Effects of cyclic tensile strain on the cytoskeletal arrangement and activation of focal adhesion kinase in murine myoblastic precursors

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Abstract

Mechanical stimulation has been shown capable of modulating cell behaviour. By mechanotransduction cells are able to translate extrinsic stimuli into chemical signals that can affect migration, proliferation, transcription and apoptosis. Focal adhesion kinase is a focal adhesion-associated protein kinase which is involved in binding of the cell to extracellular compartments. Recently, focal adhesion kinase has been associated as key factor in translating extrinsic signals into cellular response. In this work, the effect of cyclic tensile strain on myoblasts ability to assemble focal adhesions and in turn recruit and activate focal adhesion kinase was analysed with immunofluorescence techniques. Subconfluent mouse myoblastic precursors were cultured on flexible-bottomed culture plates and subjected to uniaxial or equibiaxial cyclic strain before stained for vinculin, focal adhesion kinase and Tyr397 phosphorylated focal adhesion kinase. Cell subjected to cyclic strain obtained elongated morphology with clear formation of focal adhesions. Co-localised to the focal adhesions were recruited focal adhesion kinase which was then phosphorylated. The cyclic tensile strains were capable of inducing cytoskeletal reorientation in myoblast as well as recruit and activate focal adhesion kinase.

Table of content

1 Introduction

In recent years, cell-based treatments have been a rapidly emerging strategy for tissue repair and regeneration. Cell-based treatment aims to replace/repair damaged tissue or enhance biological functions of tissue (Moon du et al., 2008; Rhee et al., 1994). A prospect group for cell-based treatment is patients with deficient skeletal muscle tissue, e.g. traumatic injury or removal during surgery. Commonly these patients are treated by transfer of muscle tissue from local or distal sites to the receiving area. A drawback of this is loss of muscle volume at the donor site (Badylak et al., 2012). A way to circumvent the drawbacks of present treatment methods is by utilising cell-based treatment, hence eliminating loss of volume from donor site (Usas et al., 2011). Extraction and isolation of muscle precursor cells from patients allow manipulation and expansion of muscle cells and may hold promises for future treatment options (Farini et al., 2009). Currently muscle precursor cells can be isolated and readily differentiated to mature muscle cells (Vilquin et al., 2011). The complexity of muscle tissue, however, require more than mature muscle cell to achieve the functions needed of mature muscle (Moon du et al., 2008). Driven by knowledge of *in vivo* regulation of muscle development novel methods have been established to enhance the myogenic capacity of precursor cells. A promising approach is mechanical stimulation of precursor cells by subjecting these to different strain parameters, enhancing the myogenic capacity of cells (Kumar et al., 2004; Pennisi et al., 2011b). Current investigation focuses on optimising mechanical stimulation to achieve the highest myogenic potential (Pennisi et al., 2011b). However, more knowledge on how the cells translate the applied strain into cellular signalling events is needed.

2 Background

2.1 Skeletal muscle development

Skeletal myogenesis is the process in which functional skeletal muscle tissue is developed. Myogenesis begins with determination of muscle precursor cells to the myogenic lineage (Mok and Sweetman, 2011). The committed precursor cells (myoblasts) align, exit cell cycle, and fuse to form a multinucleated syncytium termed myotubes (Chen and Olson, 2004)**(Fig 1).** The myotubes further differentiate by creating contractile apparati to facilitate contraction of the terminally differentiated myotubes (Charge and Rudnicki, 2004). To initiate and regulate myogenesis a group of muscle-regulatory factors, MRFs, have been identified (Braun and Gautel, 2011). The MRFs are members of a family of basic helix-loop-helix DNA-binding transcription factors and their ectopic expression can induce myogenesis in non-myogenic cells (Chanoine et al., 2004). During early determinationa of precursor cells, two MRFs are increased in expression, namely

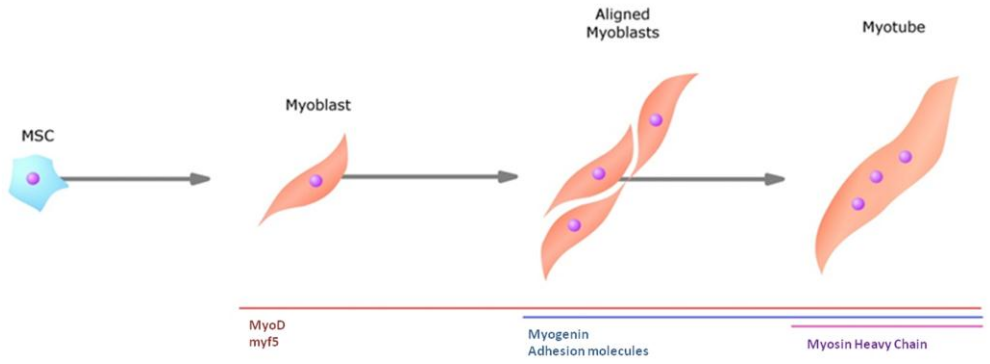


Figure 1 | Myogenic differentiation process from myoblast to terminally differentiated myotube. (a) Mononucleated spherical MSC in an undifferentiated, multipotent state. (b) The myoblast changes morphology and elongates. In early differentiation MRF, MyoD and myf5 are expressed and the cell are committed to the myogenic pathway. (c) The myoblast synthesise adhesion proteins that link the cell to the ECM and surrounding cells, thus the myoblasts align. During differentiation myogenin is active and take part in the synthesis of the contractile apparatus. (d) Terminal state of differentiation where myoblasts fuse and form long, multinucleated myotubes. These contain MHC and α-actin, both parts of the contractile apparatus. MSC = mesenchymal stem cell. MRF = myogenic regulatory factors. ECM = extracellular matrix. MHC = myosin heavy chain.

myogenic factor 5 (Myf5) and myogenesis determining factor (MyoD)(Bentzinger et al., 2012). Myf5 and MyoD binds E-box sequences in promoters of downstream muscle genes, forcing the cells towards the myogenic lineage by transcription of these genes. MyoD/Myf5 double knockout mice have shown a complete lack of myoblasts and skeletal muscle, hence suggesting that MyoD and Myf5 have a redundant effect on the formation and survival of myoblast and are crucial for early determination (Sabourin and Rudnicki, 2000). To negate the myogenic effect of MyoD/Myf5 in non-differentiating cells, inhibitor of differentiation (Id) heterodimerises with the MRFs effectively blocking their DNA-binding capacity (Yokoyama and Asahara, 2011). After introduction of MyoD/Myf5 the committed precursor cells, termed myoblasts, undergo a stage of proliferation during which myogenin is upregulated (Ohkawa et al., 2007). Myogenin, also a MRF, promotes the transcription of myogenic proteins needed for differentiation of the committed myoblasts (Andres and Walsh, 1996; Ferri et al., 2009). Myogenin knockout mice have shown complete lack of skeletal muscle, where myoblasts occupied the normal locations of muscle tissue. This suggests that myogenin is not necessary for myoblasts determination, but contrarily essential for the later differentiation and fusion (Sabourin and Rudnicki, 2000). Myogenin furthermore mediates the withdrawal from cell cycle by induction of the general cyclin-dependent kinase inhibitor 1 (p21) (Andres and Walsh, 1996). P21 functions as a potent cell cycle inhibitor and exerts its effect by forcing the cell to exit cell cycle at G1, thus enabling further differentiation of the myoblasts (Walsh, 1997). To further differentiate the myoblasts begin synthesising proteins essential for later fusion (Abmayr and Pavlath, 2012). The postmitotic myoblasts are divided into founder cells and fusion competent cells. Founder cells functions by attracting fusion competent cells, enabling the initial fusion. Fusion competent cells can in turn fuse with already multinucleated myotubes and are responsible for maintenance and hypotrophy of existing myotubes (Chen and Olson, 2004). Fusion is a multistep process which starts by migration of fusion competent cells towards founder cells. When close, the fusion competent cells extend membrane protrusions towards the founder cell (Knudsen and Horwitz, 1978). Membrane proteins enable cell-cell adhesion, and the two cells begin to align and elongate along their long axis. In turn specialised vesicles are recruited to the cell-cell junction and dissolve the membrane forming the multinucleated myotubes (Horsley and Pavlath, 2004). After fusion, the multinucleated myotubes begins the formation of the contractive apparati. From

vinculin-rich focal adhesions, described later, bundles of actin and nonmuscle myosin premyofibrils are formed along the sides and ends of myotubes (Rhee et al., 1994). Recruitment of titin and muscle myosin converts the premyofibrils into myofibrils capable of generating force contraction of the fully differentiated myotubes (Sanger et al., 2010).

To investigate myogenesis *in vitro*, well defined cell models have been established. C2C12 is a readily available murine myoblast cell line, which by deprivement of serum can be induced to undergo myogenic differentiation (Andres and Walsh, 1996; Olson, 1992). To enhance the myogenic capacity, C2C12 has been subjected to mechanical stimulation in addition to serum starvation (Pennisi et al., 2011b). To understand how the cell reacts to mechanical stimulation, a basic knowledge of how the cell binds to extracellular cues is needed.

