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Effect of RGD functionalization and stiffness modulation of polyelectrolyte multilayer films on muscle cell differentiation ☆

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ABSTRACT

Skeletal muscle tissue engineering holds promise for the replacement of muscle damaged by injury and for the treatment of muscle diseases. Although arginylglycylaspartic acid (RGD) substrates have been widely explored in tissue engineering, there have been no studies aimed at investigating the combined effects of RGD nanoscale presentation and matrix stiffness on myogenesis. In the present work we use polyelectrolyte multilayer films made of poly(L-lysine) (PLL) and poly(L-glutamic) acid (PGA) as substrates of tunable stiffness that can be functionalized by a RGD adhesive peptide to investigate important events in myogenesis, including adhesion, migration, proliferation and differentiation. C2C12 myoblasts were used as cellular models. RGD presentation on soft films and increasing film stiffness could both induce cell adhesion, but the integrins involved in adhesion were different in the case of soft and stiff films. Soft films with RGD peptide appeared to be the most appropriate substrate for myogenic differentiation, while the stiff PLL/PGA films induced significant cell migration and proliferation and inhibited myogenic differentiation. ROCK kinase was found to be involved in the myoblast response to the different films. Indeed, its inhibition was sufficient to rescue differentiation on stiff films, but no significant changes were observed on stiff films with the RGD peptide. These results suggest that different signaling pathways may be activated depending on the mechanical and biochemical properties of multilayer films. This study emphasizes the advantage of soft PLL/PGA films presenting the RGD peptide in terms of myogenic differentiation. This soft RGD-presenting film may be further used as a coating of various polymeric scaffolds for muscle tissue engineering.

1. Introduction

Regenerative medicine and tissue engineering make use of injected cells or of biomaterials to support cell attachment and to provide them with the appropriate cues to guide their differentiation. Skeletal muscle tissue engineering holds the promise of the replacement of muscle damaged by injury following surgery or due to trauma and for the treatment of muscle diseases, such as muscular dystrophy or paralysis. Adult skeletal muscle progenitor cells are considered a powerful source for the generation of several tissues, especially skeletal muscle, [1] but also smooth muscle [2], bone [3,4] and fat tissue [5,6]. The process of muscle formation requires that muscle precursor cells become activated, proliferate, differentiate, and fuse together to form multinucleated myotubes. Proliferation and differentiation of skeletal myoblasts are mutually exclusive events, which are governed by the up-regulation of transcriptional activators [7]. A major limitation to the clinical application of muscle progenitors is rapid loss of their muscle stem cell properties once they are removed from their in vivo environment [8].

The development of skeletal muscles is known to depend on the interaction of muscle cells with their surrounding extracellular matrix (ECM) [9]. Transmembrane receptors like the dystrophin–glycoprotein complex are known to be important [10,11]. However, other transmembrane receptors of the integrin family [12] have been shown to be crucial for skeletal muscle development and function [13,14].

Tissue engineering requires a combination of engineering methods, cell biology and materials. Within this context, a goal of biomaterials scientists is to design biocompatible scaffolds in which

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cells can adhere, proliferate, differentiate and synthesize their own matrix to regenerate tissues. Adhesive properties can be provided either by grafting or by physically adsorbing cell adhesion molecules.

The tripeptide sequence arginylglycylaspartic acid (RGD) is present in many ECM proteins, including fibronectin, vitronectin, fibrinogen, von Willebrand factor, thrombospondin, laminin, osteopontin, bone sialo protein, and some collagen isoforms [15]. It binds to a wide range of integrin receptors in a non-selective manner, i.e. not specifically to a given integrin receptor. Several strategies can be applied to achieve better selectivity and/or target only one type of integrin receptor (for a review see Gribova et al. [16]). In vitro ligands containing the RGD peptide have already been used to increase the early adhesion of anchorage-dependent cells in the field of biomaterials [17]. This was especially targeted to osteoblasts on peptide-grafted poly(ethylene glycol) hydrogels [18], to fibroblasts on ethylene–acrylic co-polymer film with immobilized peptides [19] and to endothelial cells on polyurethane [20]. Self-assembled monolayers have also emerged as a method that allows the creation of discrete regions of controlled peptide identity and density within a bioinert background [21–23]. However, so far studies of muscle cell differentiation in vitro have used full-length ECM proteins such as fibronectin [24], laminin [25] or collagen [26], which are difficult to couple to synthetic or natural biomaterials. In addition, the functionality of these ECM proteins may be altered by adsorption or chemical coupling to materials. Mooney and co-workers [27] showed that RGD coupling improved the initial adhesion and enabled the differentiation of myoblasts cultured on two-dimensional (2-D) or inside three-dimensional (3-D) alginate gels.

Besides biochemical cues, matrix stiffness has also been shown to be an important parameter in regulating the function of various tissue and cell types in vivo and in vitro [28,29]. Indeed, a number of pathologies, including muscle pathologies, involve changes in matrix properties. In dystrophic muscles a more fibrotic tissue and increased stiffness of the diaphragm have been observed compared with a normal diaphragm [30].

It is now acknowledged that the mechanical properties of the substrate in vitro can affect muscle cell adhesion, spreading, proliferation and differentiation. This has been shown using different types of synthetic and natural materials, such as model synthetic polyacrylamide (PA) gels coated with collagen [26], poly(ethylene glycol) (PEG) hydrogels [31], alginate gels of varying stiffness [27] and polyelectrolyte multilayer films made of biopolymers [32]. Recently Post and co-workers [33] showed, using PA gels of varying stiffness and protein coating, that proliferation was influenced only by stiffness, whereas differentiation was influenced by both stiffness and protein coating.

However, to date there have been no studies aimed at investigating the combined effects of RGD nanoscale presentation and matrix stiffness in myoblast adhesion, proliferation and differentiation. Polyelectrolyte multilayer films [34] are currently emerging as a new kind of biomaterial coating that can be used to guide cell fate [35,36]. Advantageously, the architecture of the films, their biodegradability and bioactivity can be controlled [37]. The films can also be micropatterned to have (X–Y) architecture by combining them with microfabrication techniques such as photolithography, microcontact printing or microfluidics [38–40]. Furthermore, they can be deposited on various types of supporting materials, including metals, polymers and ceramics, which are already approved as implantable materials [37].

In this study we investigated the influence of substrate stiffness and RGD nanoscale presentation alone or in combination on C2C12 myoblast adhesion, proliferation and differentiation. To this end we cultured myoblasts on polyelectrolyte multilayer films made of poly(L-lysine) (PLL) and poly(L-glutamic) acid (PGA) whose stiffness can be tuned by chemical cross-linking [41]. Moreover, such films are of particular interest since they are made of biodegradable polymers and appear to be biocompatible [42].

In addition, presentation of a RGD-containing peptide was achieved by chemically grafting the peptide to PGA [43] and adsorbing it as the final layer of the film. Such covalent grafting provides a good control of surface composition, a stable link and limits release of the functional group into the culture medium.

We studied the combined effects of RGD nanoscale presentation and matrix stiffness on early adhesion of the myoblasts to their late differentiation in myotubes after 9 days in culture, until the formation of sarcomeres.

2. Materials and methods

2.1. Covalent grafting of RGD adhesion peptide to PGA (poly(L-glutamic) acid)

The type I collagen-derived peptide was chosen according to a published sequence that was shown to induce adhesion of human primary osteoblasts in vitro [43]. The 15 amino acid peptide containing a central RGD (Arg–Gly–Asp) sequence (Cys–Gly–Pro–Lys–Gly–Asp–*Arg–Gly–Asp*–Ala–Gly–Pro–Lys–Gly–Ala, CGPKGD*RGD*AGPKGA) was purchased from GeneCust (Dudelange, Luxembourg). The peptide was grafted as described previously [43]. Briefly, the first step consisted of grafting maleimide groups onto PGA (P-4886, Sigma) chains. To accomplish this grafting 60 mg of PGA were dissolved in 3 ml of a 10 mM HEPES buffer (pH 6.5) solution containing 20 mg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and 3 mg of N-hydroxysulfosuccinimide (sulfo-NHS) under an inert atmosphere (nitrogen gas) under magnetic stirring. Then 24 mg of N-(2-aminoethyl) maleimide trifluoroacetate was added. The reaction was allowed to proceed at room temperature for 24 h. After removal of the by-products by dialysis against water the PGA–maleimide was freeze-dried. The average number of maleimide groups bound to PGA was 16% (i.e. on average 16 maleimide groups every 100 repeating PGA units), as determined by ^{1}H NMR analysis. In the second step the PGA–maleimide was reacted with the peptide to form the PGA–RGD: 5 mg of PGA–maleimide were mixed with 5 mg of peptide in 1.5 ml of 10 mM HEPES buffer (pH 7.4) and maintained for 24 h under magnetic stirring at room temperature. An excess of mercaptopropionic acid was used to neutralize the unreacted maleimide groups. The solution was dialyzed against water and freeze-dried. The quantitative grafting ratio of the peptide was determined by ^{1}H NMR, and the effective degree of grafting was found to be 10%.

