

The combination of glycosaminoglycans and fibrous proteins ~~improves~~~~improve~~ cell proliferation and early differentiation of bovine primary skeletal muscle ~~cells~~~~cells~~.

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Abstract

Primary muscle cell model systems from farm animals are widely used to acquire knowledge about muscle development, muscle pathologies, overweight issues and tissue regeneration. The morphological properties of a bovine primary muscle cell model system, in addition to cell proliferation and differentiation features, were characterized using immunocytochemistry, western blotting and real-time PCR. We observed a reorganization of the Golgi complex in differentiated cells. The Golgi complex transformed to a highly fragmented network of small stacks of cisternae positioned throughout the myotubes as well as around the nucleus. Different extracellular matrix (ECM) components were used as surface coatings in order to improve cell culture conditions. Our experiments demonstrated improved proliferation and early differentiation for cells grown on surface coatings containing a mixture of *both* glycosaminoglycans (GAGs) and fibrous proteins. We suggest that GAGs and fibrous proteins mixed together into a composite biomaterial can mimic a natural ECM, and this could improve myogenesis for ~~in-vitro~~in vitro cell cultures.

Abbreviations: ~~used~~ECMextracellular (The author would like to point out that there should be a space between the abbreviations and the subsequent explanation.) matrix; ~~GAGsg~~Lucosaminoglycans:~~MRF~~~~GAGg~~lucosaminoglycans:~~MRF~~, myogenic regulatory transcription factor; PGproteoglycan

Keywords: Extracellular matrix; Glycosaminoglycans; Bovine primary skeletal muscle cells; Proliferation; Differentiation; Myogenesis

1 Introduction

Currently primary cell model systems from skeletal muscle cells exist for several farm animals, including pig (Perruchot et al., 2012; Wilschut et al., 2010), sheep (Wu et al., 2012), dog ((ZhuZhu et al.-et al.), 2010), and turkey (Velleman et al., 2000), as well as in rodents (summarized in (Yablonka-Reuveni and Day, 2011)). In addition myogenic cell lines have contributed to important insight into the skeletal muscle biology, and the C2C12 mouse cell line is the most commonly used model for myogenesis. It is, however, important to recognize that these cell lines deviate from freshly isolated muscle cells (Langelaan et al., 2011; Yablonka-Reuveni and Day, 2011). Likewise, differences are also observed between primary cell model systems from different species. The neural cell adhesion marker (NCAM) is expressed in proliferating human and rat cells (Boldrin et al., 2010), but not in mice muscle cells (Capkovic et al., 2008). Cattle are evolutionary closer to humans than rodents, and the entire bovine genome is fully sequenced in order to provide an important supplement for human medical research (Tellam et al., 2009). To establish and characterize a bovine primary cell model system is thus desirable to acquire information about muscle development.

Muscle stem cells will upon injury, exercise or disease receive molecular triggers that enable the cells to undergo myogenesis i.e. the formation of muscle tissue. The muscle stem cells are, in adults, quiescent cells that upon activation develop into myoblasts. The myoblasts will continue proliferating as long as growth factors are accessible. When the growth factors are depleted the myoblasts will exit the cell cycle and begin the process of differentiation. During differentiation membrane glycoproteins mediate alignment of the myoblasts. Eventually the myoblasts fuse and form the multinucleated myotubes unique to muscle tissue. Activation of muscle stem cells into myoblasts, proliferation and differentiation involves the upregulation of myogenic regulatory transcription factors (MRFs). These include myogenic determination factor 1 (MyoD) and myogenin (Yablonka-Reuveni and Day, 2011). MyoD serves as the master transcription factor required to regulate genes associated with myogenesis, and it is commonly known to represent one of the earliest stages of myogenesis. As a downstream target of MyoD, myogenin regulates the transition from myoblasts into

myotubes. Once the cells have fused into myotubes, there is an increase in the expression of structural proteins such as desmin (Yablonka-Heuveni and Day, 2011).

