cyclic strain on the assembly and differentiation of mammalian myogenic precursors

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Abstract

BACKGROUND Human skeletal muscle myoblasts are exposed to a variety of mechanical forces during *in vivo* conditions. These forces affects both cell orientation, proliferation and differentiation. Different models for mechanical stimulation have been tested, but one, uniaxial cyclic tensile strain (CTS), has to our knowledge not been tested on human primary skeletal muscle myoblasts. Previous research on this model has provided knowledge about cell alignment, signaling, protein expression and gene transcription when tested on murine myoblast cell line, C2C12. However, whether the same findings is existing for a human primary cell line is of great interest to explore. In this thesis we demonstrate that primary human myoblasts, HSMM, are comparable to murine myoblasts, C2C12, in a model, that applies CTS to the cells. **RESULTS** The effects CTS on HSMM were thoroughly investigated and compared to those of C2C12. Both HSMM and C2C12 subjected to uniaxial CTS acquired a uniform orientation perpendicular to the direction of strain. Myogenic markers, myogenin and MHC showed significant enhancement of differentiation in both HSMM and C2C12 cultures, despite no significant difference was detected for early myogenic markers, Myf-5 and MyoD1. Cells fusion was observed already at day 2 for C2C12 and day 5 for HSMM. Assembly of sarcomeric structures was found within both control and CTS groups for both HSMM and C2C12 in form of actin and myosin cross-striations. **CONCLUSION** The effect of CTS on myogenic differentiation was verified for both C2C12 and HSMM cell lines, with an enhanced myogenic profile. Cytoskeleton rearrangement in response to elongation of the stress fibers aligned the cells uniformly in a perpendicular angle to the direction of strain. The enhanced myogenic properties experienced in this thesis may prove valuable in future experiments trying to unravel the true potential of primary human skeletal muscle myoblasts in regenerative medicine and tissue engineering.

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Introduction

Patient groups with lost or damaged skeletal muscle tissue have often been exposed to traumatic injuries, tumour ablation or functional damage due to myopathies.[1] Loss of functional skeletal muscle tissue often results in deficits with poor treatment options. Only few alternatives exist today providing possibility of repair and restoration of the native muscle tissue.[2] Surgical repair, either transplantation or transposition of muscle tissue, only has a limited degree of success.[1] Tissue engineering, on the other hand holds a more promising future and prove to be an alternative, but beneficial option.[3] Several hurdles are to be overcome, when understanding basic aspects of engineering of skeletal muscle *ex vivo*: Firstly, high quantity of fully differentiated cells must be obtained and, secondly, these cells have to be orientated in the same direction to establish a uniform contractible syncytium.[4] Given the complexity of *in vivo* myogenic differentiation *in vitro* models are crucial in understanding skeletal muscle regeneration and repair. Many studies of *in vitro* culturing of myoblasts is based on the murine immortalized myoblast cell line, C2C12. These cells readily proliferate when cultured in medium supplemented with fetal bovine serum and differentiates when deprived of mitogens and growth factors; although, the exact mechanisms involved remain unknown.[5] Besides their murine origin, an important issue to address concerning immortalized myoblasts is their capability of expand infinitely. Due to immortalization, thus altering in the genome, they cannot be directly compared to primary myoblasts. Primary myoblasts reaches senescence after 60-70 population doublings in culture, when extracted from fetus and even less as the donor age increases.[6] Thus these cells have a limited proliferative capability and execution of *in vitro* models utilizing primary myoblasts, in the study of disease processes and therapeutic potentials, becomes complicated. Most data on differentiation of native skeletal muscle cells is obtained using primary chick and mouse myogenic cells.[7] However, it is established that animal myogenic cells do not differentiate through the same pathways as the human myogenic cells.[8] Therefore, investigation of human myoblasts is highly valuable in unraveling the precise details and exact mechanisms of human myogenic cells and their potential. One problem with primary human myoblasts is that they are not easily immortalized, and, even if immortalization is successful, the human cells lose much of the differentiation potential

and do not form myotubes.[9] So alternative solutions, e.g. medium composition and external stimuli ensuring maintained proliferation and differentiation, is needed to near a competent model in the study of human skeletal muscle repair and engineering. One promising alternative is mechanical stimulation in the form of uniaxial cyclic tensile strain (CTS), though this approach has only been tested on murine cell lines.[10] Therefore it is interesting to implement this model on primary human myoblasts, thus the objectives of this work is to compare a CTS model[10] performed on mouse myoblasts C2C12 on primary human myoblasts, highlighting differences and similarities on the effect of CTS on cell alignment and differentiation.

Background

Skeletal Muscle Tissue Organisation

Skeletal muscle tissue is one of three major muscle types and is unique due to its connections to bones through tendons. Furthermore, skeletal muscles are controlled by the somatic nervous system, thus rendering it susceptible to voluntary movements. A vital component of skeletal muscle tissue are the myocytes. Myocytes are a result of developmental myoblasts fusing together into cylindrical and multinucleated cells, also known as myofibers. These myofibers are composed of myofibrils, actin and myosin fibrils, which are intertwined with another in a repeated pattern, thus forming sarcomeres. Sarcomeres, the main component of a fully contractile apparatus, are responsible for skeletal muscle contraction, due to interactions between actin and myosin. Other important components in a fully contractible muscle fibre are calcium-storage units and acetylcholine receptors cobbling the fibres to the somatic nervous system. Multiple myofibers are held together with connective tissue forming the individual muscles of the body, se Figure 1.

Myogenic Differentiation, Fusion and Muscle Repair

The process of myogenic differentiation from skeletal muscle progenitor cells into fully mature myofibers is a complex and multiphase process. During early myogenesis the myoblasts undergo a period of proliferation, in which expression of transcription factors from the myogenic regulatory factor (MRFs) family, also known as basic helix-loop-helix family increase.[11] This transcription factor family include MyoD, Myf-5, myogenin and MRF4. During embryogenesis, MyoD and Myf-5 are expressed in proliferating myoblasts even before terminal differentiation phase is initiated. Myf-5 is expressed first, then MyoD follows, shortly before Myf-5 expression disappears. Myogenin expression starts after Myf-5, but in advance of the expression of proteins involved in the sarcomeric architecture. Myogenin expression are initiated when the myoD gene is activated. Myogenin expression is directly controlled by Myf-5 and MyoD,

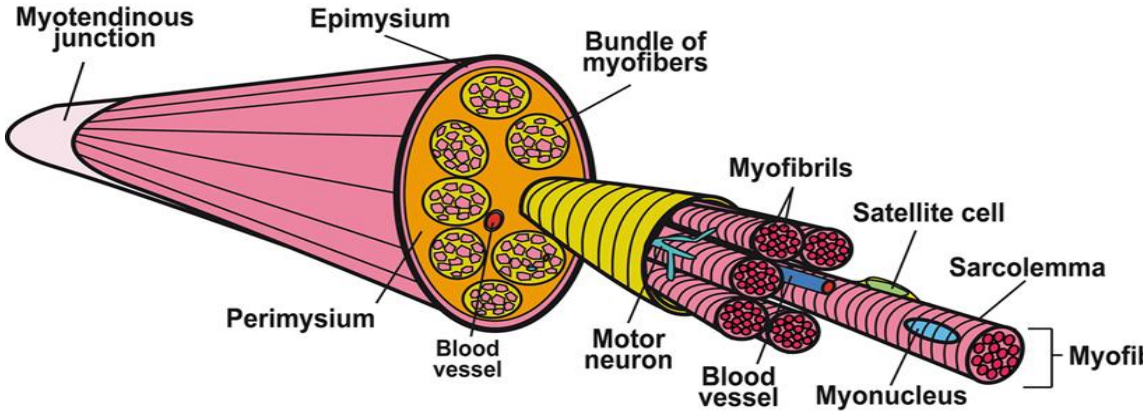

Fig. 1. Composition and structure of muscle with from myofibril to myotendinous junction.

thus, the Myf-5 and MyoD are responsible for early myoblast formation and commitment to myogenic differentiation, including regulation of myogenin expression. The role of myogenin is more prominent in terminal differentiation, where it maintains the cells in a differentiating state.[12] When the cell are committed to the myogenic differentiation pathway it enters cell cycle arrest, an irreversible state, which are caused by increased cdk-inhibitor p21 expression, along with other Cip1/Kip1 family members, p57 and p27.[13] These are responsible for inhibition of numerous cyclin-dependent kinases crucial for cell proliferation. Once p21 is expressed, MyoD plays a major role in its activation, hence forcing the cell into cell cycle arrest. p21 expression will increase after myogenin expression is initiated.[14] At this post-mitotic state the cells still appear mononucleated, but now expressing both myogenin and p21. The mononucleated myoblast pair and align with adjacent myoblasts and fusion into multinucleated skeletal muscle myotubes, essential for generation of muscle fibers, begins.