2.2. Polyelectrolyte solutions and PEM film build-up

Poly(L-lysine) (PLL) (P2636, Sigma) and PGA (P-4886, Sigma) were dissolved at 0.5 mg ml^{-1} in HEPES–NaCl buffer (20 mM HEPES, 150 mM NaCl, pH 7.4). For all experiments films were manually constructed in 96-well plates starting with a first layer of poly(ethyleneimine) (PEI) at 5 mg ml^{-1}. To deposit the subsequent polyelectrolyte layers 50 μl of the polyelectrolyte solution was deposited in each well and left for 8 min before being rinsed twice for 30 s and 5 min, respectively, with 100 μl of 150 mM NaCl (pH 6.5). This sequence was repeated until a $(PGA/PLL)_6$ film built up. Then the last layer of PGA (0.5 mg ml^{-1}) or of PGA–RGD (0.5 mg ml^{-1}) was added, giving $(PGA/PLL)_6$–PGA and $(PGA/PLL)_6$–PGA–RGD films.

In order to increase the stiffness the $(PGA/PLL)_6$–PGA films were chemically cross-linked to give $[(PGA/PLL)_6–PGA]_{CL}$ films. To obtain the cross-linked films functionalized with PGA–RGD cross-linking

was carried out after $(PGA/PLL)_6$ formation and PGA–RGD was added after cross-linking, giving $[(PGA/PLL)_6]_{CL}$–PGA–RGD films. For the cross-linking 100 μl of EDC/sulfo-NHS solution in 150 mM NaCl, pH 5.5 (mixed v/v with final EDC and S-NHS concentrations of 30 and 11 mg ml^{-1}, respectively) were deposited in the wells and incubated at 4 °C overnight. Finally the films were thoroughly washed with HEPES–NaCl buffer. The nomenclature of the films is given in Table 1.

2.3. Quartz crystal microbalance with dissipation monitoring (QCM-D)

Film build-up was followed using an in situ quartz crystal microbalance (QCM D300, QSense, Sweden) using a previously published procedure [44]. PLL, PGA and PGA with grafted RGD peptide prepared at 0.5 mg ml^{-1} in HEPES–NaCl buffer were successively injected into the cell. They were left to adsorb for 8 min and rinsed for 5 min with HEPES–NaCl buffer.

When a mass Δm is adsorbed on a crystal and the measurements are conducted in air the resulting decrease Δf typically obeys the Sauerbrey equation:

$$\Delta m = -C\Delta f/n$$

where C is the mass sensitivity constant (17.7 ng cm^{-2} Hz^{-1} at 5 MHz) and n is the overtone number.

2.4. C2C12 culture

C2C12 cells (from ATCC, used in passages 5–15) were maintained in polystyrene dishes in an incubator at 37 °C and 5% CO_2 and cultured in growth medium (GM) composed of Dulbecco's modified Eagle's medium (DMEM)/F12 medium (1:1) (Gibco Invitrogen, Cergy-Pontoise, France) supplemented with 10% fetal bovine serum (PAA Laboratories, Les Mureaux, France) containing 10 U ml^{-1} penicillin G and 10 μg ml^{-1} streptomycin (Gibco Invitrogen, Cergy-Pontoise, France). Cells were subcultured prior to reaching 60–70% confluence (approximately every 2 days). For all experiments C2C12 cells were first allowed to adhere in serum-free medium (SFM) composed of DMEM/F12 (1:1) supplemented with antibiotics. After 1 h adhesion the cells were fixed or the SMF was replaced by GM, depending on the type of experiment (see below). Cell were differentiated in a differentiation medium (DM) composed of DMEM/F12 (1:1) supplemented with 2% horse serum (PAA Laboratories, Les Mureaux, France) and antibiotics.

2.5. Cell adhesion, proliferation, migration and differentiation assays

For cell adhesion tests C2C12 cells were seeded at 15,000 cells cm^{-2} in 96-well plates and allowed to adhere in SFM for 1 h. For the short-term adhesion tests (1 h) cells were then fixed in 3.7% formaldehyde. For adhesion tests over 4 h the medium was changed to GM after 1 h and the cells were fixed after 4 h.

Cell proliferation was quantified by a 5-bromo-2′-deoxyuridine (BrdU) assay (Cell Proliferation Kit RPN20, GE Healthcare) following the manufacturer's instructions. Three time points were chosen, 4, 24 and 48 h. The cells were incubated with BrdU-containing labeling reagent for 1 h at 37 °C. Nuclei were counter-stained with Hoechst 3342 (Invitrogen). The images of BrdU-positive nuclei taken by phase contrast microscopy and the images of Hoechst-labeled nuclei taken using an inverted fluorescence microscope were merged to determine the ratio of BrdU-positive nuclei.

To follow cell migration C2C12 cells were mixed 1:1 with C2C12 HB1 GFP cells (kindly provided by E. Gomes, Institut de Myologie, Paris, France) and seeded at 15,000 cells cm^{-2} in 96-well plates. Images of the fluorescent nuclei were taken every 30 min for 5 h. For analysis at least 20 cells were tracked using ImageJ (v.1.45d, NIH, Bethesda, MD).

For differentiation assays cells were seeded at 30,000 cells cm^{-2} and allowed to adhere for 1 h in SFM. Cells were then grown for 1 day in GM and then switched to DM. The medium was changed twice a week. For the proliferation and differentiation tests in the presence of ROCK kinase inhibitor (Y-27632, Calbiochem) 5 μM inhibitor in DM was added on day 0. Fresh drug was then added every 24 h.

For the quantitative analysis of adhesion and differentiation at least 50 cells in at least 10 different fields (430 × 320 μm) were analyzed per condition. To characterize cell adhesion, cell number per field, cell area and circularity were quantified. Cell circularity is a parameter defined by the formula circularity = $4\pi(A/P^2)$, where A is the cell area and P the perimeter, which allows characterization of the cell morphology: a circularity value of 1.0 indicates a perfect circle, while decreasing values towards 0 indicate an increasingly elongated polygon.

Differentiation was characterized by the fusion index, which is the ratio of nuclei contained in myotubes to total number of nuclei [45], and by the percentage of striated myotubes.

2.6. Transfection by siRNA

Cells were transfected with siRNA against the β_1 and β_3 integrins (ON-TARGETplus SMARTpool®, respectively mouse ITGB1 and mouse ITGB3, Thermo Scientific Dharmacon) individually or at the same time, with a scrambled siRNA (All Stars negative control siRNA, Qiagen) being the control. For this the cells were seeded at 30,000 cells cm^{-2} in a 6-well plate and cultured in GM (2 ml per well) for 15 h. The transfection mix for one well was prepared by adding 6 μl of lipofectamine RNAiMAX Reagent (Invitrogen) to 305 μl of Opti-MEM medium (Gibco) and 0.72 μl of 1 mM siRNA to another 305 μl of Opti-MEM medium. The lipofectamine-containing mix was added to the siRNA-containing mix and incubated for 20 min at room temperature. Prior to transfection the GM in the wells was replaced by GM without antibiotics. Then 610 μl of the final mix were added to each well. After 24 h incubation at 37 °C the cells were transfected for a second time following the protocol described above and incubated for another 24 h. Then the cells were detached using trypsin–EDTA, seeded in GM at 20,000 cells cm^{-2} on the films built up in a 96-well plate and allowed to adhere for 4 h. Then the cells were fixed and their area quantified.

2.7. Immunostaining

Cells were first rinsed in phosphate-buffered saline (PBS) and fixed in 3.7% formaldehyde for 30 min at room temperature before

Table 1
Summary of the conditions used for the buildup of the films.

Film design			Film architecture	Film nomenclature
PGA	Cross-linking	PGA- RGD		
×			$(PGA/PLL)_6$-PGA	NCL
		×	$(PGA/PLL)_6$-PGA-RGD	NCL-RGD
×	×		$[(PGA/PLL)_6\text{-PGA}]_{CL}$	CL
	×	×	$[(PGA/PLL)_6]_{CL}$-PGA-RGD	CL-RGD

being permeabilized in 0.5% Triton X-100 for 4 min. After rinsing with PBS the samples were incubated for 1 h in 0.1% bovine serum albumin in Tris-buffered saline (TBS) (50 mM Tris, 150 mM NaCl, 0.1% NaN_3, pH 7.4). Actin was labeled with phalloidin–TRITC (1:800, Sigma) for 30 min. Cell nuclei were stained with Hoechst 33342 (Invitrogen) at 5 μg ml^{-1} for 10 min. After incubation with the primary antibodies (diluted in 0.2% gelatin in TBS) for 30 min at room temperature the cells were washed three times with TBS and incubated for 30 min with the secondary antibodies. Primary antibodies: rabbit anti-FAK pY397 antibody (1:200) (Invitrogen), rabbit anti-myogenin antibody (1:30) (Tebu-Bio) and mouse anti-myosin heavy chain (1:500) (Sigma). Secondary antibodies: Alexa-Fluor 488- or Alexa-Fluor 568-conjugated antibodies (Invitrogen) were used at 1:1000. Images were taken using a Zeiss Axiovert 200 inverted or Zeiss LSM 700 confocal microscope.