2.2 Cellular Adhesion

Focal contacts are specialised complexes which facilitate the binding of the cytoskeleton to extracellular components, e.g. extracellular matrix or the bottom of a culture flask (Hynes, 2002)**(Fig 2).** The actual binding to the extracellular component are mediated by integrins, a transmembrane heterodimer cell-surface receptors, which are capable of binding to various different extracellular ligands (Brakebusch and Fassler, 2003). After binding to a ligand in the matrix, the cytoplasmic tail of integrins binds to intracellular anchoring proteins including tensin, talin, and α-actinin (van der Flier and

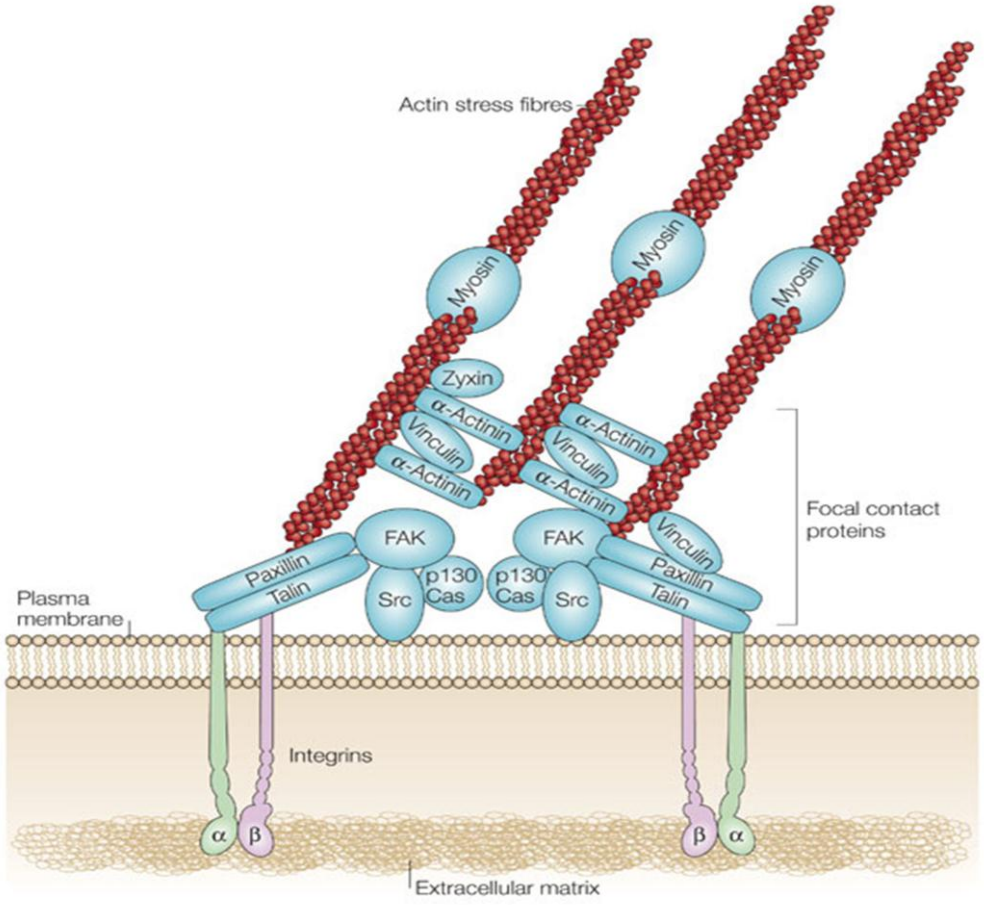


Figure 2 | Schematic overview of focal contacts. Transmembrane cell-surface receptor integrin binds to the extracellular matrix. This enable the intracellular tails of integrins to bind anchoring proteins, e.g. talin, which in turn bind actin fibres efficiently anchoring the cell cortex to the extracellular matrix. Closely associated with the created complexes are regulatory proteins controlling the maturation of focal contracts into focal adhesions.(Mitra et al., 2005)

Sonnenberg, 2001). These proteins in turn either bind other anchoring proteins, e.g. vinculin, or directly to actomyosin fibers. Crosslinking of actomysin fibers, by α-Actinin, form bundles of contractable fibers termed stress fibers. These stress fibers function as the motor units of the cell enabling movement of the cell (Pellegrin and Mellor, 2007). Focal contacts are in a state of constant flux were the focal contacts can either be disbanded, as seen in the dynamics of cell migration, or lead to a clustering of more integrins forming a mature focal adhesion enabling a more permanent cell-matrix binding (Zamir and Geiger, 2001). In addition to the anchoring abilities of focal adhesions numerous intracellular signalling pathways are associated with integrin mediated cell adhesion.

2.2.1 Focal Adhesion associated Signalling Pathways

As with conventional receptors, integrins can activate intracellular signalling pathways when bound to its ligands (Kumar, 1998). Integrins however do not possess a kinase domain or enzymatic activity and therefore relies on recruitment of proteins able of activating subsequent signalling pathways (Schwartz and Ginsberg, 2002). The intracellular tail of integrin can bind anchoring proteins which in turn can phosphorylate focal adhesion kinase (FAK) one of the most dominating mediators of integrin signalling (Parsons, 2003). Phosphorylation of FAK can take place at different sites where each site can affect downstream signalling cascades (Liu et al., 2002). Clustering of integrins, and subsequently anchoring proteins, phosphorylates FAK leading to formation of phosphorylation docking sites. Phosphorylation at Tyr397 creates high affinity binding sites for proteins containing a SH2 domain, including Src (Parsons, 2003). Src phosphorylates CAS recruiting Crk which activates DOCK180/ELMO complexes. DOCK180/ELMO have been closely associated with myoblast differentiation, where they are theorised to modulate the rearrangement of the cytoskeleton during fusion (Chen and Olson, 2004). Moreover DOCK180/ELMO functions as a guanine nucleotide exchange factor capable of activating Rac (Ouyang et al., 2008), an important regulator of cell motility, by affecting the maturation of focal adhesions (Parsons, 2003). This dynamic regulatory effect of FAK on cell motility and maturation of focal adhesions has been shown in experimental setups (Parsons et al., 2000). In FAK knockout mice a decreased rate of migration was seen with a decreased amount of focal adhesions implying a lack of

maturation of focal adhesions. However overexpression of FAK has shown a similar migratory tendency indicating that a delicate balance of FAK is needed to sustain normal cell migration and adhesion (Ibid.). Rac can further activate RhoA which has direct effect on the polymerisation of actin fibres driving the assembly and orientation of the cytoskeleton (Frame, 2004). RhoA can also influence the activity of certain transcription factors, and active RhoA have been shown crucial for the upregulation of MyoD during myogenesis (Carson and Wei, 2000).

Phosphorylation of FAK at Tyr397 can furthermore recruit phosphatidylinositol-3 kinase (PI3K), a SH2 domain containing protein. PI3K is a kinase able to phosphorylate PIP3 which in turn can activate Akt/PKB, a well know mediator of cell survival signalling

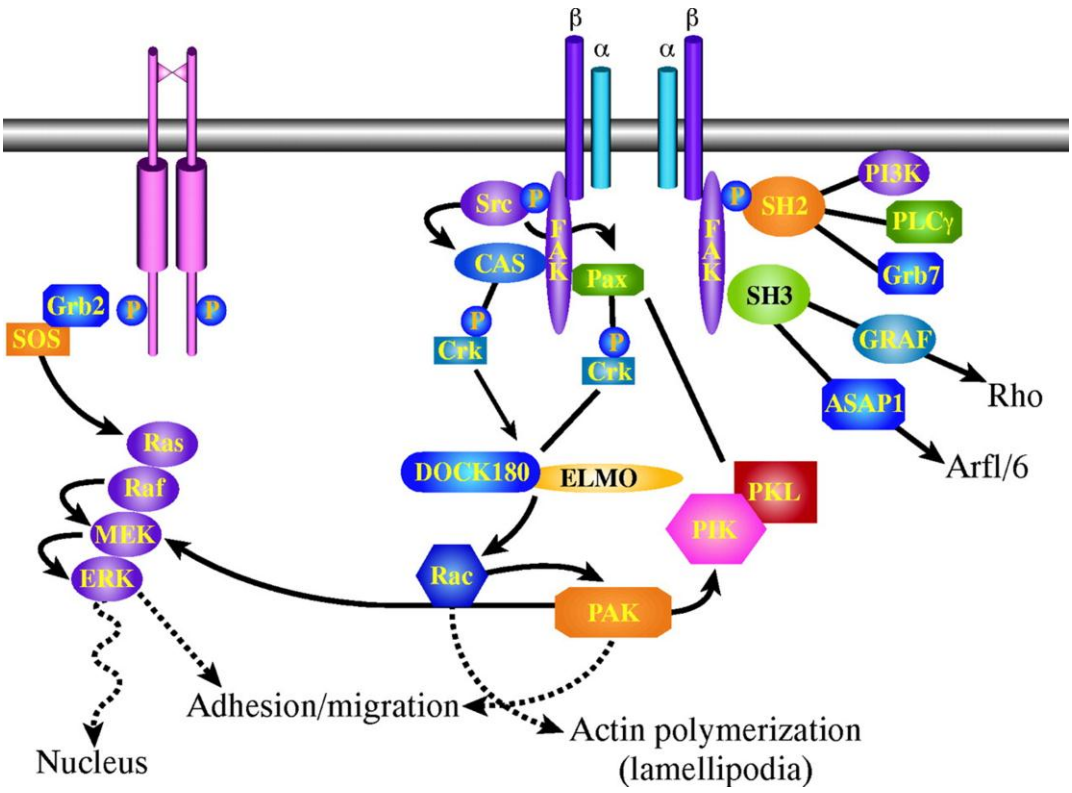


Figure 3 | Integrin signalling pathways. Integrin themselves do not possess any enzymatic effect and therefore relies on adaptor proteins to mediated signals originating from the extracellular matrix. The main mediator of integrin signalling is focal adhesion kinase (FAK) which through various cascades can activate different events in the cell, e.g. migration, proliferation or differentiation (Parsons, 2003).