The fusion begins with formation of an asymmetrical in the wall between aligned myoblasts, se Figure 2. During progressing fusion gaps in the actin wall appears, linked to vesicle accumulation, fusion pore formation and transmembrane pairing, before the cells merge together forming myotubes. In late myogenic differentiation events myogenin is responsible for myotube differentiation and activation of another bHLH member, MRF4. MRF4 is believed to participate the final events of differentiation into a myofiber. Having reached cell cycle arrest and increased expression of MRF4, the late and phenotypic differentiation marker, myosin heavy chain (MHC), are synthesized, and

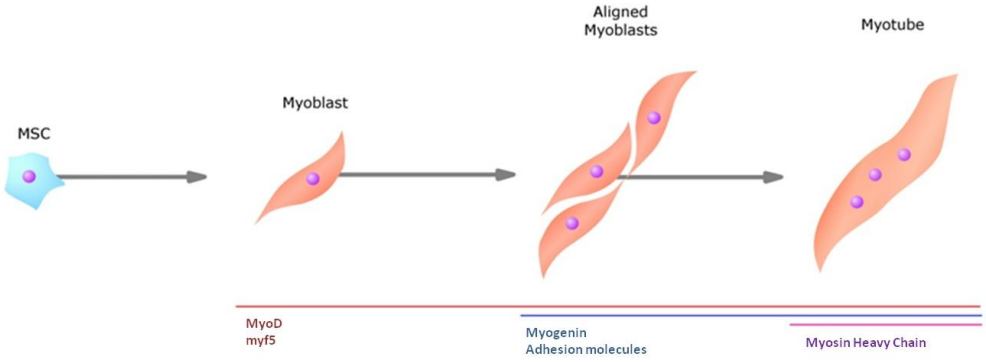

Fig. 2. Myogenic differentiation process from myoblast to myotube. Markes are displayed so they depict the their introduction point in the myogenesis.

assembly of the contractile mechanisms are starting to form.[14] MHC and α-actin are assembled in sarcomeres to form contractive myofibrils. Myofibrils consist of thick filaments, actin connected by Z discs, and thin filaments, myosin joined in a M line, thus forming sarcomeres that, in functioning tissue, are aligned and enables contraction. The approximately 2.3 μm long sarcomeres are assembled to form long striated myofibers, a definitive hallmark of skeletal muscle tissue.[15]

In relation to myogenesis, it is important to distinguish between embryonic and adult myogenesis. During fetal development, satellite cells, a heterogeneous group of stem cells and progenitor cells, are generated and positioned around myofibers.[16] These skeletal muscle satellite cells are mononucleated and located between the sarcolemma and basement membrane of terminally differentiated muscle fibers. They can be characterized by their Pax7 expression.[17,18] In adult skeletal muscle tissue, satellite cells is the main resource and participant in myofiber repair, homeostasis and senescence. Due to asymmetric division these cells are capable of self-renewing, but also generate differentiated progeny through asymmetric division.[19] The satellite cells are mitotically quiescent and have limited expression of genes and production of proteins, but in response to stress, e.g. weight bearing or traumas, several signal pathways trigger activation the satellite cells.[20] Upon activation the satellite cells move outside of the

basal lamina, and divide multiple times, giving rise to a new cell population, the skeletal muscle myoblasts, expressing Myf-5, MyoD and myogenin. In turn, these myoblasts will differentiate as result of these MRFs and fuse to form multinucleated myofibers, similar to what happens during embryonic myogenesis. Some myoblasts will cease expressing MRFs, leave cell cycle and regain their Pax7 expression, thus becoming quiescent satellite cells once again.[21]

In Vitro Research on Satellite Cells Progeny, the Myoblasts

Myoblasts are capable of undergoing myogenic differentiation when cultured *in vitro* provided that they are stimulated properly. As mentioned earlier, myoblasts can be maintained in a differentiated state, fuse and synthesize specific muscle proteins, similar to what occurs occur *in vivo* during muscle repair and maintenance. Many new approaches of *in vitro* models has been developed to increase and improve the understanding of *in vivo* conditions of myogenesis. Among these are e.g. *in vitro* engineering of skeletal muscle tissue[22,23] and electrical stimulation.[24] Mechanical strain is another important factor influencing myogenesis *in vivo*[25], thus an interesting subject to investigate *in vitro*.

Mechanotransduction and Sensation of Surrounding Environment

One of the big diversities between myogenesis *in vivo* and *in vitro* is the environment surrounding the cells called the extracellular matrix (ECM). The ECM provides structural support to cells in different tissues, but also supports a series of several other important cell functions, such as adhesion spreading and migration.[26] The native ECM in muscle tissue consists of various different, highly complex and tissue specific proteins and polysaccharides.[27] Cells respond in many different ways to signals provided from their native environmental cues. Signals intercepted by the cells are typically of chemical, mechanical or topographical origin, and in combination these stimuli invoke responses affecting key processes like growth, differentiation and programmed cell death.

Myogenic differentiation of myoblasts require a specific combination of environmental signals in order to transform into mature myotubes. Chemical signals, such as growth factors and several signalling molecules, reside in the ECM and are involved in maintenance of muscle tissue, but do also trigger the cells during tissue damage and repair. Thus, integration of these signals are important when myoblasts are cultured *in vitro* in order to mimic the native conditions. An example is choice of medium composition, which, when supplemented with mitogens and growth factors, stimulate myoblast proliferation or deprived of these reach cell cycle arrest and initiate differentiation.[28] Other extracellular stimuli interfere with the development of the growing and differentiating myoblasts, and one such stimulant is the surface on which the cells adhere. Coating the culture surfaces with ECM proteins often serves to induce chemical signals affecting differentiation, though less is known whether the coating also induce topographical signals to the cells. Because of the complex nature of ECM in native skeletal muscle tissue, *in vitro* studies often settle for a single purified protein as surface-coating. Collagen is a main component in skeletal muscle tissue and undoubtedly involved in myogenic differentiation, hence a frequently used coating protein. Myoblasts are also capable of perceiving and processing external mechanical stimuli through focal adhesion complexes. For instance, elongation of bones during embryonic development applies a passive stretching of myoblasts and result in myogenic differentiation. Adhesion molecules are one of the key elements in understanding how the cells sense and interact with their surroundings. During myogenic differentiation myoblasts begin synthesising additional adhesion proteins protruding through the membrane and linking the ECM to the cytoskeleton and intracellular signalling molecules.[15,26] Integrins, a family of cell surface receptors and some of primary sensors of extracellular ligands. Integrin-mediated signalling results in downstream activation of adaptor proteins called focal adhesion kinase (FAK) and Rho GTPases. When stimulated by their ligands integrins regulate the activity of MRFs through FAK signalling cascades.[29,30] Moreover, FAK signalling is a key activator of downstream signalling cascades, that are responsible for assembly of focal adhesion complexes. Focal adhesion complexes are sites with clustering of integrins in which the cell connect and anchor to the ECM.[29] Integrin interactions with Rho GTPases are crucial for phosphorylation of FAK and the assembly of focal adhesion complexes, but are also important in myogenic differentiation.[31] Active

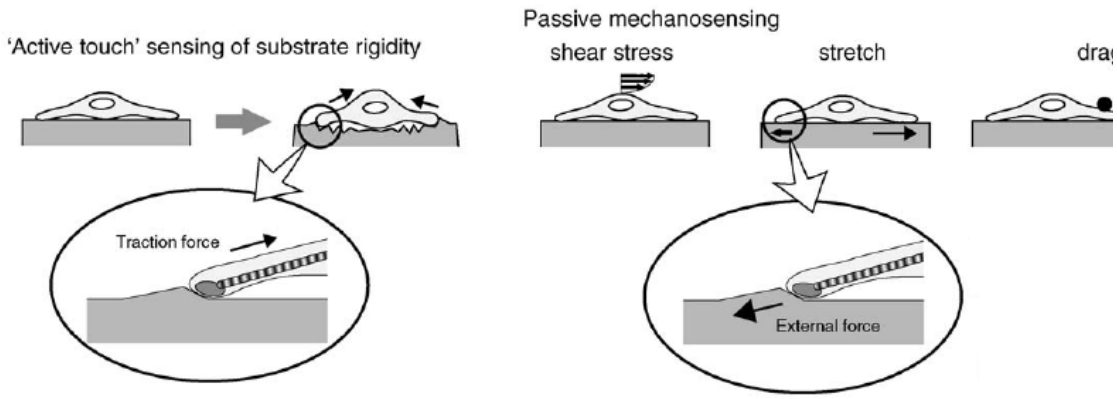

Fig. 3. Schematic illustration of mechanosensing. 'Active mechnosensing' where the cell probe and 'feel' the underlying substrate and its stiffness and passive mechanosensing caused by external factors, like for instance strain or fluid shear

RhoA is essential for expression of MyoD and key differentiation factor serum response factor (SRF)[32], whereas other Rho family members are involved in organisation of the actin skeleton during mechanosensing and migration.[29]

Recent science highlights the importance the rigidity of the substrates on which the cells are grown and differentiated.[33] Migration, proliferation and differentiation, among others, are influenced by substrate rigidity.[34,35] Substrates with intermediate stiffness are thought be ideal for muscle tissue differentiation, while the more rigid substrates are suited for osteogenic differentiation and softer substrates for neurogenic differentiation.[36,37] Thus, the cells seems to be able to "sense" and dynamically adapt to their environmental cue. Mechanosensing of external mechanical stimuli, such as stretching, are termed passive mechanosensing, but when the cells are probing the substrate rigidity via actomyosin motors in stress fibers and their connected focal adhesion complexes it is termed active mechanosensing[36], se Figure 3. Therefore, it seems that both external forces, both stretching and substrate rigidity, plays quite an important role in both growth and differentiation of myoblasts and is an interesting subject to explore and validate.

Potential of Mechanical Stimulation of Myoblasts

To orientate and enhance differentiation of myoblasts, several different approaches have been used. Myotube formation can take place on substrates of various stiffness, however, by culturing of myoblasts on substrates with an elastic modulus ranging from 8 to 17 kPa, it is possible to enhance myogenesis.[37] At such stiffness, Ca^{2+} signaling, important in regulating the actomyosin contractility, is considerably higher than in substrates of less stiffness.[38] Additionally, Ca^{2+}-dependant cell-cell adhesion molecules are important in fusion of myoblasts.[39] Interestingly, external mechanical stimulation are considered ideal to orient and differentiate myoblasts into mature myotubes.