2.8. Statistics

The results represent three independent experiments. Data are reported as means ± standard deviations. Statistical comparisons were performed using SigmaPlot v. 11.0 software and based on an analysis of variance (ANOVA) followed by an appropriate pairwise comparison or comparison versus control group procedure ($P < 0.05$ considered significant). Statistically different values are reported in the figures.

3. Results

3.1. Density of RGD peptide on the film surface

The principle of film build-up with or without subsequent cross-linking (CL) and/or functionalization with the RGD peptide is depicted in Fig. 1A. The four different film architectures studied are given in Table 1: NCL, NCL-RGD, CL and CL-RGD. The film build-up was followed in situ using a quartz crystal microbalance with dissipation monitoring (QCM-D) (Fig. 1B), which allowed measurement of the film thickness using the Voigt model [46]. Film thickness was 120 nm for a film made up of six layer pairs.

Knowing the grafting density of the RGD peptide to PGA (10%), the amount of RGD peptide present on the film surface was quantified. The adsorbed mass of PGA–RGD was 400 ng cm^{-2}, which corresponded to a RGD surface density of 0.78 molecules of peptide per nm^2 (300 pmol cm^{-2}). This is a relatively high surface coverage. The Young's modulus of these films deposited on a thick polyelectrolyte cushion has previously been measured to be 51 ± 17 kPa for NCL films and 230 ± 70 kPa for CL ones [41].

3.2. Effect of RGD functionalization and film cross-linking on C2C12 myoblast adhesion, spreading and morphology

Adhesion is the first and a very important step in cell–substrate interactions, which is especially important for anchorage-dependent cells. To evaluate the effect of film stiffness and RGD functionalization on C2C12 myoblast adhesion cells were cultured on the four different types of films, NCL, NCL-RGD, CL and CL-RGD. The cells were allowed to adhere for 1 h in serum-free medium (SFM) to eliminate any effect of serum on early adhesion. The number of adherent cells as well as their spreading and morphology (circularity) were evaluated. Actin and nuclear staining of C2C12 cells (Fig. 2A) revealed the presence of adherent cells on the NCL-RGD, CL and CL-RGD films, but only very few on the NCL film. In addition, these cells were poorly spread. Quantitative measurements of the number of adherent cells confirmed the microscopy observations, with no statistical differences between NCL-RGD, CL and CL-RGD (Fig. 2B). However, after 1 h the cell area was significantly higher on films presenting PGA–RGD (Fig. 2C) compared with those with PGA only. Cells also spread over about twice the area on CL films compared with NCL ones. Thus both substrate stiffness and RGD functionalization had an effect on cell spreading. The circularity index was significantly lower for films containing the RGD

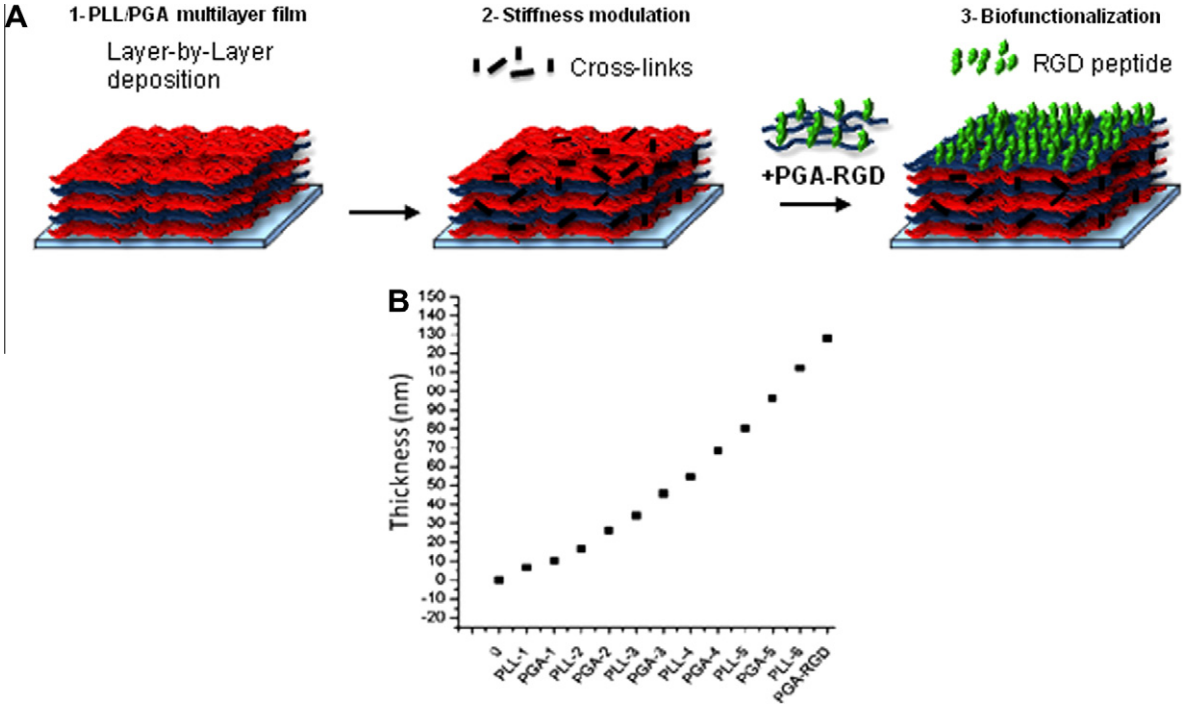

Fig. 1. Design of a biomimetic thin film combining physical and biochemical cues. (A) (1) A polyelectrolyte multilayer film (PEM) is built up on a substrate by alternating deposition of PLL and of PGA. (2) The PEM film can be covalently cross-linked using a water-soluble carbodiimide to modulate its stiffness. (3) Biochemical functionality is provided by adding a final layer of PGA on which is grafted a RGD-containing peptide. (B) Exponential growth of the film followed by QCM-D.

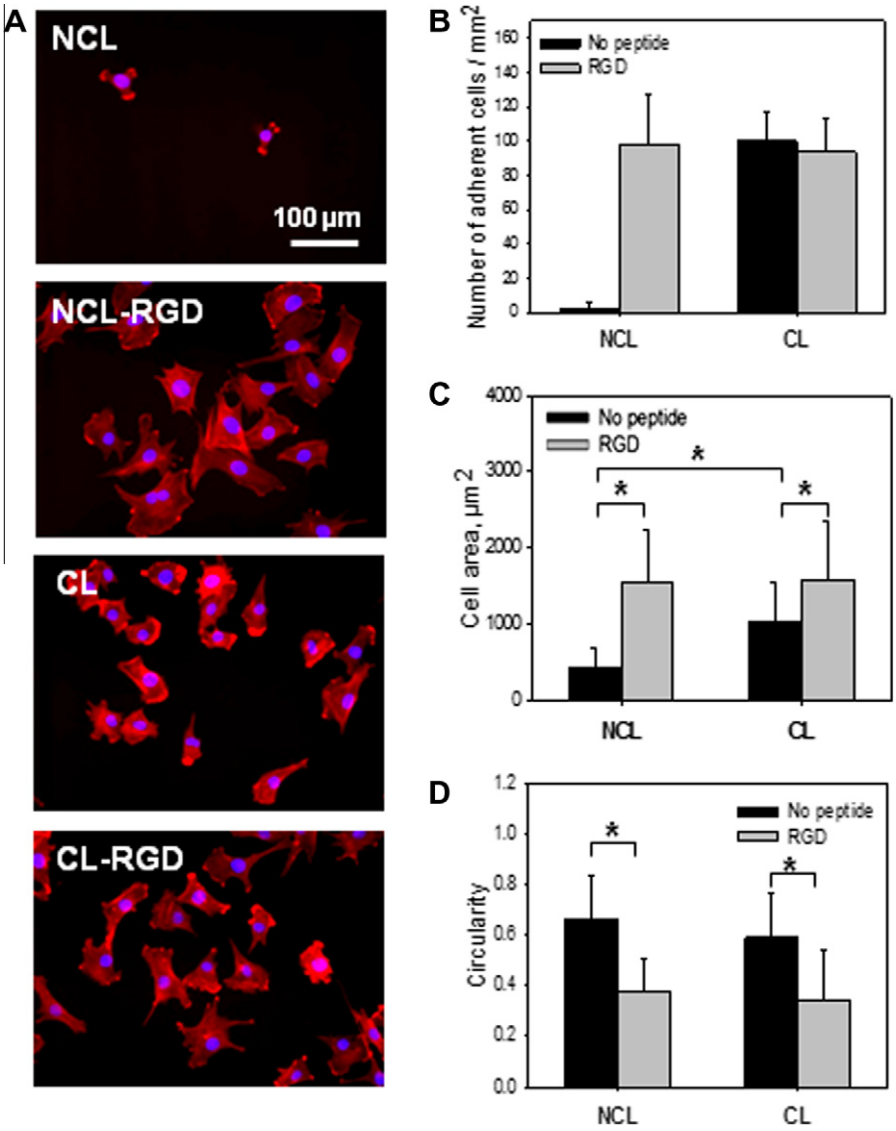

Fig. 2. Adhesion and spreading of C2C12 myoblasts at early times. Initial C2C12 cell adhesion and spreading were observed 1 h after plating the cells on NCL, CL, NCL-RGD and CL-RGD films. (A) Actin (red) and nuclei (blue) staining of C2C12 cells to visualize adhesion and spreading on the four types of films. (B) Number of adherent cells. (C) Spreading area. (D) Cell circularity quantification. Error bars correspond to SD, $^{*}P < 0.05$.