(Xia et al., 2004).

Phosphorylation of FAK at Tyr925 creates a SH2 domain binding site for growth factor receptor-bound protein 2 (Grb2). Grb2 is an adaptor protein which can affect the Ras-ERK pathway downstream. The Ras-ERK pathway leads to activation of different transcription factors involved in maintaining cellular proliferation (Schlaepfer et al., 1994). The effect of ERK has been observed in committed myoblasts, were it inhibits further differentiation by proliferation signalling. The activity of ERK decreases transiently when myoblast differentiation commits, but is in turn activated during the later phases of differentiation, and peaks during fusion events (Knight and Kothary, 2011).

During myogenesis, the FAK signalling pathway has been shown to have an important role. ECM-integrin binding, and subsequently Tyr397 phosphorylation of FAK, promotes proliferation of myoblasts through cyclin-dependent kinases. When conventionally induced for differentiation, by deprivement of serum, a dephosphorylation of FAK takes place. The inhibition of FAK has been associated with SH2 domain-containing protein-tyrosine phosphatase (SHP-2) which is capable of directly inactivating FAK. This decreased activity of FAK is crucial as it forces the determined myoblast to withdraw from cell cycle, by increased p21 activity, to allow differentiation. The decrease in FAK activity, however, is only a transient reduction. After a short period, phosphorylation of FAK again takes place and is sustained to ensure cell survival (de Oliveira et al., 2009).

As shown ECM-integrin binding can affect a wide range of signalling cascades, enabling the cell to sense the surrounding environment. Along with the binding cues supplied by the ECM, a constant mechanical stimulation is affecting the cell.

2.3 Mechanotransduction

Mechanotransduction is the process through which the cell senses mechanical stimuli from the environment and translates this into biochemical signals (DuFort et al., 2011). Mechanotransduction can be divided into either active or passive mechanosensing (Jaalouk and Lammerding, 2009)**(Fig 4).** Active mechanosensing is the process where the cell actively "probes" the surrounding matrix to examine the substrate rigidity and reacts to this (Holle and Engler, 2011). The active mechanosensing is achieved by active pulling of the stress fibers linked to the ECM via focal adhesions. This traction deforms the extracellular substrate. Soft substrates are greatly deformed, with diminishing deformation as substrate stiffness increases (Kobayashi and Sokabe, 2010). In passive mechanosensing the cell reacts to mechanical stimuli exerted from the ECM. As focal adhesions function as links between the cell and substrate many of the pathways involved in both active and passive mechanosensing are shared (Geiger et al., 2009). When focal adhesions are mechanically stimulated integrin change, exposing binding sites for anchoring proteins. Talin, one of the anchoring proteins, can also undergo force-dependent conformational changes unravelling vinculin binding sites. The net result of both integrin and talin conformational change is further clustering of integrins strengthening the exposed focal adhesions (DuFort et al., 2011). As aforementioned, clustering of integrins phosphorylates FAK enabling activation of the downstream pathways regulating cell motility, survival, and gene transcription. Worth noting is the FAK mediated activation of RhoA which leads to actin remodelling. This signalling pathway enables the cell to react to extrinsic mechanical stimuli by enforcing and rearranging the cytoskeleton in accordance with the direction of the mechanical stimuli (Wang et al., 2009).

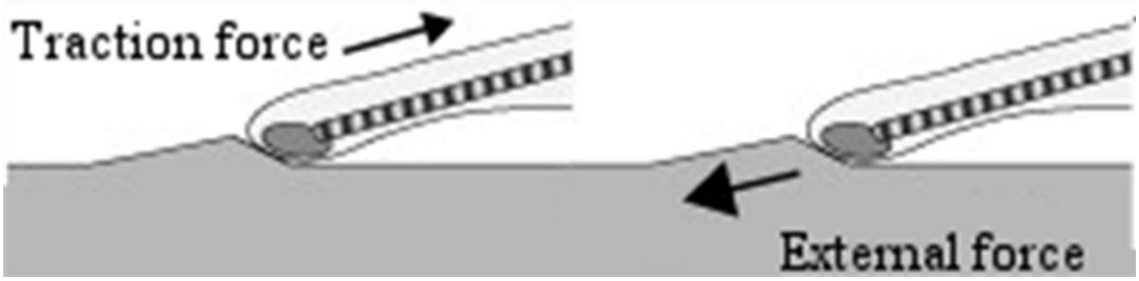


Figure 4 | Schematic illustration of passive and active mechanosensing. **(Left)** Active mechanosensing were the actomyosin fibers actively pull the substrate, thereby sensing the environment. **(Right)** Passive mechanosensing were external forces pull the cell (Kobayashi and Sokabe, 2010).

As force is generated, either in the ECM or by traction of stress fibres, the integrin binding cues can be shifted. The differential movement of focal adhesions can affect the cell membrane. Imbedded in the membrane are specialised stretch activated channels (SACs) (Sadoshima and Izumo, 1997). SACs are protein gates which are hypothesised to physical separate when the membrane is strained enabling influx of specific ions, e.g. Ca^{2+} (Denker and Barber, 2002). Ca^{2+} serves as an intracellular second messenger and is involved in many cellular processes. Increased intracellular Ca^{2+} levels activate calcineurin which in turn can activate transcription factor family NFAT (Formigli et al., 2007; Hai, 2007).

To investigate how mechanotransduction affect cellular responses, controllable experimental setups have been established. In the following a symposium of recent findings of how to affect myogenic fate by extracellular stimulation will be reviewed.

2.4 Extrinsic Stimulation of Myogenesis

The effect of extracellular stimulation of muscle tissue has been studied intensively, however the cellular events translating the stimuli are still largely unidentified. Novel methods have therefore been established to investigate this effect on myogenesis. By culturing myoblasts on substrates with rigidity modulation properties the different pathways in passive mechanosensing can be modulated (Engler et al., 2004). As myoblasts are anchor-dependent, certain substrate stiffness is necessary for formation of focal adhesion and cell survival (Frisch et al., 1996). Myoblasts are capable of forming adhesions on substrates ranging from 1 kPa to around 45 kPa, where the formation speed and strength of focal adhesions increases with increasing stiffness. Early myogenesis can be initiated throughout the whole span in substrate stiffness, however fusion is absent on substrates with the lowest modulus (Nemir and West, 2010). The late differentiation, in form of creation of contractile apparati, has been found to be significantly increased on 11 kPa substrates, interestingly near the same stiffness as myoblast cells. This was elucidated by Engler et al. by seeding myoblasts on glass plates with a layer of myoblasts on top. The bottom layer of myoblasts remained unstrained whereas the top layer showed pronounced striation (Engler et al., 2004). This indicates that substrate stiffness, through active mechanosensing, greatly influences cellular events and has been closely associated with integrin mediated matrix-cell anchoring and its downstream effectors. To clarify the entity of the effect of mechanotransduction on myogenesis, concurrent research has focused on active mechanosensing.

Research in active mechanosensing seeks to apply controlled mechanical stimulation to manipulate cellular responses. Commonly the mechanical stimulation is applied as CTS of stretchable membranes on which myoblast are seeded. The strain can be divided into equibiaxial- or uniaxial strains which have been shown to affect the cell differently (Pennisi et al., 2011b). Myoblasts subjected to equibiaxial strain have shown to enhance proliferation, thus inhibiting differentiation (Kumar et al., 2004). The proliferative response was found to be mediated by activated FAK and the downstream effector Rac effectively inhibiting myogenesis (Zhang et al., 2007). Recent data from our laboratory supports these finding by a decrease in myotube formation in comparison to non-stretched cells. Furthermore the equibiaxial strain was observed to disrupt the cell membrane

implying that the cell was sustaining damage. However, the antimyogenic effect observed seems to be limited to equibiaxial stimuli. Myoblasts subjected to uniaxial stimulation revealed promyogenic response in the form of increase number of myotubes (Pennisi et al., 2011b). In addition, cells were subjected to uniaxial strain arranged in a uniform direction perpendicular to the strain field. The mechanism for the uniform alignment has been reported to be regulated by the frequency of which the cyclic strain is applied (Ahmed et al., 2010). Stimulating cells at a frequency of 1 Hz or above causes the cell orientate away from the strain direction. This is due to a latency found in the actomysin sliding, which cannot relax quickly enough to dampen the applied strain. This result in an increased turnover of stress fibers, which when repolymerised rearrange perpendicular to the strain field results in lower stress generation (Hsu et al., 2010). It has further been shown that during the first minute of cyclic strain phosphorylation of FAK takes place. As previously mentioned, FAK is involved in mechanotransduction and can modulate the polymerisation through Rho downstream. The connection between FAK and orientation in accordance with strain has been clarified by FAK knockout cells. These cells are unable to transduce the applied strain, and are unable to reorientate and mitigate the forces generated to the stress fibers (Seko et al., 1999).