In vivo, an obvious example of the effect of mechanical stimulation is physical exercise, which causes muscle hypertrophy while immobilization causes atrophy.[40] To mimic the principle of these effects *in vitro,* different methods have been developed. Elastic silicon substrates designed for growth and differentiation of myoblasts makes it possible to apply stain to the cells.[41] Types of strain used is commonly of uniaxial and equiaxial origin, depending on the directionality of the tensile forces. At a glance, the directionality of the strain may not seem that important, but cells are believed to be capable of distinguishing between uniaxial and equiaxial strains.[42] An advantage of applying cyclic uniaxial strain is that the cells orientate homogeneously perpendicular to the direction of strain, thus aligning the cells.[43,44] This reorganization of the cells possibly occur to minimize the forces afflicted to the cells. Stretching of the substrate upon which cells grow extends stress fibers beyond their average length. The cells respond by actively rearranging their cytoskeleton striving to return the optimal tension of the stress fibers.[10,43] When utilized *in vitro* it becomes possible to align the cells in parallel to each other. Though this scenario is present when cells are cultured *in vitro* the same might not be the exact case *in vivo*, where the ECM consists of a 3D meshwork in contrast to the 2D environment of a culture substrate. When cells subjected to uniaxial tensile strain is cultured in a 3D environment, they arrange accordantly to the direction of the strain, suggesting that use of scaffolds are required to attain results closer to native conditions.[45]

Various cell types, including mesenchymal stem cells, fibroblasts and the aforementioned C2C12 mouse myoblasts, exhibits an enhanced myogenic profile when subjected to strain.[46,47] Few studies investigate the effect of cyclic strain on primary animal myoblasts, whereas straining of primary human myoblasts is left unexplored.

Aim

As most of the research regarding the effect of uniaxial cyclic tensile strain (CTS) has been performed on the C2C12 cell line, the objectives of this report is to investigate a similar model conducted on primary human skeletal muscle myoblasts. The purpose is to validate and compare the positive effect of assembly and differentiation on C2C12 cells when applied to human skeletal muscle myoblasts (HSMM). The basis of the comparison will be on the expression of key factors, such as Myf-5, MyoD, myogenin and MHC, involved in the transformation from myoblasts into myotubes. Therefore, the hypothesis of this study is that uniaxial CTS has significant positive effect on alignment and differentiation of C2C12 as well on HSMM. We will be utilizing fluorescent staining and reverse transcriptase-real time polymerase chain reaction (RT-qPCR) to detect specific myogenic markers and their mRNA expression levels.

Materials and Methods

Cell Culture

C2C12 cells, derived from a murine myogenic cell line, were acquired from American Tissue Type Culture Collection (LGC Standards, Sweden) and cultured in growth medium consisting of 89% Dulbecco's Modified Eagle Medium, 10% fetal bovine serum, 0.5% penicillin/streptomycin, and 0.5% gentamycin (Invitrogen). Human skeletal muscle myoblasts (HSMM; Lonza Walkersville, USA) were grown in growth medium consisting of 84% F12 nutrient mixture (HAM), 15% fetal bovine serum, 0.5% penicillin/streptomycin, 0.5% gentamycin, 10 ng/ml human recombinant epidermal growth factor, 1 ng/ml fibroblast growth factor, and insulin. Differentiation medium consisted of DMEM supplemented with 2% heat-inactivated horse serum, 0.5% penicillin/streptomycin, and 0.5% gentamycin, independent of cell line. Cultivation of cells was performed in T175 culture flasks. C2C12 cells were passaged when confluence reached 80-90% and HSMM at 50-70%, which corresponds to approximately every 3-4 days. HSMM from passages 2 to 4 and C2C12 from passages 3 to 4 were seeded in 6-well flexible-bottom Bioflex culture plates precoated with collagen-I (Flexcell International Corporation, #BF-3001C). Seeding density for C2C12 and HSMM was approximately 5.000 cells/cm^2 and 10.000 cells/cm^2, respectively. When cells were seeded for PCR, a custom designed seeding chamber was used, so only mRNA from cells affected directly by the CTS were being harvested. When cells grew 90% confluent, cell differentiation was initiated by substituting growth medium with differentiation medium (day 0). Differentiation medium was replaced every 2 to 3 days.

Mechanical Stimulation

Subsequently to differentiation induction at day 0 the cells were subjected to mechanical stimulation for 48 hours using a stretching device, Flexcell FX-5000 Tension System (Flexcell International Corporation). The mechanical stimulation regime was CTS achieved through rounded rectangular pistons and vacuum suction of the

membranes of the pre-coated flexible-bottom culture plates. The mechanical stimulation procedure consisted of semi-sinusoidal tensile strain pulses with a peak amplitude of 15% at 0.5 Hz. To prevent the influence of CTS to control cultures valved rubber inserts were used.

Cell Staining

Prior to staining, cell samples were washed with phosphate-buffered saline solution (PBS) and fixated in 4% formaldehyde for 30 minutes. Nuclei were stained by incubation with Hoechst 33342 (Molecular Probes®, #H-3570) diluted in PBS (1:1000) for 30 minutes. Cells were permeabilized with 0.1% Triton X-100 in PBS for 5 minutes and subsequently blocked with 2% bovine serum albumin in PBS for 30 minutes to prevent unspecific binding of antibodies. A primary anti-myosin monoclonal antibody (1:500; Clone MY-32; Sigma-Aldrich®, #MFCD00145920) was labelled with Zenon Mouse IgG Labeling Kit (Alexa-fluor 647 Mouse IgG1; Invitrogen™, #Z25008).

Staining of skeletal muscle MHC and myogenin was performed using a solution containing Zenon-labelled primary anti-myosin monoclonal antibodies (1:500) and Alexa Flour® 488 conjugated monoclonal anti-myogenin antibodies (1:100; eBioscience®, #53-5643-82). The staining solution was incubated with the samples for 45 minutes, followed by a second fixation in 4% formaldehyde for 15 minutes. F-actin was stained by incubation in Bodipy 558/568 Phalloidin (1:40; Invitrogen™, #B-3475) for 20 minutes. The samples rinsed in PBS and stored in PBS at 4°C until observation time.

Microscopy, Image Analysis and Counting

Phase contrast and fluorescent images were obtained with a AxioCam MRm and a Zeiss Axio Observer.Z1 microscope (Carl Zeiss). The software AxioVision rel. 4.7 (Carl Zeiss) were used to image the samples. For each sample 9 mosaic (2x2) images were taken at 10x magnificationin the centre of the area of strain and used for analysis of cell

orientation and cell counting. Directionality and alignment of the cells were analysed using a Directionality plugin for Fiji/ImageJ (National Institutes of Health) on images of actin filaments. Two blinded independent skilled observers used Cell Counter plug-in for ImageJ to quantify myotube formation and grade of differentiation. The percentage of nuclei in myosin positive myotubes were calculated from the total number of nuclei inside myosin-positive myotubes in the myosin channel divided by the total amount of nuclei present on the Hoechst channel. The percentage of myogenin-positive nuclei were calculated from the total amount of myogenin-positive nuclei in the myogenin channel divided by the total amount of nuclei in the Hoechst channel. High magnification images were captured at 64x magnification to analyzed the existence of actin and myosin cross-striations using a tool in AxioVision to measure pixel intensities of the actin and myosin channels.

RNA Purification and cDNA Synthesis

For each time point, day 2, 5, 8 and 11, for the cell cultures, the cells were harvested and mRNA extracted using Aurum™ Total RNA Mini Kit (Bio-Rad, #732-6820) and cDNA prepared using an iScript™ cDNA Synthesis Kit (Bio-Rad, #170-8891) both according to manufacturers protocol. mRNA and cDNA concentrations were measured using NanoDrop® ND-1000 Spectrophotometer (Fischer Scientific). cDNA was synthesized from approximately 150 ng for each sample determined by the smallest mRNA concentration.

Primer Design and Optimization

Primer3 software (v. 0.4.0; SourceForge.net®) was used to design two primers, forward and reverse, from sequences of human Myf-5, MyoD1, myogenin and MYH2 genes. In the same way were two primers from sequences of mouse genes made for each Myf-5, MyoD1, myogenin and MYH2. A pair of primers were designed for housekeeping genes PPIA and YWHAZ from both human and mouse genes. The primers were ordered

and delivered from DNA Technology A/S (Risskov, Denmark). Oligo Analysis Tool (Eurofins MWG Operon) was used to check for potential primer dimer. Each primer was tested for annealing specificity using NucleotideBLAST (NCBI) and assure annealing to desired product, E value < 0.0004. Optimal PCR temperatures and conditions were determined for all primer sets.

Real-Time Reverse Transcription-Polymerase Chain Reaction

A reaction mix of 25 µL containing IQ SYBR® Green Supermix (Bio-Rad, #170-8882), cDNA (diluted 1:50 in MilliQ water) and 10 pmol primer was mixed for each sample. The final reactions and blanks, as control, were aliquoted in doublets and carried out in a sealed PCR-plate. The plates was run in a 2-step real-time qPCR program using a MyiQ5 Single Color Real-Time Detection System (Bio-Rad). The program was set to following: Initial denaturation for 3 min at 95°C followed by 50 amplification cycles for 15 sec at 95°C and 30 sec at the specific annealing and extension temperature of the individual primer. To confirm product specificity a melting curve analysis was performed simultaneously. A fourfold serially standard curve, consisting of cDNA from all samples, was used to calculate the relative expression for each gene. The expression values of each gene was normalized to the geometric mean calculated the expression of housekeeping genes PPIA and YWHAZ.