peptide compared with films without peptide (NCL and CL) (Fig. 2D). This indicates that the presence of RGD but not film stiffness influences the cell circularity.

As only a few cells adhered to NCL films these films were discarded from subsequent experiments on cell differentiation. It is interesting to note that they constitute a "blank slate" [35] for the specific effect of RGD grafting on PGA.

3.3. Characterization of adhesion via integrin receptors and cell migration

The cells are mechano-sensors that actively sense their environment via specific cell surface receptors, especially integrins [47]. Several integrins can interact with the RGD ligand, especially $\alpha5\beta1$ and $\alpha V\beta3$. Cell sensing and integrin activation lead to the formation of focal adhesions (FA), sites where the cells are in contact with the matrix and exert cell traction [48,49]. FA, which are enriched in integrins and in several other proteins, are linked to actin stress fibers.

To characterize cell interaction with the substrate via integrin receptors the formation of stress fibers and the presence of focal adhesions were first analyzed in C2C12 cells cultured on the different films for 4 h (Fig. 3A and B). Focal adhesions were visualized by labeling phosphorylated focal adhesion kinase (pFAK), an important component of mature FA (Fig. 3A). Robust focal adhesions or even fibrillar adhesions (small dashes at the cell periphery) only formed on the NCL-RGD films, while only small and thin focal adhesions or focal complexes (small dots) were visible on the CL and CL-RGD films (Fig. 3B). These results showed that both film stiffness and RGD functionalization played a role in the organization of the actin cytoskeleton and also in the formation of focal adhesions. The presence of the adhesive ligand on soft films led to the formation of numerous focal and fibrillar adhesions, while only focal complexes formed on the stiffest films, even in the presence of RGD.

As stress fibers and focal complexes/adhesions play a key role in cell migration we investigated whether cell migration is influenced by the substrates. To this end cell migration on the different films 5 h after cell seeding was followed by tracking individual cells. The

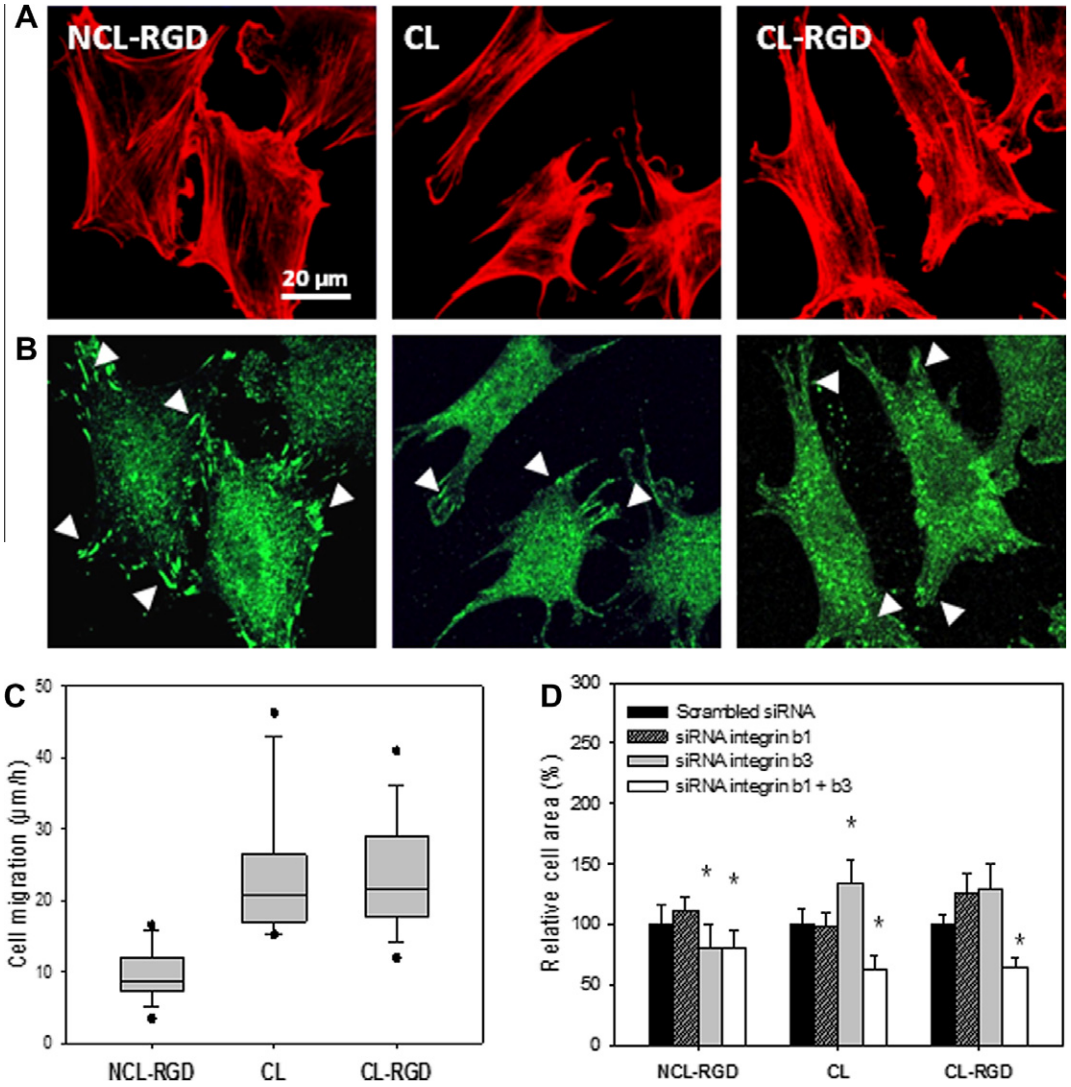

Fig. 3. Effect of film stiffness and RGD functionalization on cytoskeletal organization, focal adhesion and migration. (A) Staining of actin cytoskeleton (red) after 4 h culture. (B) Staining of phosphorylated focal adhesion kinase (pFAK Y397, green) after 4 h culture. (C) Myoblast migration measured over 5 h after seeding. (D) Effect of blocking β_1 and/or β_3 integrins using siRNA: quantification of the cell area after 4 h adhesion ($^*P < 0.05$ compared with scrambled siRNA). Focal adhesions/complexes are indicated by white arrowheads.

migration speed results on the different films are shown in Fig. 3C. Cells migrated at ~20 μm h^{-1} on stiff films (CL and CL-RGD), which was about twice as fast as cells on NCL-RGD films (10 μm h^{-1}). Thus the decrease in focal adhesions on stiff films (CL and CL-RGD) correlated with enhanced migration on these films.

In order to investigate the possible role of β-chain integrins in cell adhesion knockdown of β_1 or β_3 integrin or both using the siRNA approach was studied. These integrins are known to be involved in cell mechano-sensing and both are present in C2C12 myoblasts [50,51]. The cell area of the transfected cells was quantified after 4 h adhesion (Fig. 3D). On NCL-RGD films only β_3 blocking, not β_1 blocking, led to a slight decrease in cell spreading. Double tranfection of β_1 and β_3 siRNA gave similar results. These data suggest that myoblast interaction with the RGD-containing peptide partly involves β_3 integrins. On CL and CL-RGD films siRNA against β_1 or β_3 alone did not decrease cell spreading, and even significantly increased it in the case of siRNA β_3 treatment on the CL film. However, when the cells were transfected with both β_1 and β_3 siRNA the cell area decreased significantly on the CL and CL-RGD films, suggesting that both the β_1 and β_3 integrins are used exchangeably by the cells to interact with these films. Thus it appears that the integrins involved in cell spreading on the different films differ.