3 Aim

Though much progress have been done, the pathways by which cells translate mechanical stimulation into chemical signalling are still poorly described, however key mediators have been identified. Focal adhesions function to anchor the cell and are therefore also important in mediating extric stimuli by downstream cascades. It is therefore hypothesised that cyclic mechanical stimulation can modulate cell behavior by interaction with focal adhesion and key mediator FAK leading to changes in cytoskeletal arragement and differentiation. The aim of this experiment is to explore how different types of cyclic strain can recruit and activate FAK in a C2C12 myoblast model. This will be done by immunofluorescence analysis of focal adhesion protein vinculin, FAK, and Tyr397 phosphorylated FAK.

4 Methods

4.1 Cell Culture

Mouse myogenic cell line C2C12 (CRL-1772™) from American Tissue Type Culture Collection (LGC Standards) were cultured in murine growth medium(GM) (90% Dulbecco's Modified Eagle Medium (DMEM)(Lonza Group Ltd), 10% fetal calf serum (Invitrogen™), 0.5% penicillin/streptomycin and gentamycin (Invitrogen™)). C2C12 myoblasts were cultured in T175 and T75 Tissue Culture Flasks (Greiner Bio-One) and passaged when 80-90% confluency was reached. The cells were seeded on either a 24 well tissue culture plastic plate or in 6-well flexible-bottom Bioflex® Culture Plates precoated with collagen-I (Flexcell International Corporation) at a cell density of 5,000 cells/cm^2. Differentiation was induced with differentiation medium (DM)(DMEM (Lonza Group Ltd), 2% heat-inactivated horse serum (Invitrogen™), 0.5% penicillin/streptomycin and gentamycin (Invitrogen™)).

4.2 Mechanical Stimulation

C2C12 cells were subjected to a predetermined mechanical stimulation regime for 24 or 48 hours utilising a Flexcell® FX-5000™ Tension System (Flexcell International Corporation). The construction of the tensions system was arranged so either rounded rectangular pistons applied cyclic uniaxial strain or round piston applied equibiaxial strain to the flexible bottoms of the Flexcell plates by vacuum. Vacuum was prevented from applying strain to control wells using FlexStop™ valved rubber inserts (Flexcell International Corporation). The CTS stimulation was set to apply 15% strain impulses at 0.5 Hz.

4.3 Fixation and Cell Staining

For immunofluorescence analysis, cell samples were washed with phosphate-buffered saline (PBS) and fixated in 4% formaldehyde for 15 minutes. The cells were permeabilised with 0.1% Triton X-100 (Sigma-Aldrich®) for 5 minutes and blocked with 1% bovine

serum albumin (BSA) for 30 minutes prior to incubation with antibodies against vinculin, focal adhesion kinase and Tyr397phosphorylated focal adhesion kinase. During the course of this project two different sets of antibodies were used.

Zenon: Anti-FAK (clone D1; Santa Cruz® # sc-271126)(1:100) visualised with Alexa Fluor 350 conjugated anti-mouse IgG_1 Zenon labelling kit (Invitrogen® #Z-25000), anti-pFAK (Tyr397; 1:150; Santa Cruz® # sc-11765-R)(1:100) visualised with Alexa Fluor 488 conjugated anti-rabbit Zenon labelling kit (Invitrogen® #Z-25302) and anti-vinculin(1:100) visualised by Cy5 anti-rabbit.

Indirect: Anti-FAK visualised by Alexa Fluor 488 donkey anti-mouse IgG (Invitrogen® #A-21202)(1:150), anti-pFAK visualised by Cy5 anti-rabbit and vinculin (N-19, Santa Cruz® #Sc-7649)(1:100) visualised by Alexa Flour 350 donkey anti-goat IgG (Invitrogen® #A-21081)(1:150).

All reagents were diluted in PBS containing 1% BSA. For both kits visualisation of actin was done with Bodipy 558/568 conjugated Phalloidin (eBioscience® #53-5643-82)(1:100). After stain the flexible membranes wells were cut out and stored in a petri dish containing PBS at 4°C.

4.4 Microscopy and Image Analysis

Fluorescence images were obtained with an AxioCam MRm (Carl Zeiss International) attached to a Zeiss Axio Observer.Z1 microscope (Carl Zeiss International) using the AxioVision rel. 4.7 software (Carl Zeiss International). High magnification images were taken at 64x magnification. For each sample a representative z-stack of 10 stacks was obtained. To equalise focal plane variance and improve quality of the images, samples were 3D deconvolved using AutoQuant 2.2 (Media Cybernetic, Inc). 3D deconvolution was performed using a theoretical point spread function with 10 iterations.

5 Results

5.1 Formation of focal adhesions in myoblasts

Firstly the basic formation of focal adhesions in proliferating myoblasts was addressed. C2C12 were sparsely seeded on a 24 well tissue culture plastic (TCP) plate and kept in GM. After 48 hours, the cells were fixed and focal adhesions were indirectly visualized by staining focal adhesion anchoring protein vinculin in conjunction with actin fibers and nucleus (**Fig 5a).** The myoblasts were found to have fully spread and formed a spindle-like shape. Distinct actin cytoskeleton was observed for all cells with clear formation of stress fibers. Each stress fiber contained clusters of vinculin localized at the ends of the fibers (**Fig 5b**, **c**). Nearly all cells contained a markedly higher density of vinculin in the periphery, which was most evident in cells with a visual leading edge. No myotubes were observed, however few myoblasts had begun aligning. Situated around the aligning cells a higher accumulation of vinculin was seen.

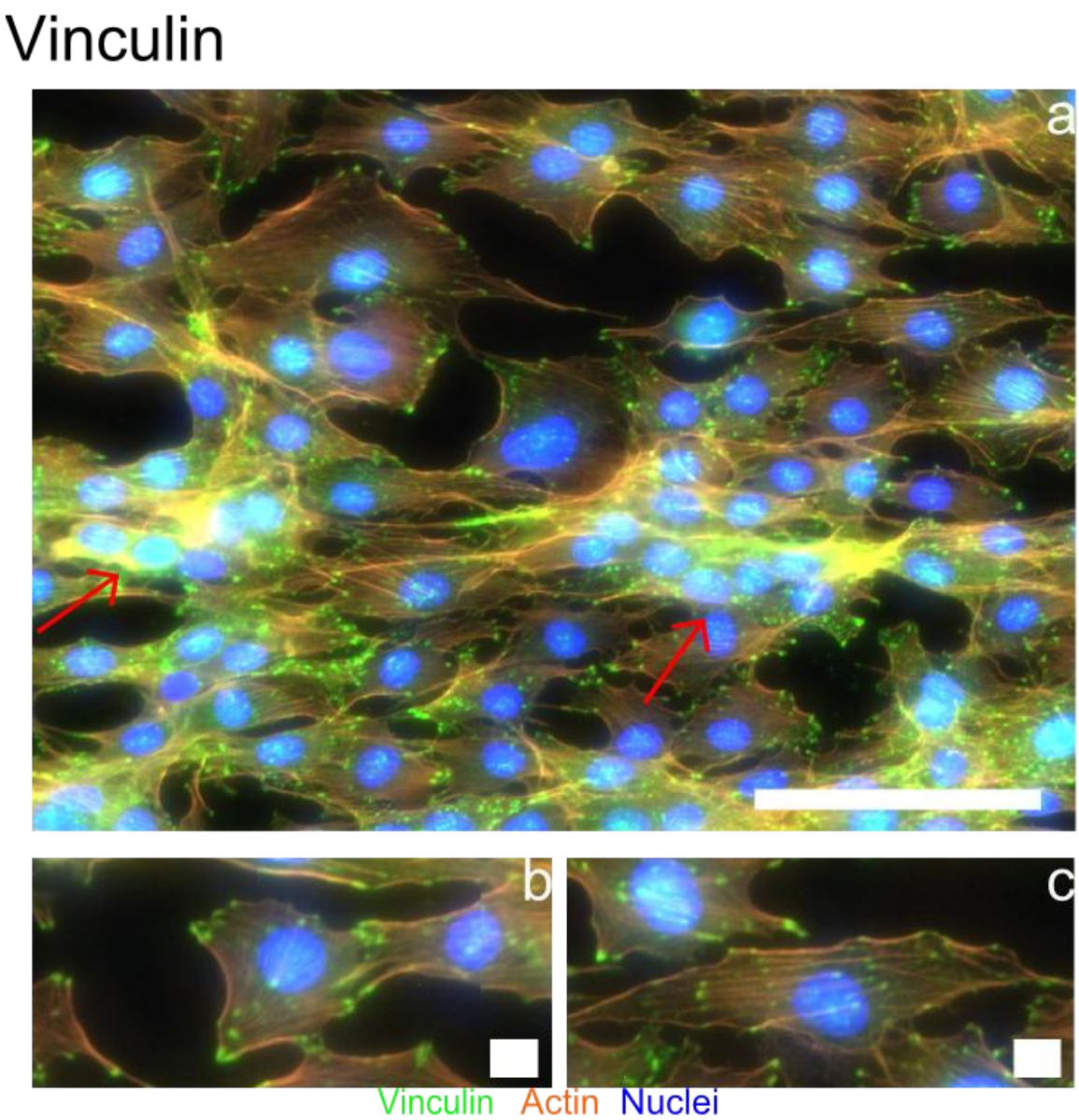


Figure 5 | Initial C2C12 response on tissue culture plastic. 48 hours after seeding cells were stained with Hoechst 33342 nuclear stain, Bodipy 558/568 phalloidin, and Cy5 labelled anti-vinculin. (a) Representative fluorescence micrographs of myoblasts. Arrows indicate aligning nuclei. Scale bar denotes 100 µm. (b) Example of peripheral clusters of vinculin. Scale bar denotes 10 µm. (c) Example of stress fibers terminating in cluster of vinculin. Scale bar denotes 10 µm.