Statistical Analysis

To ensure reproducibility of the experiment, two biological replicates were made for the counting analysis. Statistical analysis were performed using SPSS Statistics 19 (IBM). Differences in orientation angle were tested using the non-parametric Kolmogorov-Smirnov test. Comparison of differences in counting results between groups were performed using one-way ANOVA. Unequal variances were assured and confirmed by Levene's homogeneity-of-variance test. Tamhane's T2 test were used as post-hoc comparison among the groups.

Results

Myotube Orientation and Alignment

Cells from day 0 displayed a dense monolayer of undifferentiated myoblasts whereas at day 2 the cells displayed a more elongated morphology and minority of the myoblasts were starting to fuse into multinucleated myotubes. Cells allowed to differentiate for 5 days contained more multinucleated myotubes and fewer myoblasts. Orientation of HSMM myotubes were analyzed in reference to strain direction and compared to a similar model performed on C2C12 myoblasts. Representative inverted phase contrast images displaying cell distribution and orientation are presented in Figure 4a. At day 0 both strained and control samples displayed a random cell orientation. Immediately after the end of 48 hours of stretching (day 2) both HSMM and C2C12 subjected to uniaxial CTS displayed an normal distribution on the orientation around a perpendicular angle to the direction of strain. The same findings applied to all stretched cells at day 5 and 8 of differentiation. For the control cells at all time points of differentiation a random distribution of orientation angle was found, similar to that of day 0. The histomorphometric analysis showing that strained cells align in 90° angle to the direction of strain are displayed in Figure 4b.

To compare the difference in distributions between groups for each cell line individually, Independent Samples Non-Parametric Kolmogorov-Smirnov test was performed. Statistical significant difference was found between C2C12 control and CTS conditions ($p<0.001$) and between HSMM control and CTS conditions ($p<0.001$) demonstrating a difference in distribution. To compare the effects of CTS on alignment between the two cell lines the kurtoses were calculated. C2C12 control cells had a platycurtic appearance with a kurtosis value of -1.405, as did HSMM control cells with a kurtosis value of -0.758. Both strained C2C12 and HSMM had a leptocurtic appearance with kurtosis values of 0.830 and 1.923 respectively.

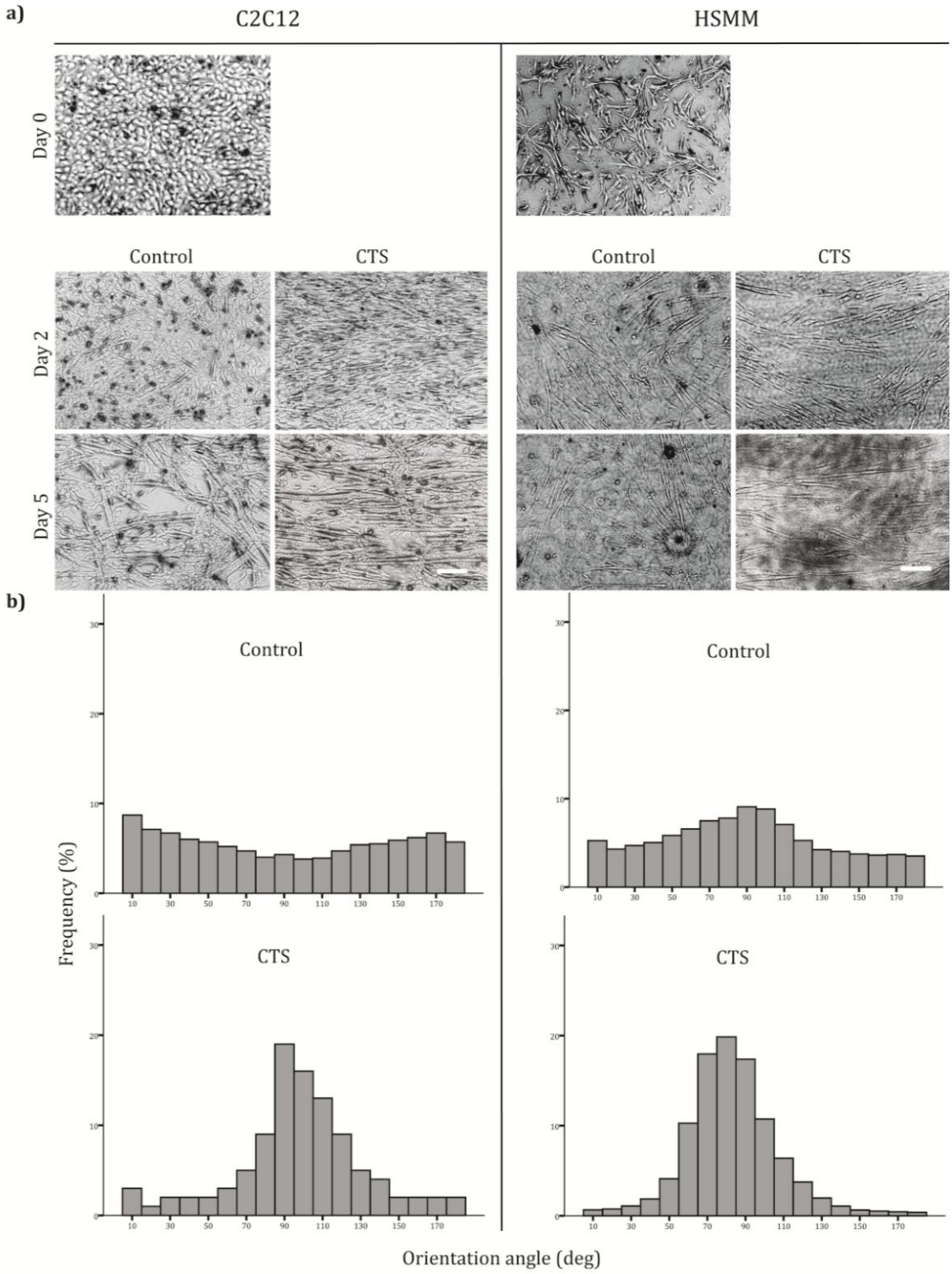

Fig. 4. Comparison of strain on cell orientation and morphology. **(a)** Inverted phase contrast images showing C2C12 and HSMM after 0 (start of induction/stretching), 2 (end of stretching) and 5 days of differentiation under different conditions. Scale bar denotes 200 µm. **(b)** Histograms displaying the percentage of cell counted in a given angle interval throughout 9 images from the same sample at 5 days of differentiation. 90° represents the perpendicular angle to the direction of uniaxial cyclic tensile strain.

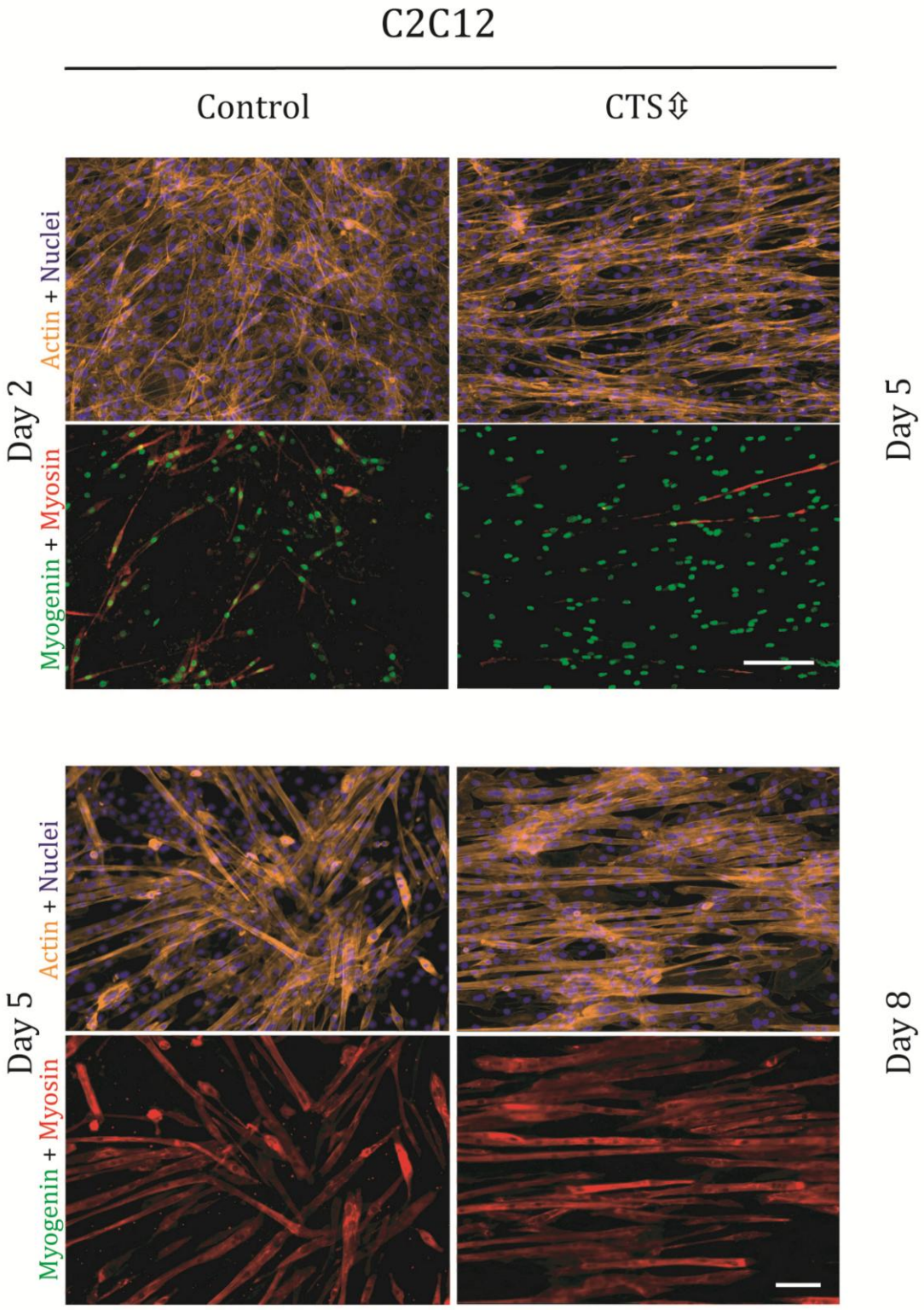

Fig. 5. Representative fluorescent micrographs of cells following uniaxial cyclic tensile strain and their corresponding controls. Cells are stained Hoechst 33342 nuclear stain (blue), Bodipy 558/568 phalloidin (orange), Alexa Fluor 488 anti-myogenin antibody (green), and Alexa Fluor 647 anti-myosin antibody (red). Scale bar denotes 200 μm.