3.4. Effect of film cross-linking and RGD functionalization on myogenic differentiation

C2C12 myoblasts are a well-known model for the in vitro study of myogenic differentiation due to their ability to reproduce processes that take place during in vivo differentiation of skeletal muscle progenitors [52]. The effect of film stiffness and presentation of the RGD ligand on myoblast differentiation in myotubes were studied over 9 days. Phase contrast microscopic images of myogenic differentiation are shown in Fig. 4A. The formation of myotubes was observed on NCL-RGD films, while cell aggregation followed by detachment occurred on both the CL and CL-RGD films, after 1–2 days on CL-RGD films and after 2–3 on CL ones. Some detached cells were able to form aggregates that remained adherent until day 9 of differentiation.

Staining of the myosin heavy chain (MHC), a late marker of myogenic differentiation [7], was used to characterize myogenic differentiation and the formation of myotubes. MHC was well-

expressed only in myotubes cultured on NCL-RGD films, while its expression on the CL and CL-RGD films was very weak (Fig. 4B). The fusion index was 25% for NCL-RGD films and only 8% for CL and 3% for CL-RGD films (Fig. 4C). Thus only small fractions of the remaining cells adherent on CL and CL-RGD films were able to fuse. Moreover, the multinucleated cells on these films did not have the typical elongated morphology of myotubes nor were they striated. Only cells grown on NCL-RGD films reached 67% striation of the myotubes, indicating very good maturation (Fig. 4B, upper image of right column).

These results show that the soft RGD functionalized film used in our study was the only film architecture enabling myoblast fusion and differentiation in an efficient manner. Conversely, the CL and CL-RGD films were inappropriate for long-term differentiation of myotubes.

3.5. C2C12 myoblast poor differentiation on stiff films is associated with decreased myogenin expression and enhanced proliferation

For skeletal myoblasts cell cycle arrest is necessary to undergo differentiation. During myogenic differentiation a highly ordered process of temporally separable events takes place, that begins with the expression of myogenic transcription factors and is followed by cell cycle arrest [7]. In order to further understand the origin of the inappropriate differentiation on stiff films we quantified expression of the transcription factor myogenin at early times in the differentiation process (days 1–3) (Fig. 5). On NCL-RGD films myogenin was already expressed on day 1 (4% of nuclei) and steadily increased until day 3 (13% of nuclei). On CL films only 1% of nuclei were myogenin-positive on day 1 and 5% on day 3. On CL-RGD films no positive nuclei were detected on day 1 or 3, but about 2.5%

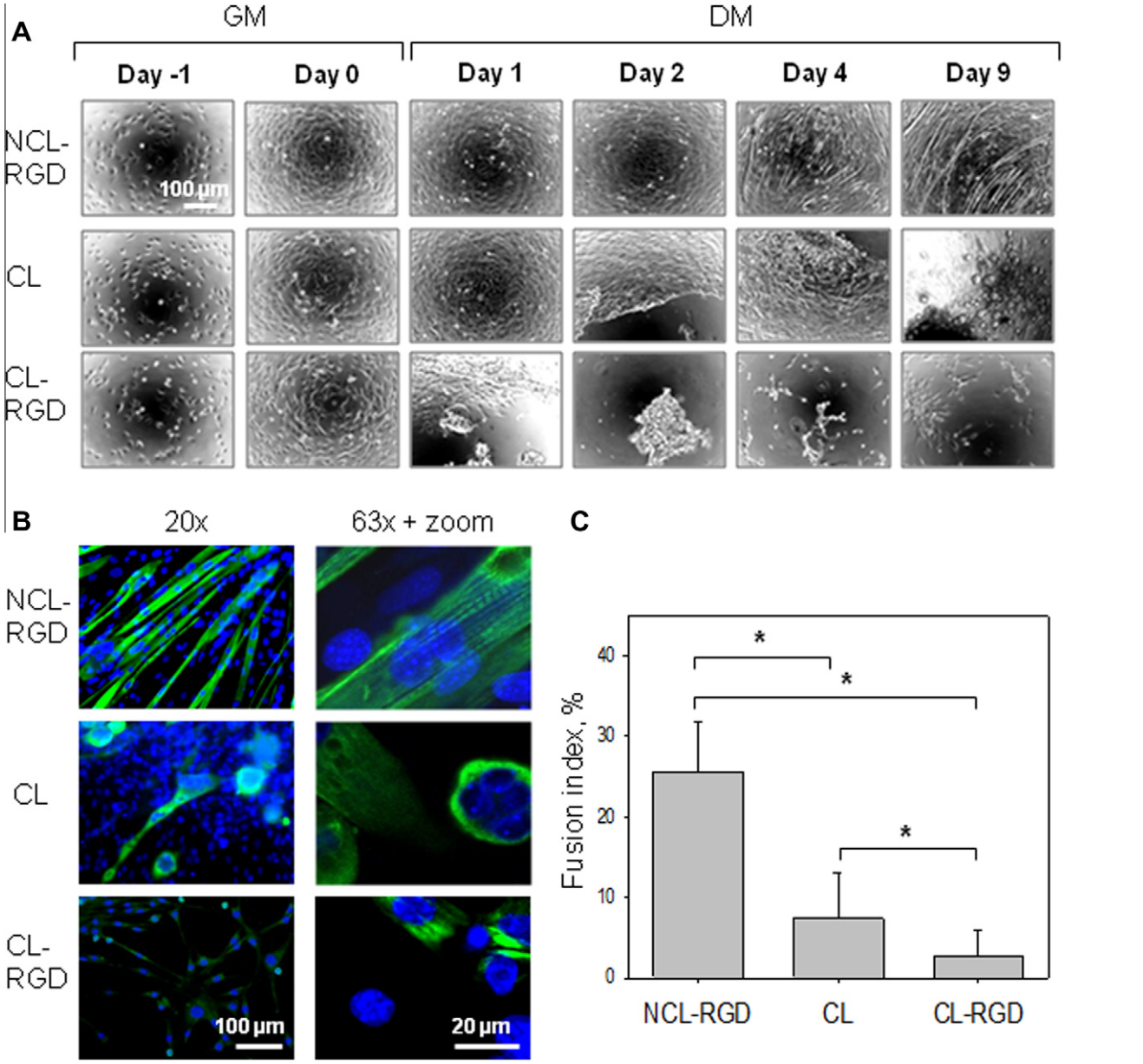

Fig. 4. Myogenic differentiation of C2C12 myoblasts is effective on NCL-RGD films. (A) Phase contrast microscopy observations of C2C12 cell differentiation on the NCL-RGD, CL, and CL-RGD films. After 24 h proliferation in GM (i.e. day −1) the cells were placed in DM (i.e. day 0) and were allowed to differentiate until day 9. Cell detachment was observed on CL and CL-RGD films after a few days in DM. (B) Myosin heavy chain (green) and nuclei (blue) labeling (20× and 63× magnification). (C) Quantification of the fusion index. Error bars correspond to SD, $^{*}P < 0.05$.

myogenin-positive cells were observed on day 2. These results show that myogenin expression is decreased on stiff films, especially on CL-RGD films, where no positive nuclei were found on day 3.

As down-regulation of proliferation is needed for myogenesis to occur [7], we also quantified cell proliferation on the different types of films before and after addition of DM (days −1 to 1) using a BrdU incorporation assay (Fig. 6). Days −1 and 0 represent the initial growth phase in GM 4 and 24 h after cell seeding, respectively, before switching to DM. No difference in the percentage of proliferating cells between the three types of films was observed in GM (Fig. 6B). However, significant differences were observed after 24 h culture in DM, termed here day 1. As anticipated, the rate of cell proliferation decreased dramatically on NCL-RGD films from 50% to 10%, which corresponded to cell cycle arrest. Conversely, on the CL and CL-RGD films these rates not only did not decrease but increased to reach ~90%. As a consequence, on days −1 and 0 the total cell number on the three types of film was similar (Fig. 6C), but on day 1 the number of cells on the stiff films was higher than on NCL-RGD. The highest cell number was found on the CL-RGD film.