5.2 Co-localization of pFAK and vinculin

To validate the presence of FAK and pFAK, myoblasts were seeded on a 24 well TCP plate. After 48 hours the cells were fixed prior to staining of actin, vinculin, FAK and pFAK according to strain protocol 1. As the spatial location of the proteins of interest is in different focal planes **(Fig 5)** series of 10 stacks was obtained **(Fig 6).** Vinculin was found to be situated closest to the substrate followed by actin. FAK and pFAK was found in same focal plane. Myoblasts were found to have fully spread with pronounced formation of stress fibers with vinculin situated at the end. At every cluster of vinculin co-localization of pFAK was found. In addition a high amount of perinuclear situated pFAK and FAK was observed. No FAK was observed to have co-localized to either vinculin or pFAK. Due to high background noise, the obtained stack was 3D deconvolved to deblur the images. The processed images revealed a more pronounced formation of stress fibers with high a degree clustering of vinculin. Co-localisation of both FAK and pFAK to the vinculin complexes were observed throughout the cell.

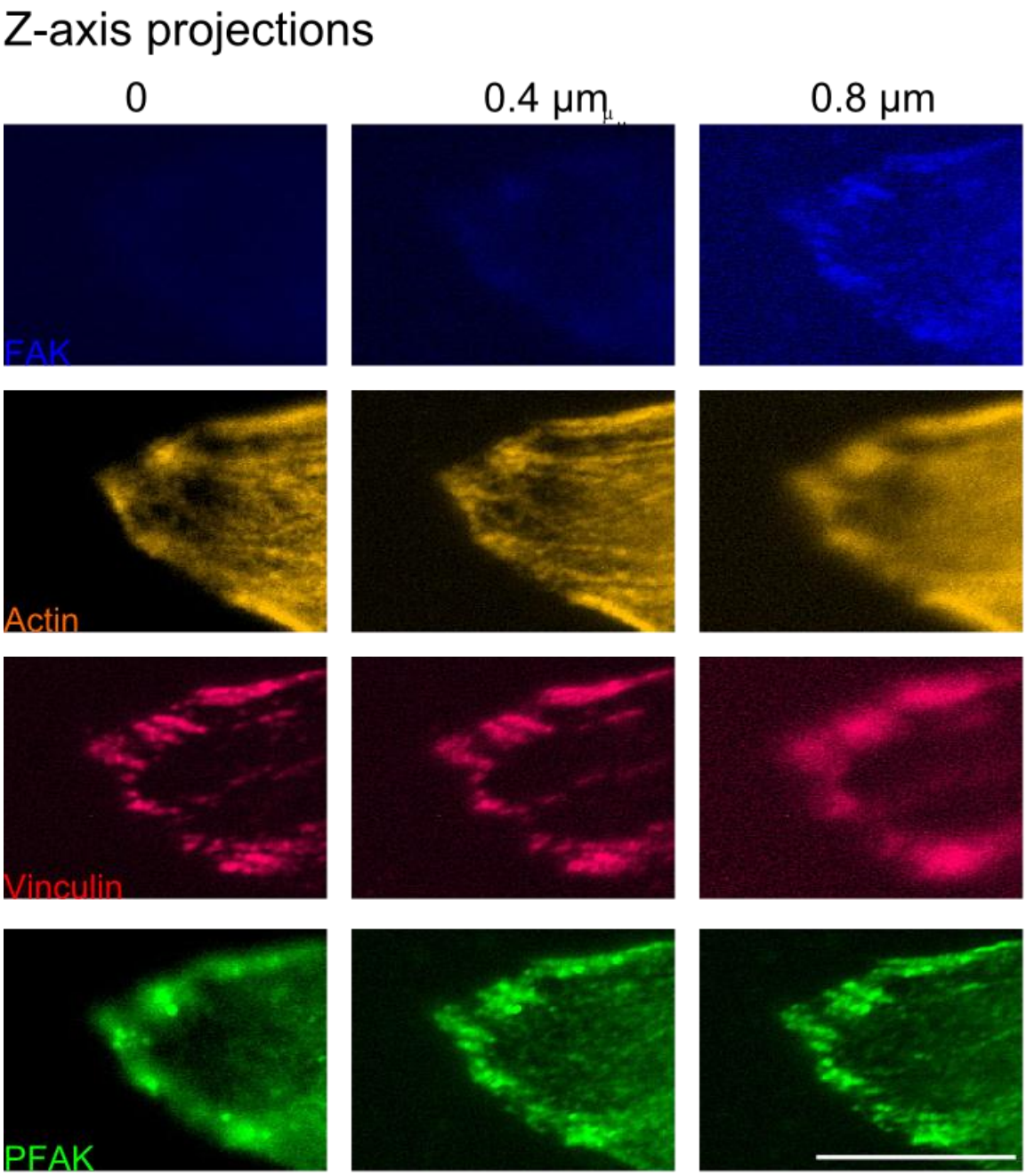
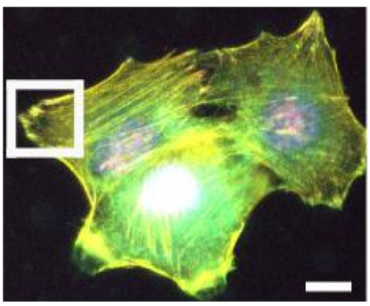


Figure 5 | Spatial arrangement of focal adhesions. High-magnification fluorescence micrographs of myoblasts 48 hours after seeding. Cells were stained with Bodipy 558/568 phalloidin, Cy5 labelled anti-vinculin, Alexa Flour 350 labelled anti-FAK and Alexa Flour 488 labelled anti-pFAK. For each sample, a z-stack of 10 0.2µm slides was obtained to assess the spatial arrangement of focal adhesions. Scale bars detonate 10 µm