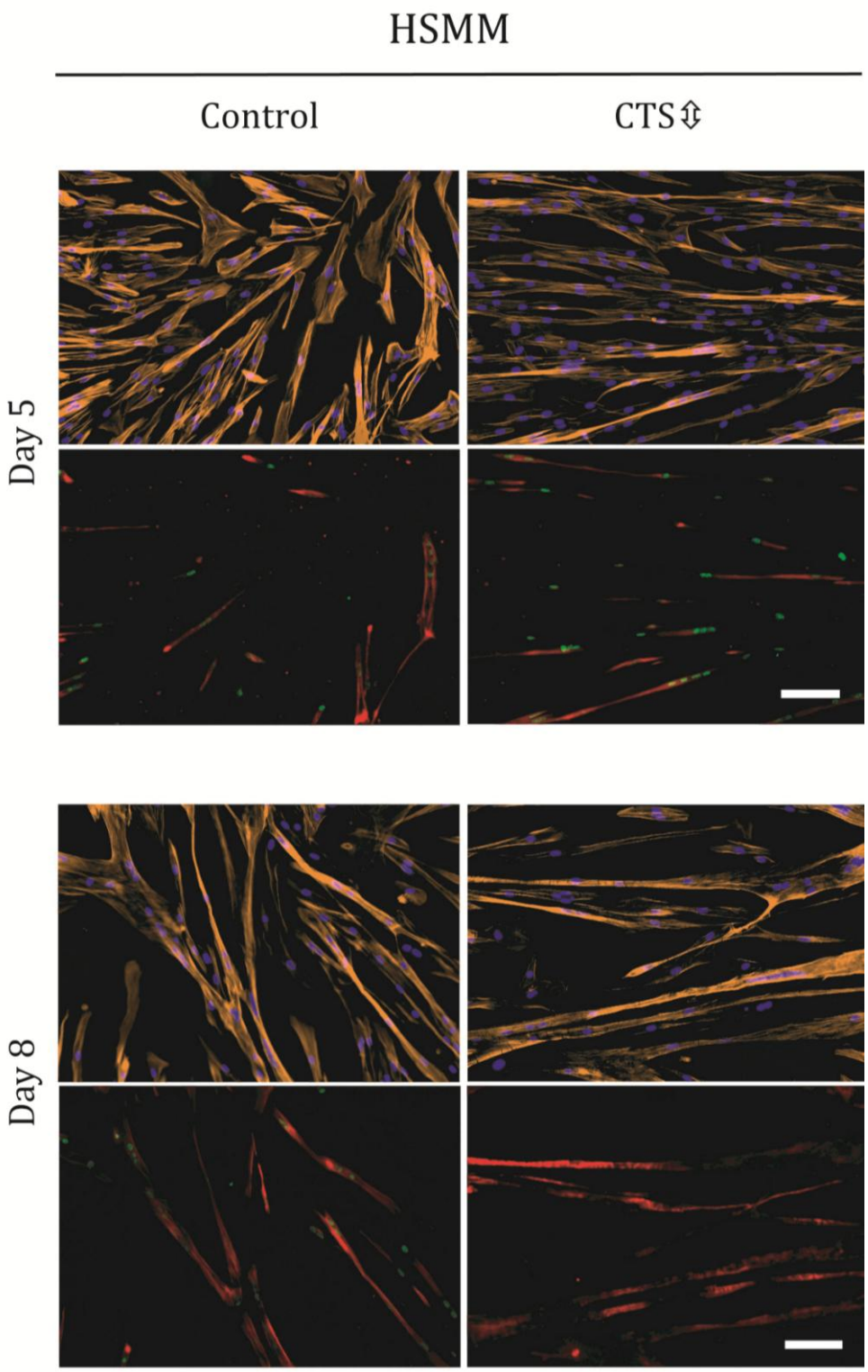

Fig. 6. Representative fluorescent micrographs of cells following uniaxial cyclic tensile strain and their corresponding controls. Cells are stained Hoechst 33342 nuclear stain (blue), Bodipy 558/568 phalloidin (orange), Alexa Fluor 488 anti-myogenin antibody (green), and Alexa Fluor 647 anti-myosin antibody (red). Scale bar denotes 200 μm.

Myogenic Differentiation and Myotube Formation

An early marker of myogenic differentiation, myogenin, and two major components of the sarcomere, actin and myosin, were analyzed *in situ*. HSMM samples at 2 and 5 days of differentiation were compared to an identical model performed on C2C12 myoblasts, but due to lack of evident presence of myogenin and myosin at day 2 of differentiation, another time point, day 8, was added to the HSMM model. After 48 hours (day 2) of stretching the alignment of the cells perpendicularly to the direction of strain is evident, but also continues to be maintained throughout an ongoing period of differentiating. Representative micrographs displaying both C2C12 and HSMM stained for nuclei, myogenin, actin and myosin are presented in Figure 5 and 6. In the C2C12 samples from day 2 a state of early differentiation was observed, with many cells displaying elongated cell morphology and alignment in parallel bundles, even with some degree of fusion present in both control and CTS samples. Many nuclei contain myogenin at this point. At day 5 of differentiation the C2C12 the extent of myotube formation is clear. Myotubes are present both in control and CTS samples, but at day 5 myogenin present are reduced to almost nothing for both conditions. Since the HSMM samples were excluded from day 2, due to lack of both myogenin and myosin, the comparison will be based on samples from day 5 and 8. Both day 5 and 8 samples display the same orientation as found for the C2C12 samples, though the density appears lower for the HSMM. The morphological shape of the HSMM at day 5 are like the C2C12 elongated. A minority of the myoblasts have fused to myotubes containing myosin and myogenin is present in most nuclei involved in myotubes. At day 8 more myoblasts have fused to myotubes containing myosin.

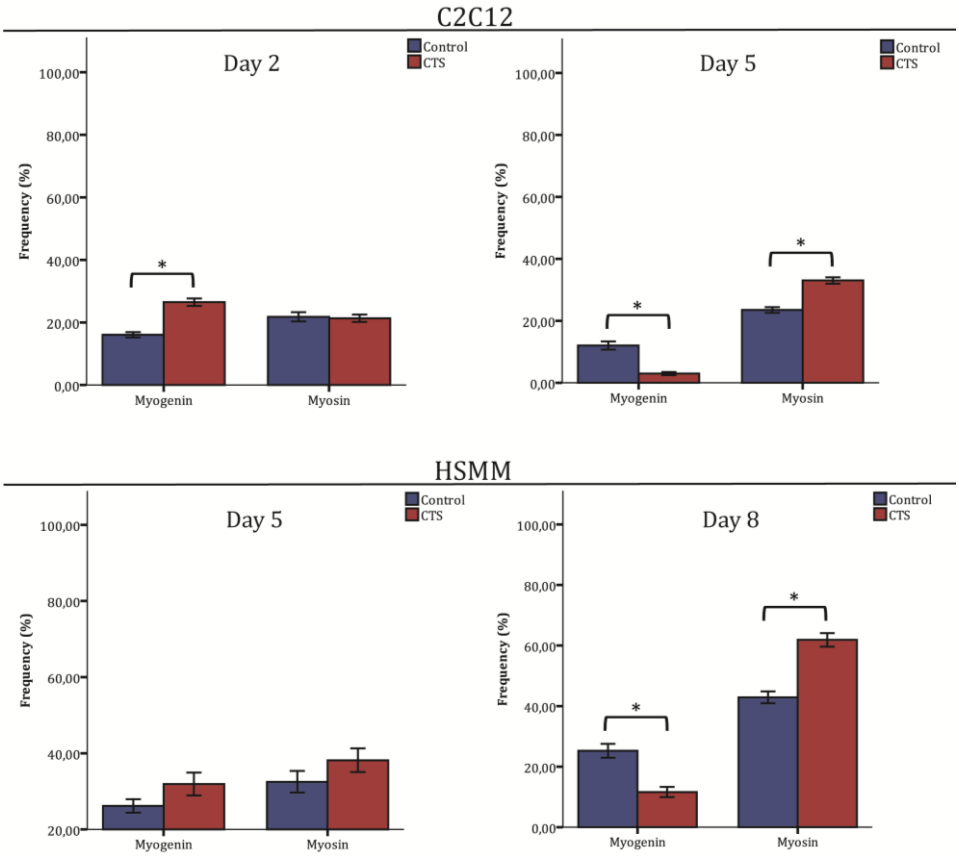

Fig. 7. Quantitative analysis of cell differentiation. C2C12 myoblasts were analyzed at day 2 and 5. HSMM cells were analyzed at days 5 and 8. Values are displayed as mean ± standard error of the mean. Statistically significant differences are indicated by an asterisk ($p<0.05$)

Quantitative analysis of differentiation revealed a stitistical significant higher presence of myogenin-positive nuclei in stretched group of C2C12 at day 2 than the control group ($p<0.001$), showed in Figure 7. In contrary the CTS group contained less myogenin-positive nuclei than the control group for the C2C12 ($p<0.001$). There was not found any significant difference between the percentage of nuclei inside myosin-positive myotubes between control and CTS groups for day 2, but at day 5 the CTS group had significant higher percentage of nuclei inside myosin-positive myotubes than the control group ($p<0.05$). HSMM control and CTS groups did not reveal any significant difference in both percentage og myogenin-positive nuclei or nuclei inside myosin-positive myotubes at day 5, Figure 3b. At day 8 the CTS group contained significantly lower percentage of myogenin-positive nuclei than the control group ($p<0.05$), 11% compared

to 25%. In addition the percentage of nuclei inside myosin-positive myotubes was significantly higher for the CTS group ($p<0.05$), surpassing 60%, compared to the control group only reaching just above 40%.