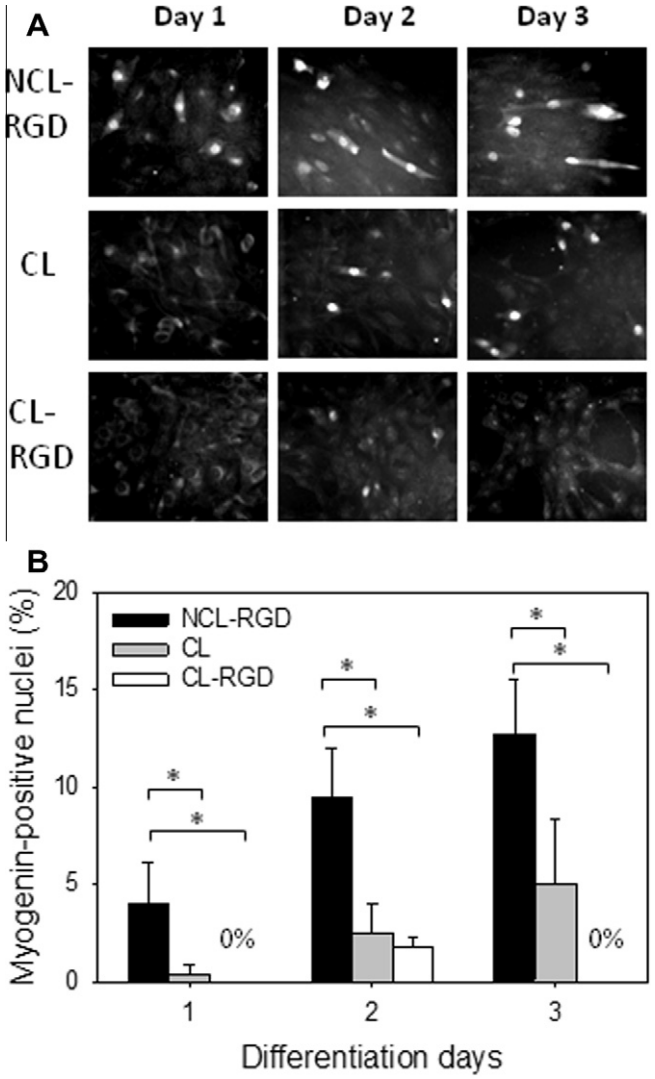

Fig. 5. Myogenin expression is decreased on stiff films. After 24 h proliferation in GM the medium was changed to DM and the cells were allowed to differentiate for 3 days. (A) Myogenin labeling on days 1, 2 and 3 of differentiation. (B) Quantification of the percentage myogenin expressing cells. Error bars correspond to SD, $^{*}P < 0.05$.

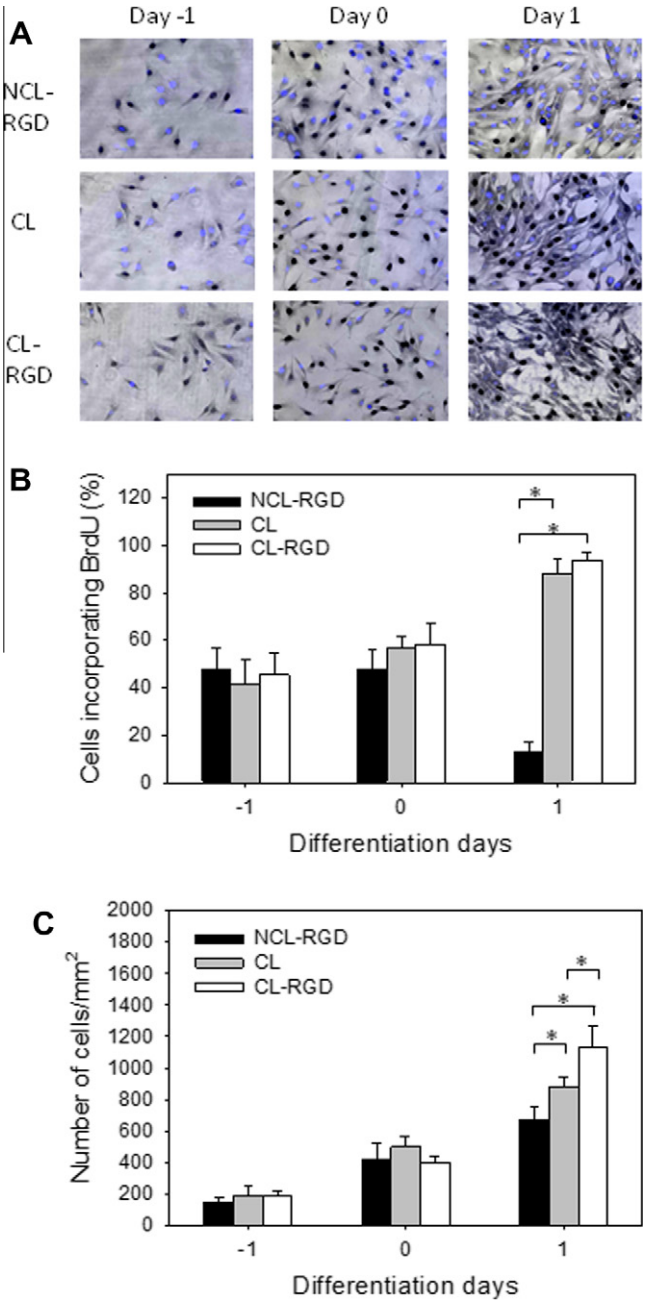

Fig. 6. Proliferation is enhanced on CL films resulting in an increased cell number. After 24 h proliferation in GM (day −1) the medium was changed to DM (day 0) and the cells were allowed to differentiate for 1 day. (A) Staining of BrdU in the nuclei (in black) associated with fluorescent labeling of the whole nucleus (blue). (B) Percentage of BrdU-positive cells. (C) Quantification of the total number of adherent cells. Error bars correspond to SD, $^{*}P < 0.05$.

These results show that two key events in myogenic differentiation, i.e. myogenin expression and cell cycle arrest, were altered on stiff films. Cells on stiff films bypass the cell cycle exit induced by growth factor deprivation. The cell detachment observed on stiff films may thus be due to enhanced proliferation leading to excessive cell confluence, and/or to enhanced cell migration.

3.6. Effect of inhibition of ROCK kinase on myogenin expression and cell proliferation and differentiation on stiff films

Previous studies have shown that myoblast differentiation is regulated through Rho/ROCK pathways that must be down-

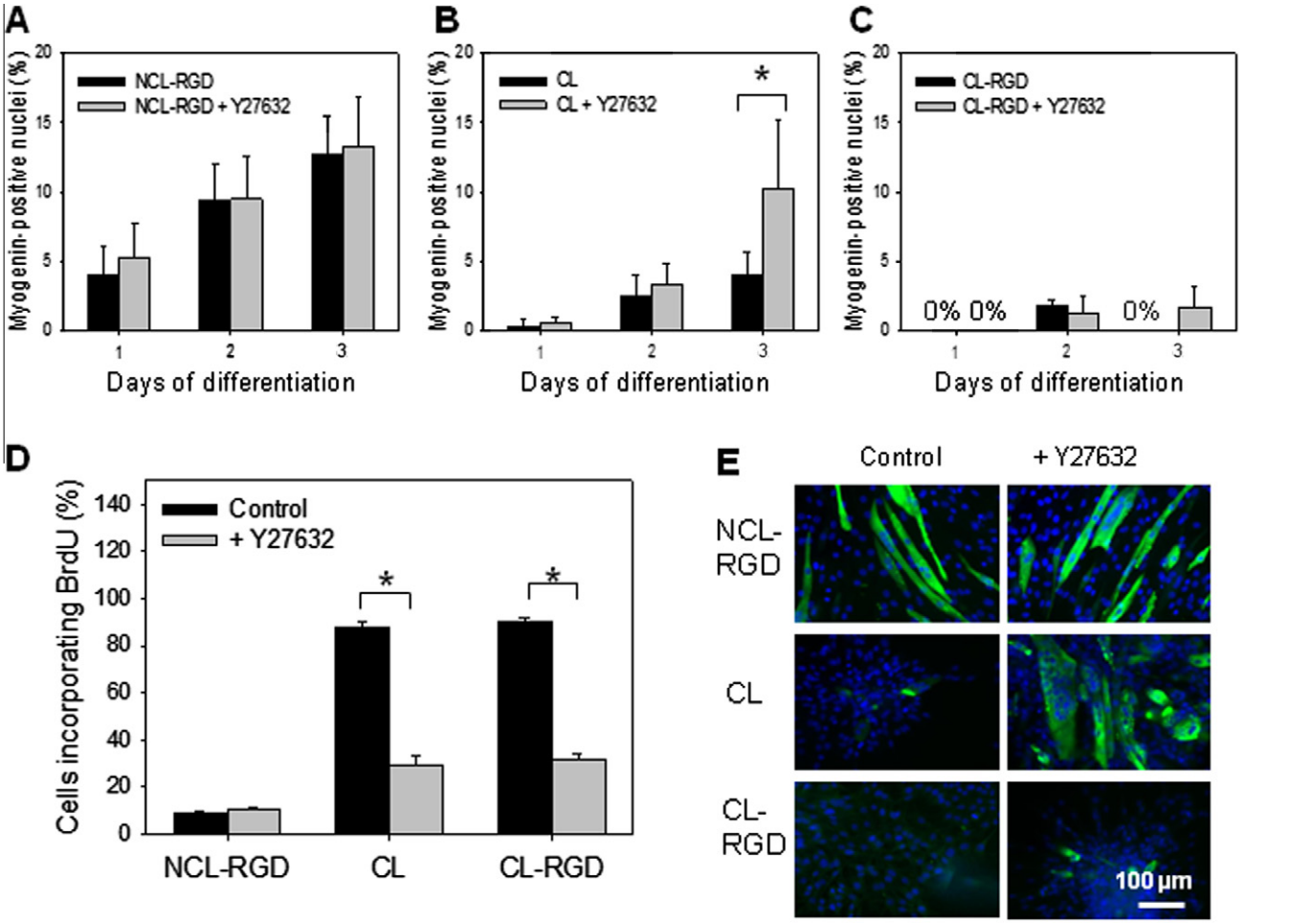

Fig. 7. ROCK kinase inhibition decreases myoblast proliferation and rescues differentiation on CL films. After 24 h proliferation in GM the cells were transferred to DM and allowed to differentiate for 6 days. (A–C) Myogenin labeling on days 1, 2 and 3 in DM. (D) Percentage of BrdU-positive cells on day 1 of differentiation. (E) Myosin heavy chain labeling on day 6 of differentiation. Error bars correspond to SD, $^{*}P < 0.05$.