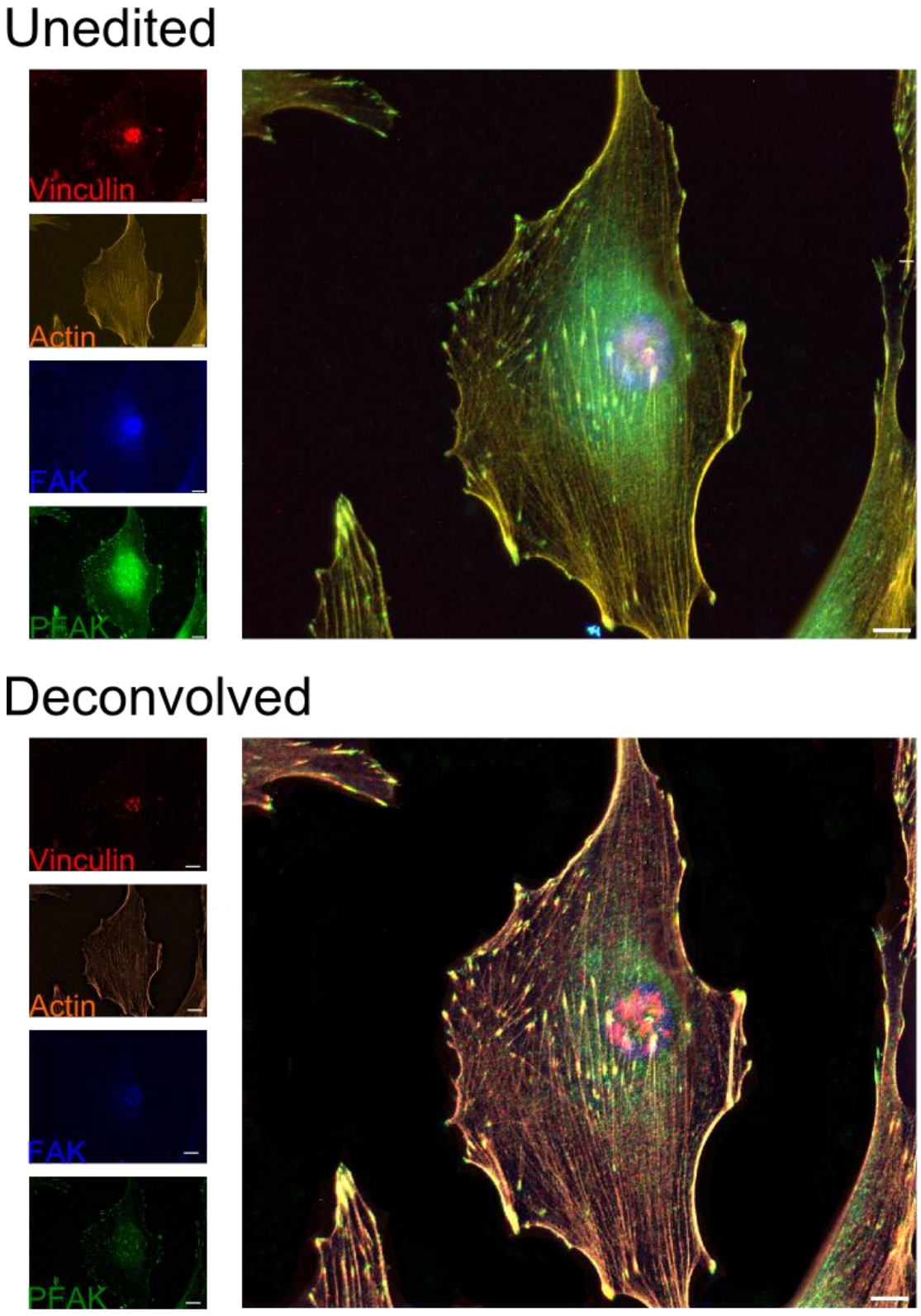


Figure 6 | Expression of FAK and pFAK in C2C12 cells. Cells were plated on TCP for 48 hours before staining with Bodipy 558/568 phalloidin, Cy5 labelled anti-vinculin, Alexa Flour 350 labelled anti-FAK and Alexa Flour 488 labelled anti-pFAK. For each sample, a z-stack of 10 0.2µm slides was obtained. **(Upper)** Unprocessed maximum intensity projection of representative image. **(bottom)** Maximum intensity projection of 3D deconvolved image. 3D deconvolvtion was performed using a theoretic point spread function with 10 iterations. Scale bars denote 10 µm.

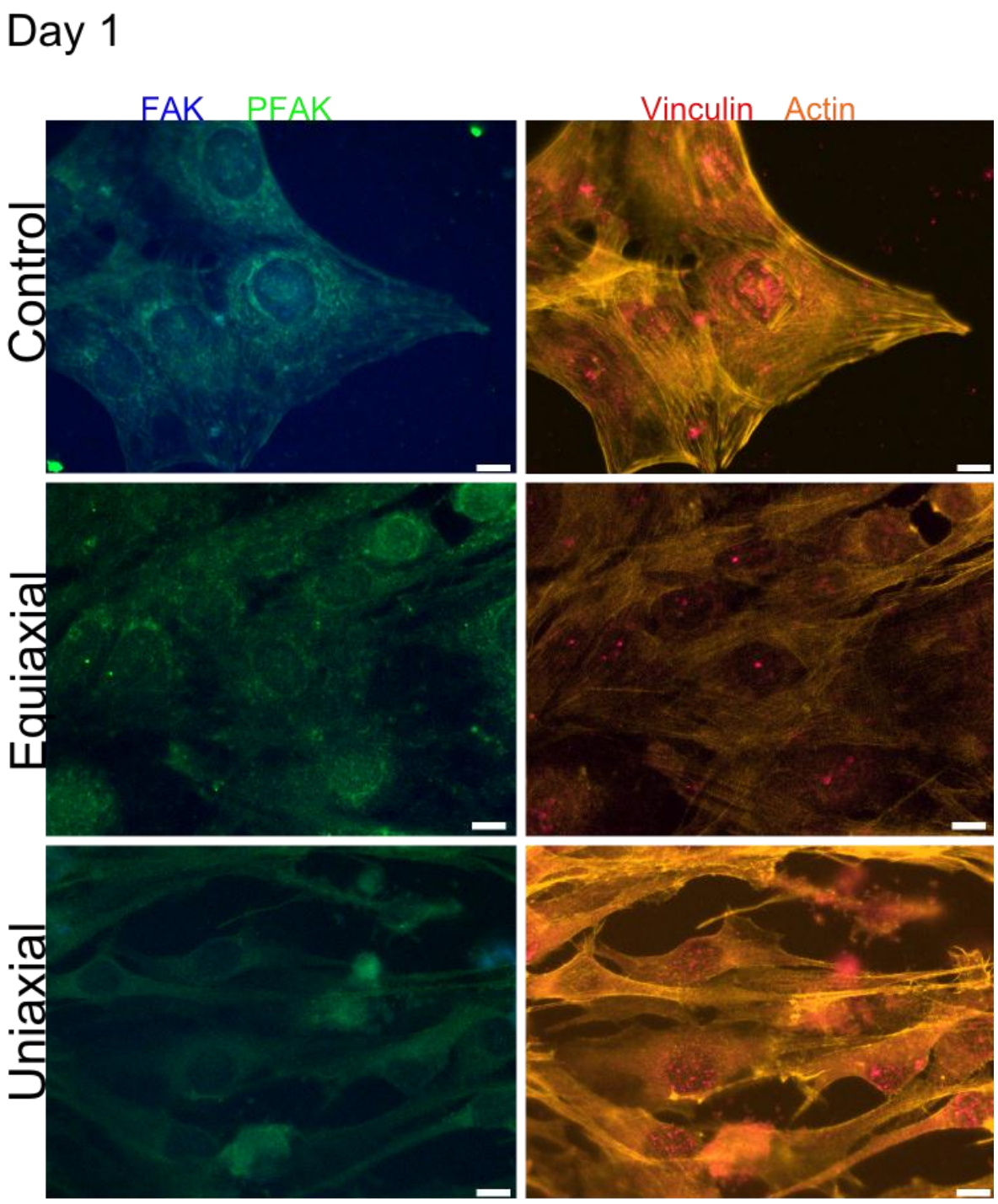
5.3 Cyclic tensile strain

To explore the effect of CTS on the assembly and activation of FAK, two experiments was conducted using two different staining protocols; Experiment 1 (Exp1) using Zenon protocol and experiment 2 (Exp2) using the indirect protocol.

In Exp1 C2C12 cells were seeded onto Flexcell plates. After 48 hours the cells were induced and subjected to CTS, either as uni- or equibiaxial strain, for 1 or 2 days. After CTS the cells were fixed and stained using the Zenon kit strain protocol **(Fig 7).**

At day 1, morphological changes, as observed through actin visualization, were evident for both CTS conditions compared to the spindle-like morphology observed in control conditions. Cells subjected to equibiaxial strain had obtained a more elongated morphology, which was even more pronounced in cells subjected to uniaxial strain. At day 2 the changes in morphology were even more evident. Most cells in both uni- and equibiaxial strain, as well as highly confluent cells in control conditions, had obtained an elongated morphology. Cells subjected to uniaxial strain was found to have reoriented in a uniform direction, which was not observed in either control or equibiaxial conditions. At day 2 formations of stress fibers was clearly observed in both strain condition. Regardless of strain or control, no clustering of vinculin to stress fibers was found at day 1 or day 2. Vinculin and FAK were both observed to be evenly distributed throughout the cytoplasm and to intranuclear structures. pFAK was distributed throughout the entire cytoplasm, with markedly higher concentrations closely situated to actin fibers. Regardless of condition no sign of differentiation was visible.

The incoherence between previous results and the results observed in cells subjected to CTS lead to a revision of the utilized method. To enhance the signal of primary antibodies, the strain protocol used was discarded, and an indirect visualization method using secondary antibodies were used for further experimentations.