Actin and Myosin Cross-Striations

In samples of C2C12 from day 5 and samples of HSMM from day 8 some matured myotubes displayed sarcomeric structures. Representative images of cross-striations in both C2C12 and HSMM control and CTS groups are presented in Figure 8a. The actin and myosin channels each shows the aligned striated fibers which fits another in between the gaps. When pixel intensities measured on a single line across a striated fiber the pixel intensities for both actin and myosin peaks for every 2.5-3 µm, showed in Figure 8b.

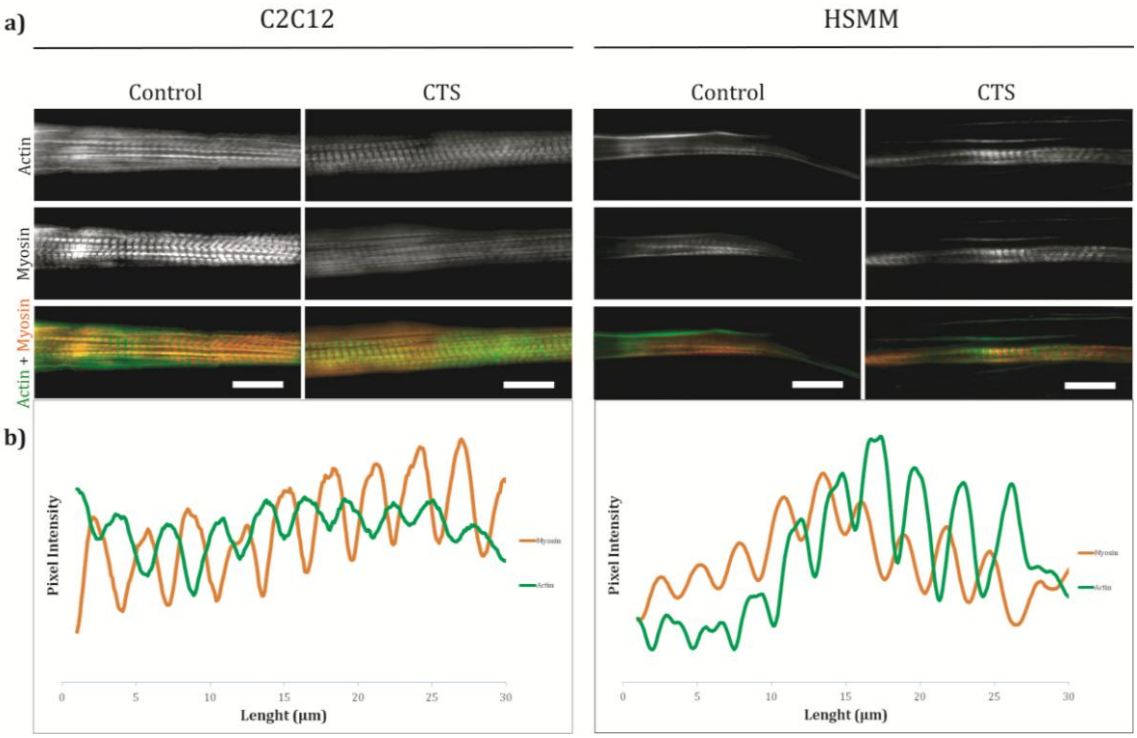

Fig. 8. a) High-magnification micrographs showing actin and myosin cross-striations. **b)** The graphs are representative of both control and CTS cells and display the frequency and interleaved pattern of the striations. Scale bar denotes 20 µm.

RT-qPCR of HSMM

RT-qPCR analysis results are displayed in Figure 9. Myf-5 expression, after being normalized to housekeeping genes, shows a decrease in both control and CTS conditions in days succeding day 0. The decrease almost follows a exponentially decreasing pattern as the differentiation process goes on. Error bars indicate that the biological replicates closer to being identical. No statistical differences was found in between the two conditions during the differentiation process from day 0 to 11 for Myf-5. MyoD1 expression at day 2 control and day 5 CTS show huge standard deviations, but at day 8 and 11 the expression levels give more reliable outcomes, being just below that of the day 0 baseline. At day 11, there is a tendency that controls express more MyoD1 than CTS, though no significant differences could be confirmed due to the non-parametric nature of the underlying data. Myogenin expression during ongoing differentiation show an initially low expression at day 0 baseline, 2 and 5 after which expression levels increase above 5-fold, except from day 8 CTS. Notably, most error bars for late differentiation time point of myogenin expression are huge and complicate proper analysis between different time points and conditions. Similar to myogenin expression, levels of MYH2 expressed at day 2 and control do not deviate from baseline at day 0. When reaching 8 and 11 days of differentiation MYH2 expression increases above 5-fold, but, as seen for myogenin expression, huge error bars complicate comparison and analysis of this late expression peak. Both data sets for myogenin and MYH2 expressions show no significant difference between condition groups at any time of differentiation, due to their non-parametric nature. In general for expression of the myogenic markers of differentiation, except Myf-5, is, that the data sets contain relative large gaps in between biological replicates in groups with same conditions, thus rendering an actual comparison of myogenic marker expression impossible.

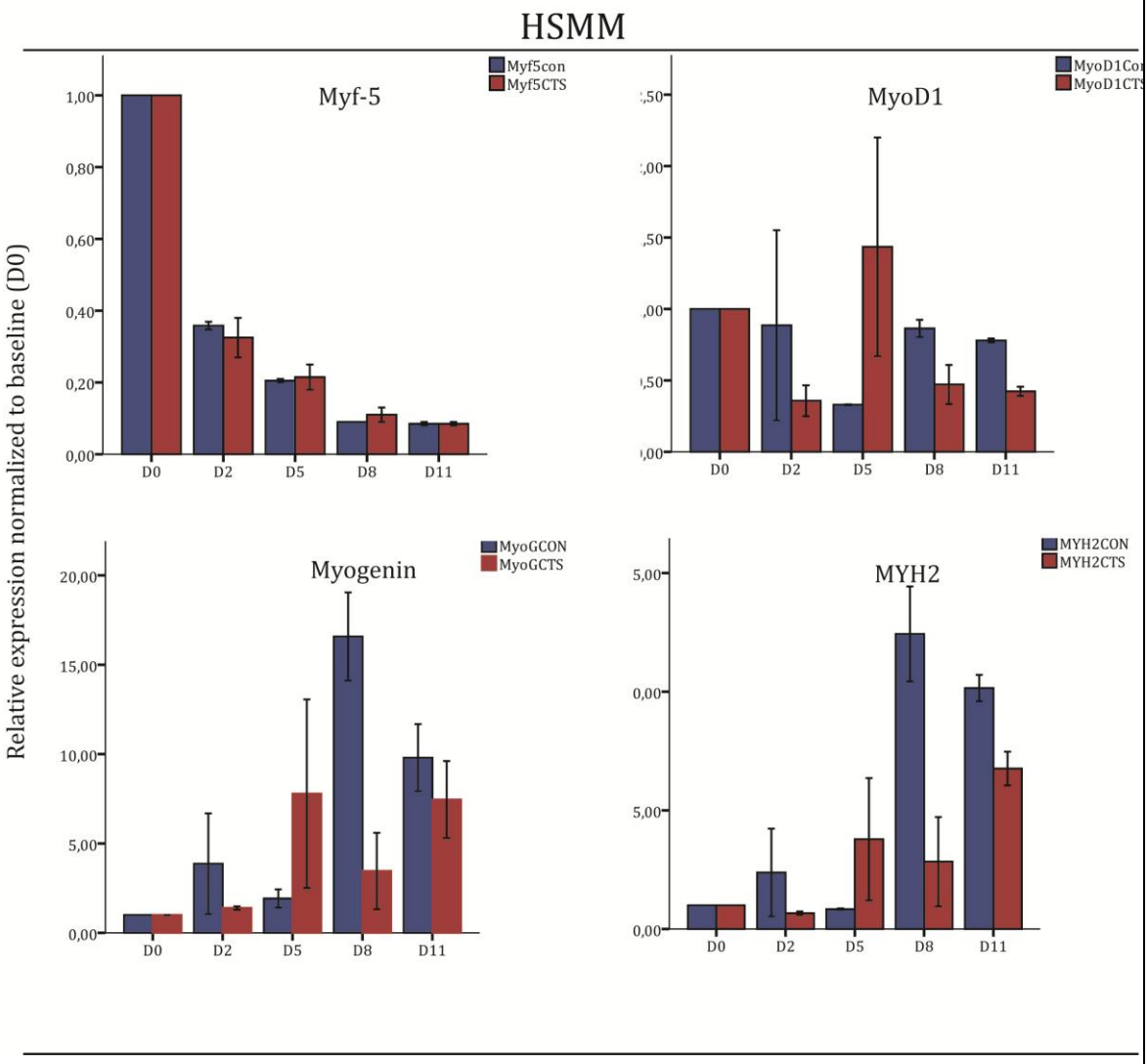

Fig. 9. HSMM relative expression patterns of Myf-5, MyoD1, myogenin and MYH2 after being normalized to day 0. Geometric mean of PPIA and YWHAZ was used to calculate expression levels. Error bars is ± SE of mean.