regulated to allow myogenesis [53]. These authors showed that constitutive activation of ROCK resulted in decreased myogenin expression and inhibition of myogenic differentiation in C2C12 cells, while inhibition of ROCK led to an accelerated exit from the cell cycle and induced myogenin expression. These data suggest that ROCK is involved in maintaining myoblast cycling and prevents commitment to differentiation [54]. We thus hypothesized that decreased myogenin expression and excessive proliferation on CL films, resulting in poor differentiation, may be due to enhanced ROCK activity. To investigate whether the ROCK pathway was involved in inappropriate cell differentiation on stiff films, the ROCK inhibitor Y27632 was added on day 0 of differentiation. Its effect on myogenin expression was evaluated on days 1, 2 and 3, and on proliferation on day 1. In addition, MHC was labeled on day 6.

First we studied the effect of a ROCK inhibitor on myogenin expression (Fig. 7A–C). On NCL-RGD films Y27632 treatment did not alter myogenin expression at any time (Fig. 7A). On CL films a 2-fold increase in myogenin-positive nuclei was observed after 3 days treatment, reaching 10% (Fig. 7B). On CL-RGD films myogenin expression remained very weak, at less than 3% for all conditions. However, it was still present on day 3 when treated with ROCK inhibitor, while it was absent in the control (non-treated) condition (Fig. 7C).

The effect of Y27632 on C2C12 myoblast proliferation on day 1 was also tested (Fig. 7D). There was no difference for NCL-RGD films, but we observed a 3-fold decrease in BrdU-incorporating cells on the CL and CL-RGD films. This indicates that ROCK was involved in the excessive C2C12 proliferation on these films.

MHC expression on NCL-RGD films was not modified by Y27632 treatment (Fig. 7E). In contrast, on CL films Y27632 treatment was sufficient to prevent cell detachment and allow myogenic differentiation. Of note, the myotubes formed were not as elongated as on NCL-RGD films and the nuclei were more clustered. Finally, no differentiation was observed on CL-RGD films upon Y27632 treatment, suggesting that ROCK inhibition alone was insufficient to restore the differentiation program, and that a more complex molecular mechanism was involved in the cell response to these films.

4. Discussion

It is becoming increasingly clear that both the mechanical and biochemical properties of the substrate play an important role not only in cell adhesion but in many other processes, such as proliferation and differentiation [29]. However, the contribution of each type of signal, i.e. substrate stiffness and adhesive ligand, is not always easy to decouple. Mechano-sensitivity studies often use synthetic hydrogels such as PA with ECM proteins or PEG onto which are grafted ECM fragments [55]. It is already acknowledged that the mechanical properties of the substrate can affect muscle cell adhesion and differentiation. This has been observed on PA

surfaces [26], PEG hydrogels [31] and on PEM films made of PLL and hyaluronan [32,56]. However, little work has been done on the role of RGD-containing peptides in myogenic differentiation. Rowley and Mooney [57] showed that the RGD peptide is necessary to promote myoblast attachment to alginate hydrogels and that myoblasts only differentiated on alginate gels with a specific combination of monomeric ratio and RGD grafting density [57]. In addition, RGD peptides were found to significantly improve myoblast cell adhesion to grooved polystyrene substrates [58].

Here we used layer by layer films made of polypeptides as modular substrates, which can be stiffened by chemical cross-linking and can be specifically functionalized by grafting a RGD-containing peptide onto PGA [43]. In a previous study the 15 amino acid collagen type I-derived peptide containing an RGD adhesive sequence has been tested for both short-term adhesion properties and long-term proliferation of primary osteoblasts [43]. In the present work four different types of films, with or without cross-linking and with or without the RGD-peptide, allowed investigation of the effect of mechanical and biochemical signals and their combinations on important events of myogenesis. We especially focused on the sequence of events involved in C2C12 cell differentiation, including early adhesion, migration, proliferation, differentiation and fusion of myoblast into myotubes.

The results obtained by QCM-D regarding (PLL/PGA) film growth (Fig. 1) are consistent with those obtained previously by optical waveguide light mode spectroscopy on the same films [43], with, however, a higher thickness measured by QCM-D due to the water incorporated in the films.

While the cells spread more on the RGD-functionalized films, a more detailed analysis of cell interaction with the substrates showed that the stiffness was also very important: only cells on NCL-RGD films exhibited the formation of robust focal adhesions and migrated at low speed. The presence of only small focal complexes on stiff films (CL and CL-RGD) (Fig. 3) correlated with enhanced migration on these films Fig. 3. Our results also suggest that soft films with attached RGD moieties and stiff films recruit different combinations of integrin receptors: while β_3 knockdown alone had an effect on myoblast spreading on NCL-RGD films, knockdown of both β_1 and β_3 is required to affect myoblast spreading on CL and CL-RGD films (Fig. 3D). However, inhibition of cell spreading on the different films was never complete on blocking β_1, β_3 or both integrins at the same time (Fig. 3D), suggesting that other integrin or non-integrin receptors may be involved. It has been shown using epithelial cells that the β_1 and β_3 integrins promote different migration modes: adhesion induced by β_3 resulted in static cell–matrix adhesion and persistent migration, while adhesion induced by β_1 promoted highly dynamic cell–matrix interactions and random migration [59]. These results indicate a link between cell surface interactions via specific integrin receptors, focal adhesion dynamics and cell migration. Our results on myoblasts show that there is a correlation between integrin recruitment, the size of focal adhesions/complexes and cell migration speed: involvement of β_3 correlates with robust adhesion and low migration speed, while recruitment of both β_1 and β_3 are related to smaller adhesion complexes and enhanced cell migration (Fig. 3). Our results thus suggest that the control of motile strategy by integrins may be a common feature of different cell types.

Interestingly, β_3 integrin was found to be crucial for myogenic differentiation of C2C12 myoblasts and to mediate satellite cell differentiation [51], while β_1 integrin, which is constitutively expressed in skeletal muscle, was earlier shown to be dispensable for myogenesis [60]. This is in agreement with our data showing that on PEM films differentiation was only possible on soft films with attached RGD peptide. The stiffest films offered unfavorable conditions for differentiation as myogenin expression was decreased on stiff films (Fig. 7) but proliferation was enhanced (Fig. 6). Moreover, the cells detached from stiff films after few days in DM. We hypothesize that cell detachment may be due to enhanced proliferation leading to excessive cell confluence and/or to enhanced cell migration. ROCK kinase, which is known to be involved in myogenic differentiation, but also in cell blebbing, may be responsible for cell detachment (for a review see Fackler and Grosse [61]).

Interestingly, myoblast differentiation could be partially rescued on CL films by treatment with a ROCK inhibitor, which decreased the level of proliferation and increased myogenin expression (Fig. 7B). However, on CL-RGD films even those myoblasts treated with ROCK inhibitor which showed a decreased level of proliferation were still unable to express myogenin and to differentiate (Fig. 7C). Enhanced ROCK activity on stiff films may be a consequence of the engagement of β_1 integrin on these films, since it has been reported that β_1 induces a higher RhoA activity than β_3 [59]. Both RhoA and its effector ROCK play a crucial role in myogenic differentiation, as both activities must be down-regulated to allow myogenesis to occur [53,54,62,63].

In our previous work [32] we evaluated the adhesion and differentiation of C2C12 myoblasts using PLL and hyaluronan (HA) multilayer films of varying stiffnesses. On these films the formation of focal adhesions was greater on stiff films compared with soft ones. Evaluation of the adhesive behavior during the initial steps of spreading showed that blocking β_3, but not β_1, integrins inhibited cell adhesion on stiff PLL/HA films [56]. This differs from cross-linked PLL/PGA films, which are stiffer than PLL/HA films, for which blocking of both the β_1 and β_3 integrins was necessary to inhibit cell spreading. Thus the molecular mechanisms of cell–film interactions involve different integrins depending on the film type, film stiffness and presence of a specific ligand (RGD peptide).