Day 1
FAK
PFAK
Vinculin
Actin
Control
Equiaxial
Uniaxial

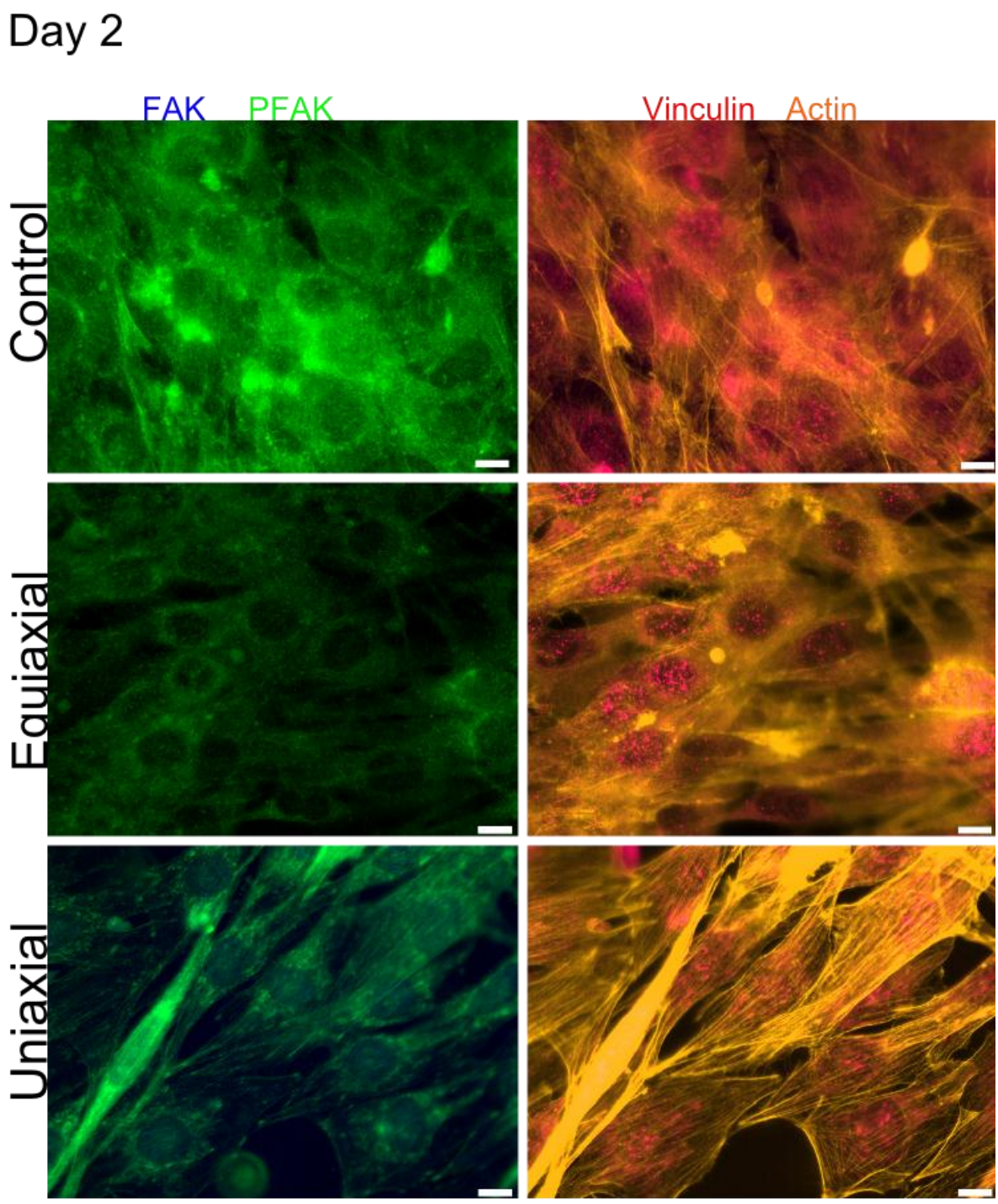
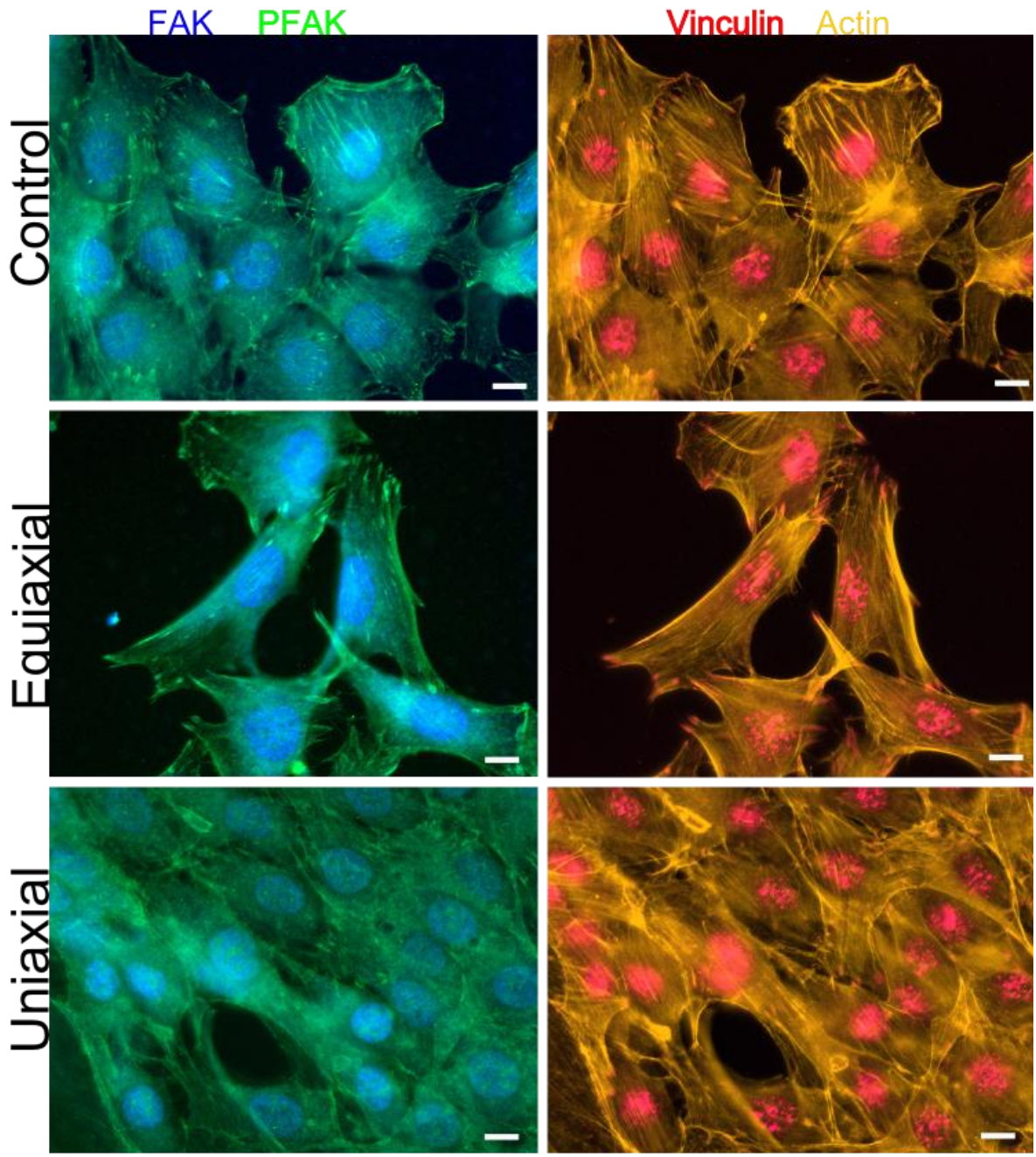


Figure 7 | Comparison of the effect of euqibiaxial and uniaxial cyclic strain on focal adhesion and FAK. Cells were seeded on a Flexcell plate and subject to 24 hours **(left)** or 48 hours **(right)** before fixing. The cells were stained for with Bodipy 558/568 phalloidin, Cy5 labelled anti-vinculin, Alexa Flour 350 labelled anti-FAK and Alexa Flour 488 labelled anti-pFAK. Red arrows depict co-localization of vinculin, FAK and pFAK. Scale bars denote 10 µm.

In Exp2 myoblasts were seeded on a Flexcell 6 well plate and after 48 hours induced and subjected to uni- or equibiaxial strain. After strain, the cells were fixed and stained for actin, vinculin, FAK and pFAK according to the indirect staining protocol **(Fig 8).**

At day 1 cells had obtained an elongated morphology for both strain conditions, while cells in control retained spindle-link morphology. It was common for all conditions that isolated cells were found to have spread to a greater extent than cells with cell-cell contact. It was further observed that isolated cells formed more distinct stress fibers. However, all cells had stress fibers with clustering of vinculin associated at the ends. Further associated with the stress fiber ends were both FAK and pFAK which, along with vinculin, formed elongated struktures that followed the curvature of the stress fiber ends. In addition to the distinguishable clusters, pFAK, FAK and vinculin was found throughtout the entire cytoplasm, and the latter two was observed in distinctively high levels in the nucleus. At day 2 cells in control conditions still retained their spindle-like shape, whereas cell subjected to equibiaxial strain had obtained a somewhat more elganted shape. The cells subjected to uniaxial strain, however, showed a non-elongated morphology similar to that of cells in control conditions. Stress fibers were easy distiguishable for all conditions, and as in day 1 a co-localization of vinculin, FAK and pFAK were observed at stress fiber ends.