RT-qPCR of C2C12

Expression of myogenic markers Myf-5, MyoD1 and myogenin is displayed in Figure 10. Myf-5 expression for CTS groups initially increases slightly, but drop to below baseline at day 5. Control groups decrease from day 0 and onward as differentiation progresses. MyoD1 expression increases for both condition groups to above 5-fold at day 5. Myogenin expression increase to above 10-fold already at day 2 and maintain the expression throughout day 5. Expression of MYH2 are not displayed, because primers

for this specific marker turned out not being specific, thus being excluded for unreliable outcome.

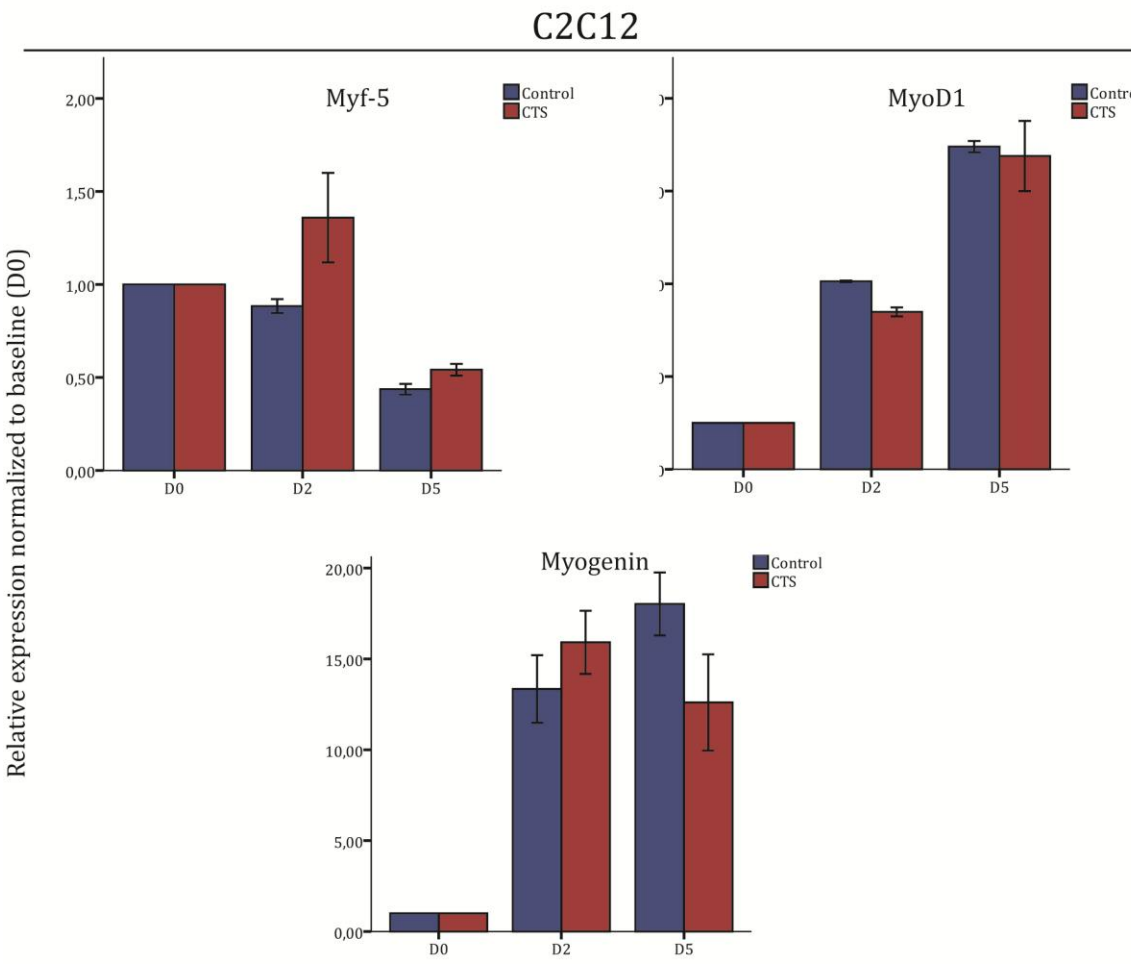

Fig. 10. C2C12 relative expression patterns of Myf-5, MyoD1, myogenin and MYH2 after being normalized to day 0. Geometric mean of PPIA and YWHAZ was used to calculate expression levels. Error bars is ± SE of mean.

Discussion

Uniaxial Cyclic Tensile Strain Align and Orientate Myoblasts

Both C2C12 and HSMM subjected to uniaxial tensile strain tends to align and orientate perpendicular to the direction of strain, showed in Figure 4, whereas nonstrained control cells, as showed in Fig. 1. The reason for this behavior is connected to the rearrangement of the cytoskeleton in attempt to return the optimal tension of stress fiber after being stretched beyond average length from straining forces. This is a phenomenon previously observed when cells cultured in a 2D environment are affected by tensile strain forces.[43] Ahmed *et al.* [44] found that myoblast orientate approximately in a 70° angle perpendicular to direction of strain. In contrast to the strain applied in this work (15% at 1 Hz for 48 hours) Ahmed *et al.* did use only 7% at 0.5 Hz 96 hours. This finding suggests, that the higher level of strain applied, and not the duration of staining period, have a bigger impact in aligning the cells perpendicular to the direction of strain. It has also been suggested, that parallel alignment of myoblasts enhance fusion through a side-by-side fusion. Aligning of myoblasts and an increased count of nuclei inside myotubes in CTS groups compared to control groups observed in this work, cannot be conclusively connected to each other, because it is unknown whether this effect is caused by mechanical stimuli or alignment or a combination of all. Similar for the mouse and human cell lines utilized in this work, is that mechanical stimulation that they both seems align and orientate perpendicularly to the direction of strain, but differs in how well they orientate around 90°, as given by their kurtosis. With a steeper distribution around 90°, a kurtosis value of 1.923, more HSMM cells tends to orientate perpendicularly to the direction of strain compared to C2C12, with a kurtosis value of 0.830, and a more widely distribution around 90°. The essence of this finding is that mechanical stimulation induce alignment and rearrange the cells in response to CTS independent on whether the cells are of mouse or human origin.

Myogenic Differentiation profile of C2C12 and HSMM

Since little is known about the effect of CTS on a primary human myoblast cell line, we chose to compare an integrated model performed on mouse C2C12 with primary HSMM. The interpretation on the effect of CTS will be carried out on the C2C12 succeeded by a comparison on similarities and diversities will be made to see if this model of mechanical stimulation is applicable to HSMM as well.

CTS Did Not Influence Myf-5 Expression Patterns Successfully

As showed in Figure 10, Myf-5 expression displays an initial maintenance at day 2 and a following slight decrease at day 5. Independent on whether mechanical stimulation was applied or not, no significant differences between condition groups was detected. One thing to point out, is that the percentage of nuclei inside myosin-positive myotubes is around 20% at day 2 and slightly increased at day 5. This indicate that the most of the nuclei left must be mononucleated myoblasts and these could still be at an early stage of differentiation, therefore still producing Myf-5 transcripts. This would also explain the slight decrease in Myf-5 expression at day 5, as nuclei inside myosin-positive myotubes increases. A maintained Myf-5 expression do not match findings of others. Lindon *et al.*[48] report of a downregulation occurring as early as at the onset of differentiation. Lindon *et al.* also reports that Myf-5 expression decreases when myogenin expression is introduced. Since myogenin already is introduced at day 2 for this thesis, the maintained expression of Myf-5 do not make sense. Previous studies by Abe *et al.*[49] found significantly different mRNA expressions in stretched C2C12 cultures (15% stretching, intensity 2 Hz) after 12 hours of stretching in differentiation medium, but not after 24 hours and further on. Considering that Myf-5 is the earliest emerging MRFs, even present in proliferating myoblasts, a difference in Myf-5 mRNA expressions might be detectable if CTS, through Rho GTPase and FAK mediated signaling, could induce earlier onset of differentiation within 12 hours. Whether CTS can induce such a response in C2C12 remains unknown for now. HSMM cultures displayed an almost exponentially decreasing Myf-5 expression from day 0 baseline until it reaches almost

1/10 at day 11, as showed in Figure 9. This pattern was not consistent with the findings for C2C12, but can maybe be partly explained by the higher percentage of nuclei inside myosin-positive myotubes for the HSMM compared to C2C12. Higher amount of nuclei inside myosin-positive myotubes would mean increased amount of surrounding myoblasts, that still might be in an early stage of differentiation. Just like the C2C12, no significant difference were found in between CTS and control groups at all time points. Interestingly, the pattern of Myf-5 expression in HSMM cultures seems to agree with what Lindon *et al.* reported, while C2C12 did not manage to.