Cell adhesion to ECM influences cell proliferation by transducing signals via cell surface integrin receptors, and proliferation is generally low in soft matrices and high in stiff matrices [64,65]. Proliferation of C2C12 myoblasts is also greater on stiff substrates compared with soft ones [27,32]. Here we have shown that the proliferation of C2C12 cells was significantly increased on stiff films via activation of ROCK (Fig. 7). The Rho/ROCK pathway is also known to be implicated in the remodeling of focal adhesions and migration of tumor cells [66]. Similarly, tumor malignancy and invasion is associated with matrix stiffening [67]. In this context, C2C12 cells on stiff films seemed to acquire some features of cancer cells: they bypassed the cell cycle exit induced by growth factor deprivation and showed an absence of mature focal adhesions and enhanced migration. Thus CL and/or CL-RGD films may be used as a model basement membrane for studies of cancer cell behavior in response to matrix stiffening. Decreased myogenin expression and the absence of exit from the cell cycle observed on cross-linked films could also be a potential tool for in vitro amplification of satellite cells while preserving their multiple differentiation potential. Indeed, these primary cells rapidly lose their stem cell properties and switch to differentiation upon removal from their niche and culture in vitro [8].

Based on our experimental data we propose a model for the interplay between mechanical and biochemical stimuli during the induction of C2C12 myogenic differentiation (Fig. 8). Adhesion on NCL-RGD films involved β_3 integrins and provided favorable conditions for myogenic differentiation of C2C12 cells. Adhesion on stiff films (CL and CL-RGD) involved β_1 integrins in addition to β_3 integrins and promoted ROCK activation. This led to a high proliferative state without myogenic differentiation. On CL films ROCK inhibition allowed myogenic differentiation. However, on CL films with attached RGD peptides ROCK inhibition was insufficient to induce myogenin expression and allow cell differentiation. We suggest that a mechanical signal (stiffness) by CL-RGD films may affect cell interaction in combination with a biochemical signal

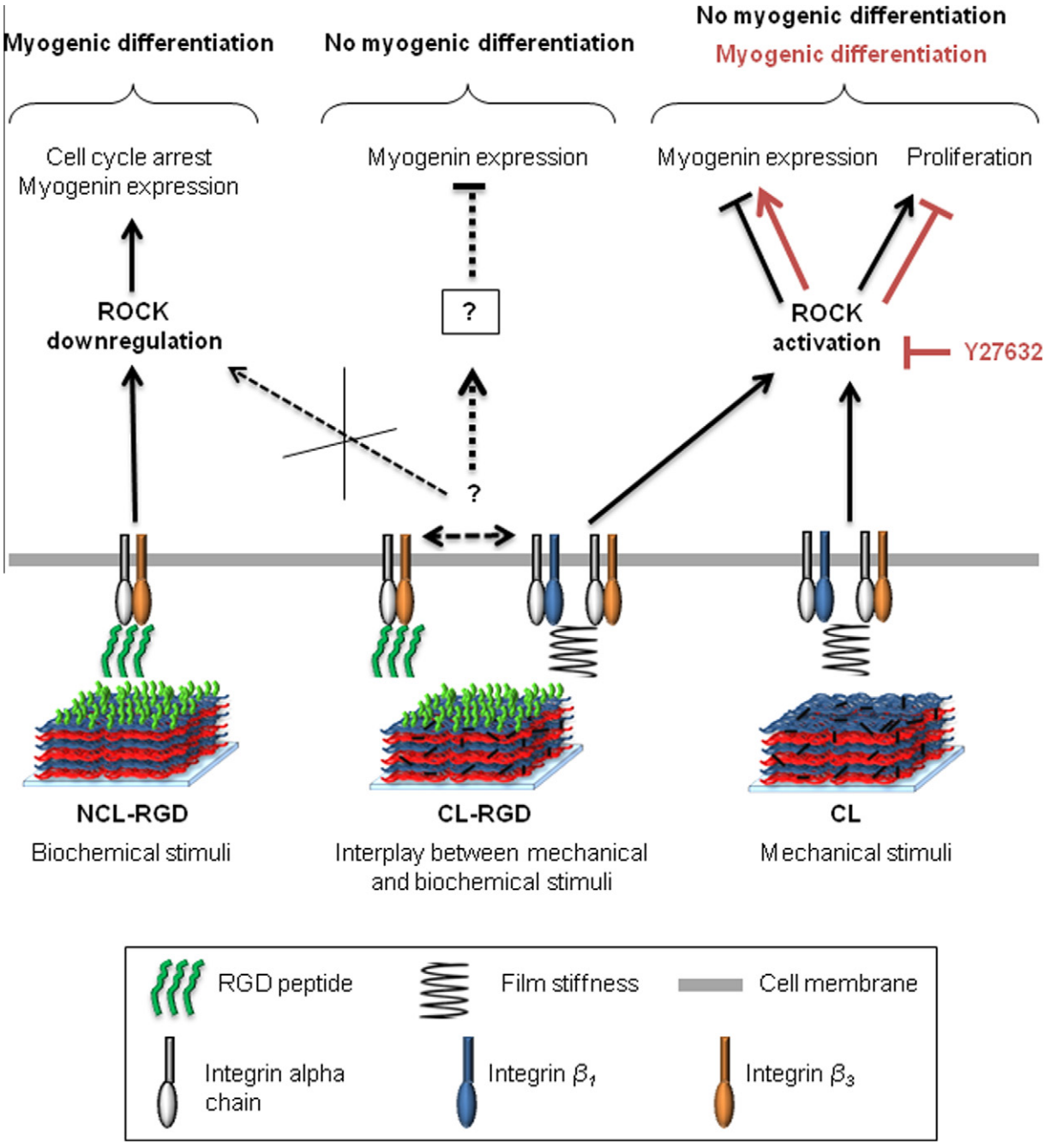

Fig. 8. Model of the interplay between mechanical and biochemical stimuli during induction of C2C12 myogenic differentiation on soft/stiff films functionalized or not with the RGD peptide. Adhesion involving β_3 integrins on NCL-RGD films (soft films with covalently attached RGD peptide) provides favorable conditions for myogenic differentiation of C2C12 cells. When the medium is changed to DM the rate of proliferation of the cells decreases and the production of myogenin increases. Adhesion on CL and CL-RGD films (stiff films) involving β_1 and β_3 integrins promotes ROCK activation, leading to a high proliferative state even in DM and to low myogenin expression. When the cells on stiff films are treated with ROCK inhibitor during differentiation the rate of proliferation decreases significantly on both CL and CL-RGD films. Additionally, on CL films myogenin expression increases, allowing the cells to undergo myogenic differentiation. However, on CL-RGD films ROCK inhibition was insufficient to induce myogenin expression and allow cell differentiation. We suggest that mechanical signals (stiffness) on CL-RGD films may affect cell interaction with biochemical signals (RGD peptide), resulting in the inhibition of β_3 integrins by RGD peptide or by β_1 integrins.

(RGD peptide), resulting in the inhibition of β_3 integrins by RGD peptide or by β_1 integrins. Thus our findings underline the importance of engineering substrates with well-controlled properties, as mechanical signals provided by the substrate can modify cell responses to biochemical cues. In this context, NCL-RGD represents a tool for the study of cell responses to RGD independently of the pathways activated by mechanical signals. In view of their excellent capacity to support myogenic differentiation, the soft films with attached RGD moieties may be used as coatings of various types of scaffolds used in muscle tissue engineering.

5. Conclusions

In the present work four different types of PEM films, with or without cross-linking and with or without the RGD peptide,

allowed an investigation of the effect of mechanical and biochemical signals and their combination on important events of myogenesis. Soft films with attached RGD peptide appeared most appropriate for myogenic differentiation of C2C12 myoblasts, while stiff films (CL and CL-RGD) induced enhanced migration and proliferation and inhibited myogenic differentiation. ROCK inhibition was sufficient to rescue C2C12 differentiation on CL films, but no significant changes were observed on CL-RGD films, showing that different signaling pathways were activated on each type of film, depending on their mechanical and biochemical properties. Our model allowed highlighting how important events in myogenesis such as adhesion, migration, proliferation, myogenin expression and fusion are regulated by substrate elasticity and the presence of an adhesive ligand.

These results suggest that thin films with tunable mechanical and biochemical properties may be a useful tool for biophysical studies of muscle progenitors in controlled 2-D microenvironments, as well as their expansion and differentiation in vitro. In addition, these films could very easily be used to coat a wide range of 2-D structured materials and 3-D scaffolds.

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Appendix A. Figures with essential colour discrimination

Certain figures in this article, particularly Figs. 1–4 and 6–8, are difficult to interpret in black and white. The full colour images can be found in the on-line version, at http://dx.doi.org/10.1016/j.actbio.2012.12.015.

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