Day 1
FAK
PFAK
Vinculin
Actin
Control
Equiaxial
Uniaxial

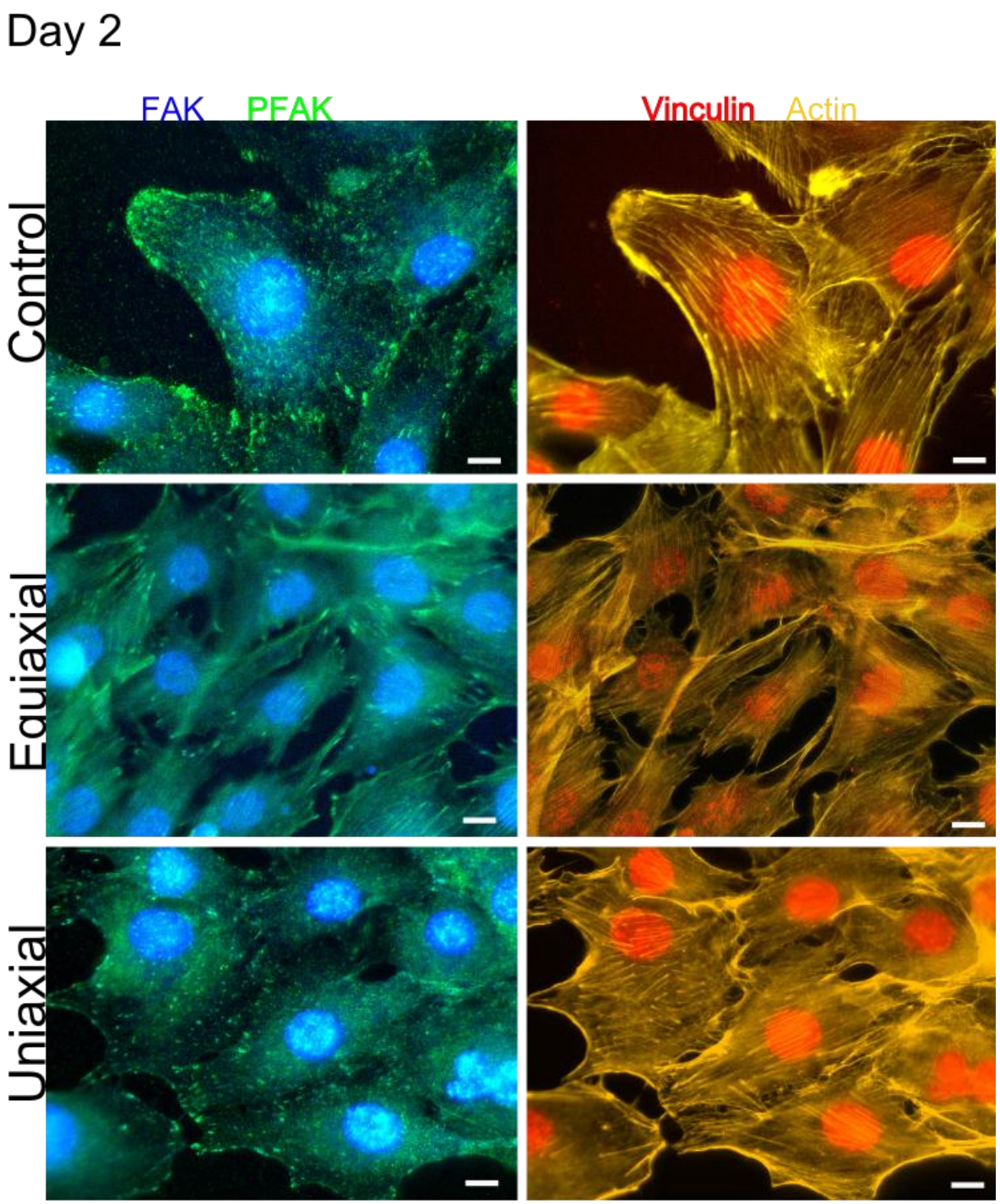


Figure 8 | Comparison of the effect of euqibiaxial and uniaxial cyclic strain on focal adhesion and FAK. Cells were seeded on a Flexcell plate and subject to 24 hours **(left)** or 48 hours **(right)** before fixing. The cells were stained for with Bodipy 558/568 phalloidin, Alexa Fluor 350 labelled anti-vinculin, Alexa Fluor 488 labelled anti-FAK and Cy5 labelled anti-pFAK. The obtained images were, after processing, pseudocoloured to facilitate comparison to earlier experiments. Red arrows depict co-localization of vinculin, FAK and pFAK. Scale bars denote 10 µm.

6 Discussion

In this work, methods for visualisation of focal adhesions and mediator of integrin signalling FAK have been established. Moreover it has been shown that the levels of these can been effected by extrinsic mechanical stimulation in form of CTS.

Myoblasts spread and form focal adhesions on plastic

To verify the presence and to establish a general comprehension of the *in situ* locations of focal adhesions in C2C12 cells, the cells were stained for vinculin. As mentioned previously vinculin facilitates actin-integrins interactions in focal adhesions, thus high density clusters of vinculin function as markers for focal adhesions. As seen in figure 5, clearly defined clusters were localized in conjunction with terminal ends of stress fibers. The presence of focal adhesions, thus anchoring the cell to the substrate, was expected since myoblasts will undergo anoikis when unable to form sufficient adhesion to the environment (Fujio et al., 2001). Studies have shown that stress fiber polymerization takes place from formed focal adhesions (Hsu et al., 2010; Katoh et al., 1995) which could explain the localization of vinculin to the ends of stress fibers. The fact that aligning nuclei was observed could indicate that the myoblasts had begun differentiating, however, as the cells only was cultured for 48 hours after seeding in GM this alignment may simply be caused by random reorientation of the myoblasts.

Focal adhesion kinase is recruited and activated in focal adhesions

As visualisation of focal adhesions, by vinculin, was established, the correlation between focal adhesions, FAK and pFAK was analyzed. As seen in figure 5 and 6, FAK and pFAK was found to co-localize to the same *in situ* locations as vinculin, hence recruited and activated in close proximity of focal adhesions, concurring with litterature (Frame, 2004; Schlaepfer et al., 1994). As the focal plane differed between the proteins of interest a z-stack was obtain to ensure optimal focus. As seen in figure 5 vinculin was observed closest to the substrate followed by actin and lastly FAK and pFAK. To

neutralize the difference in focal planes, the z-stacks were 3D-deconvolved as seen in figure 6. The obtained images revealed a distribution of both FAK and pFAK throughout the cytoplasm in addition to clearly identifiable co-localisation to vinculin. This concurs with data from Yim et. al. (2010) which has shown that in addition to the FAK and pFAK present in focal adhesion, a detectable amount is situated perinuclearly from where it is recruited to forming focal adhesions.

Myoblasts subjected to CTS change morphology and rearrange the cytoskeleton

To determine the effect different types of CTS on the focal adhesions and activativation of FAK two experiments was conducted and analysed with regards to morphology, state of differentiation, focal adhesion formation and FAK activation.

As shown in figure7 and 8 both uniaxial and equibiaxial strain affected the morphology. In both Exp1 and Exp2 cells subjected to either strain type had, from day 1, obtained an elongated shape compared to the spindle-like shape observed in control types. Furthermore the cells subjected to uniaxial strain appeared to orientate towards the same direction. These findings are coinciding with previous findings that uniaxial strained cells orientate uniformly perpendicular to applied strain (Pennisi et al., 2011a). It is theorised that this rearrangement of the cytoskeleton takes place when the strain pulsations exceed actomyosin' ability to retract after each impulse. Lack of retraction leads to degradation of the actomyosin fibers and in turn the focal adhesions. By formation of new focal adhesions the cell can rearrange perpendicular to the strain where less torsion is transferred to the actomyosin fibers (Hsu et al., 2010). For Exp1 the elongated morphology and reorientation were more profound at day 2. For Exp2 the changes in morphology and reorientation of cytoskeleton was diminished. As these features otherwise always was present, included in previously conducted experiments utilizing the exact same machine (Pennisi et al., 2011a; Pennisi et al., 2011b), it is thought that due to technical malfunction no strain was applied to the cells.

Unviable staining method for assessing focal adhesions and focal adhesion kinase

As it was shown in TCP experiments, visualisation of focal adhesions, FAK and pFAK was possible with the Zenon labelling kit. The same stain was utilized in Exp1, however, as seen in figure 7 no clear detection of clusters associated with focal adhesions was possible for vinculin, FAK or pFAK. For FAK, lack of specific signals resulted in a more pronounced background noise, making further analysis impossible. For vinculin and pFAK no clusters was found associated with stress fibers or cell membrane, instead vinculin was seen diffusely throughout the cell with dense clusters within the nuclei while pFAK showed complete co-localization with actin. These finding were contradicting to previous studies, were vinculin (Putnam et al., 2003) and FAK (Shikata et al., 2005) was found to form clusters at the cell periphery in cells subjected to CTS. Furthermore, as these findings were common for all experimental conditions the method was scrutinized. Reviewing the antibodies revealed massive bleed-through from actin to pFAK (data not shown), hence the Zenon antibody protocol was discarded. Instead the primary antibodies were detected by fluorescence labeled secondary antibodies in Exp2.

Focal adhesion is recruited to vinculin and phosphorylated

As seen in figure 8, clusters of vinculin were found associated to both stress fibers and the cell membrane with little difference between conditions or points in time. Co-localized to all vinculin clusters were both FAK and pFAK suggesting that FAK is recruited to the stress fibers where it is activated. However, a study made by Shikata et. al. (2004) contradicts this, as it was found that FAK is recruited to the cell periphery when cells were subjected to CTS. Furthermore Shikata et. al. showed that cells in control conditions had an accumulation of FAK to the stress fibers. This could imply, along with the lacking morphological changes, that the mechanical stimulation used in this experiment was insufficient to stimulate the cells.

7 Conclusion

In this work it has been shown that visualisation of focal adhesion in myoblasts is possible. Furthermore it shown has the integrin downstream effector FAK is recruited to these complexes where it is phosphorylated. Due to technical difficulties it was not possible to fully assess how these important mediators of mechanotransduction is affected by CTS and therefore further experimentations is needed.

8 Perspectives

In this work a few elements mechanotransduction have been investigated. The complexity in translating mechanical stimulus makes this an area with many years of research yet to come. Already the importance of mechanical stimulation has been shown, and it is therefore important to gain further insight into how this novel method can be used to improve cellular responses. In this experiment immunofluorescence was used to investigate protein interactions. Immunofluorescence functions as a valid tool to obtaining knowledge concerning *in situ* composition and interactions between different proteins. However, a drawback is the difficulties in quantifying observed events, especially with small structures as focal adhesions and FAK. Therefore it would beneficial to employ robust methods like western-blot or RT-qPCR as a quantifiable tool in addition to immunofluorescence. Furthermore, the long term aspects of CTS have to be investigated. Current research focuses on the immediate effect of CTS and little is known on how the cells react to longer durations. Therefore it would be beneficial to investigate how the mediators of mechanotransduction can affect long term cellular events as differentiation and proliferation.

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