CTS Did Not Influence MyoD1 Expression Patterns Successfully

Another early marker of myogenic differentiation, MyoD1, increased stepwise as differentiation continued at day 2 and 5 for both CTS and control groups. Our findings, showed in Figure 10, did not show any significant difference in between condition groups over 5 days of differentiation, though CTS groups tends to be express less MyoD1 than control groups at both day 2 and 5. The stepwise increase of MyoD1 expression may be caused by a mix of myotube and myoblast transcripts owing to the high amount of myoblasts still not fused to myotubes as seen for the Myf-5 expression. If the remaining myoblasts have entered an early differentiation state an initial peak in MyoD1 expression might be found, similar to findings of others.[50] Though, there does not seem be an agreement in literature, because others find that MyoD1 expression is maintained in differentiating myoblasts[51] and be *et al.*[49] show that MyoD expression, in cells are subjected to mechanical stretching, decreases immediately after differentiation induction. They also found significantly higher expression of MyoD in stretched groups compared to control groups within the first 12 hours of differentiation, but none in the following 36 hours. MyoD1 expression in HSMM cultures, showed in Figure 9, did not show the same incremental increase as showed for C2C12. On the other hand, the expression appears decreasing throughout the differentiation period compared to the day 0 baseline. Given the huge error bars displayed on several data sets, comparison in expression patterns between C2C12 and HSMM cannot be accomplished before additional biological replicates have been repeated.

Myogenin Peaks Earlier in Cells Subjected to CTS

On the basis of the counting analysis of the fluorescence micrographs, showed in Figure 7, a significant different myogenin profile between C2C12 CTS and control groups at both day 2 and 5 is detectable. As early as day 2, significantly elevated levels of myogenin was observed in CTS groups compared to the control groups. This early peak in myogenin activity can be connected to the following increase in nuclei included in myosin-positive myotubes at day 5, as the role of myogenin is more prominent in terminal differentiation.[12] RT-qPCR results on myogenin transcript expression, showed in Figure 10, showed a low baseline level of myogenin transcript expression, but increasing above 10-fold at day 2 and 5. Moreover, RT-qPCR does not show significantly different myogenin transcript expression between CTS and control groups at day 2 or 5. However, there is tendency that it is increased at day 2 and decreased at day 5 for CTS groups compared to control groups. Clark *et al.*[52] demonstrated that GTPases are key regulators of mechanosensing in C2C12 cultures exposed to CTS. Our findings of increased myogenin protein levels in response to mechanical stimuli can possibly be connected to increased integrin-mediated signaling and its downstream effect on adaptor proteins, such as Rho GTPases. Rho GTPases play a major role MyoD expression and in turn regulates myogenin expression[31], thus explaining the early difference in myogenin protein levels between CTS and control groups at day 2. The significantly decreased myogenin protein levels in CTS groups at day 5, in contrast to the higher protein levels in control groups, is possibly due to an earlier peak in CTS groups compared control groups and subsequently reaching expected decreases in middle and late differentiation stages faster[50]. Being an early marker of differentiation, myogenin is expected to be present at day 2 coinciding with the cells entering cell cycle arrest and p21 expression increases[13]. Ferri *et al.*[50] reports, that myogenin protein levels peaks shortly after differentiation induction is initiated, but decreases in middle and late differentiation stages. However, they also report that myogenin transcript increase shortly after induction is initiated, but peaks at day 3 and 5 of differentiation, thus stating that transcript and protein turnover are differently regulated. This could also be the case for the differences in myogenin protein levels and transcript expression in this thesis. If the number of biological replicates for RT-qPCR was increased, the possibly of

performing parametric tests could be optional and significant differences for C2C12 confirmed. Counting analysis of myogenin presence in HSMM, as showed in Figure 7, followed almost the same overall pattern of myogenin presence as seen for the C2C12 cells. Despite no significant difference could be confirmed at day 5, myogenin protein levels tend to be slightly higher in the CTS groups than the control groups. At day 8, on the other hand, a decrease in myogenin protein levels, results in statistically significant differences between CTS and control groups, with the control group having the highest amount. The pattern follows the same as for the C2C12 except it seems the myogenin protein levels is peaking later in HSMM. Unfortunately, huge error bars for most time points of differentiation in the RT-qPCR analysis made it impossible to extract anything reliable. This problem might be solved by increasing the number of biological replicates and an actual comparison can be made. It is therefore concluded, that CTS enhances early myogenesis in CTS group compared to control group for C2C12 at day 2, despite the RT-qPCR not being able to support this finding. It is also assumed, that CTS enhances differentiation of HSMM by causing an earlier myogenin peak, with significantly decreased myogenin observed at day 8 for CTS group compared to control group.

Myosin

Myosin levels are, according to the counting analysis, already present in approximately 20% of the C2C12 cells at day 2 and increases slightly at day 5, where CTS groups display significantly higher levels than control groups, showed in Figure 7. Due to its existence in the sarcomeric architecture formed in late myogenic (phenotypic) differentiation, myosin is expected to manifest its substantial presence at day 5.[14] Only the counting analysis could confirm the presence of myosin, though attempts were made to include MYH2 expression in a RT-qPCR analysis. Due to unspecific primer design, we were not able to trust the product from the RT-qPCR analysis, thus the data was excluded. The percentage of nuclei inside myosin-positive myotubes was at day 5 for HSMM almost similar to the day 2 and 5 for C2C12. At day 8 the percentage of nuclei inside myosin-positive myotubes increases markedly, having a significantly higher percentage in CTS groups (approx. 60%) compared to control groups (approx. 40%).

The same significant difference was observed at day 5 for C2C12 cell, thus suggesting that the onset of myosin expression appears later for HSMM. It is therefore concluded, that the significantly increased presence of MHC in CTS groups compared to control groups confirms, that CTS succesfully enhances differentiation at the later stages.

Actin and Myosin Cross-Striations Confirm Incipient Sarcomeric Formation

High-magnification micrographs, Figure 8, showed that both C2C12 CTS and control groups displayed actin and myosin cross-striations at day 5. This is a characteristic of mature myotubes only emerging in late differentiation and do only occur on substrate with stiffness near that of native muscle tissue (Young's modulus, E ~12 kPa).[33] When cultured on glass and softer or stiffer substrates, cross-striation do not occur. The pixel intensity measurement on both actin and myosin shows that actin is peaking in intensity myosin is reaching lowest intensity. The lenght between each peak for both actin and myosin settle at approximately 2.5-3 μm thus representing the M-lines and Z-discs of the sarcomeric architecture. Actin and myosin cross-striations were also observed in HSMM CTS and control groups at day 8, as showed in Figure 8, displaying the same interlacings in pixel intensities for actin and myosin. This finding confirm the incipient development of sarcomeric structures characteristic of maturing myotubes.

Mechanical Stimulation Model Suitable for Primary Human Myoblasts

To sum up the discussion of the effect of CTS on alignment and orientation of both C2C12 and HSMM, we successfully demonstrated that myoblasts align and orientate perpendicularly to the direction of strain, independent on whether they are of murine or human origin or primary or immortalized origin. The effect of CTS on the different myogenic markers and their expression patterns is clearly demonstrated, and serves its purpose in enhancing myogenic differentiation of the myogenic marker profiles myogenin and MHC for murine C2C12 cell line. Despite not being able to achieve any significant change in Myf-5 and MyoD1, both markers showed their entry in early

differentiation stages directly followed by introduction of increased myogenin. Significantly increased MHC presence in CTS groups in the late differentiation stages was confirmed during the counting analysis, but could unfortunately not be supported by RT-qPCR of MYH2 transcript expression. Additionally, more complex sarcomeric structures, like actin and myosin cross-striations began appearing in the late differentiation phase at day 5 for C2C12 and day 8 for HSMM. These findings are in agreement with previous findings of Pennisi et al.[10] on the effect of CTS on the myogenic profile of C2C12. The attempt to apply the exact same strain model to a primary human skeletal myoblast cell line yielded many similar effects on the myogenic marker profile. Despite the absent data from the RT-qPCR analysis, a successful counting analysis informed us that significant differences in myogenin and MHC levels was present at day 8 of differentiation for HSMM. Inclusion of significant differences from day 5 seems to be within reach, if only more batches were to be included. So conclusively, many similar effects of CTS on myogenic differentiation of myoblasts were observed for HSMM as for C2C12.

Conclusion

The main objective in this work was to investigate the effect of a uniaxial cyclic tensile strain model on myogenic differentiation of mouse C2C12, in order to compare it to the effects of the same model performed on primary human skeletal muscle myoblasts, HSMM. It has successfully been demonstrated that CTS align and orientates both C2C12 and HSMM perpendicularly to the direction of strain. Furthermore, CTS enhances myogenic differentiation of C2C12 and HSMM, though the effect on differentiation of C2C12 was seen already at day 2 in contrast to HSMM, where it was not recognizable before day 8. In conclusion, this model of mechanical stimulation of myoblast cell lines holds a promising future as a new alternative method to enhance myogenic differentiation in various skeletal muscle cell lines *in vitro*.

Perspectives

Striving of obtaining more conclusive data on the effect of uniaxial cyclic tensile strain on both cell aligning and differentiation, this experiment needs to be repeated in order to strengthen existing and possible new statistical significant findings. Acquisition of a few new reliable primers are needed for RT-qPCR to be completed. Including a western blot to quantify protein levels and not only mRNA expression, since there seems to be differences in mRNA and protein turnover. Investigation of muscle specific calcium ion channel and acetylcholine receptor changes in response to CTS could also prove to be an interesting angle on how skeletal muscle myoblasts differentiate *in vitro*.

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