The development of skeletal muscles ~~in vivo~~ in vivo is obviously much more complicated and tightly regulated than myoblast differentiation and fusion ~~in vitro~~ in vitro. Muscle growth is governed by its micro-environment, consisting of e.g. surrounding cells, vascular system and the extracellular matrix (ECM) (reviewed in Thorsteinsdottir et al., 2011). The most basic role of ECM is to provide a supportive scaffold for cells, promoting cell aggregation and migration. The ECM is, however, far from just a biological scaffold (although recognized as this for decades). The ECM can directly influence cell behavior through ECM-specific receptors on the cell surface, and indirectly by sequestering and storing soluble growth factors which are then presented at the cell surface at relevant times. In this way ECM provides mechanical support, but also signals to the interior of the cell, affecting a variety of cellular responses. The ECM is composed of two main classes of macromolecules: proteoglycans (PGs) and fibrous proteins (Frantz et al., 2010). PGs are proteins with glycosaminoglycan (GAG) chains covalently attached to the core protein. The GAG chains on the protein core are unbranched polysaccharide chains composed of repeating disaccharide units that can be divided into different subtypes (sulfated or non-sulfated GAGs) (Schaefer and Schaefer, 2010). PGs have an enormous molecular diversity due to the various combinations of protein cores in combinations with one or more GAG chains of different subtypes. The importance of PGs and GAGs in muscle differentiation has previously been demonstrated, in which inhibition of PG synthesis strongly inhibited the differentiation process (Osses and Brandan, 2002). Collagen is the most abundant fibrous protein, and constitutes ~30% of the total protein mass of a multicellular animal (Frantz et al., 2010). Laminin, entactin, fibronectin and tenascins are other proteins that constitute the ECM (Thorsteinsdottir et al., 2011). *In vitro*, various ECM components of the muscle tissue influence the muscle cell behavior (Boonen et al., 2009; Wilschut et al., 2010). Muscle cells grown on laminin-coated substrates resulted in more and thinner myotubes compared with myotubes on collagen type IV and matrigel coating (Boonen et al., 2009) and an improved myogenic differentiation (Wilschut et al., 2010). Since these differences can be both species (Boonen et al., 2009), and cell type dependent (Macfelda et al., 2007), it is important to examine how ECM components influence ~~in vitro~~ in vitro bovine primary muscle cells.

In the present study the aim was to investigate if a combination of GAGs and ECM proteins could improve bovine primary muscle cell growth and myogenesis.

2 Materials and methods

2.1 Antibodies

Rabbit anti-MyoD and goat anti-myogenin (G-20) were from Santa Cruz Biotechnologies Inc. (Santa Cruz, CA, USA), and rabbit anti-desmin was from Abcam (Cambridge, UK). Mouse anti-NCAM (5.1H11) was from Developmental Studies Hybridoma Bank (Iowa city, IA, USA). Mouse anti-TE7 and mouse anti-myogenin (MAB3876) were from Millipore (Billerica, MA, USA). Mouse anti- α -tubulin was from ~~Sigma-Aldrich (St. Louis, Sigma Aldrich (St. Louis,~~ MO, USA). Alexa 647-conjugated donkey anti-goat was from Invitrogen (Carlsbad, CA, USA). Alexa ~~488-conjugated~~ ~~488-conjugated~~ goat anti-mouse and DyLight 549-conjugated mouse anti-rabbit were from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA, USA). Cy5-conjugated goat anti-rabbit and Cy-3-conjugated goat anti-mouse were from GE Healthcare (Chalfont St Giles, UK). CellLight™ Golgi-GFP ~~*BacMam 2.0*~~ ~~*BacMam 2.0*~~, DAPI and ~~Alexa 488~~ ~~Alexa-488~~ Phalloidin were from Molecular probes (Invitrogen, Carlsbad, CA, USA).

2.2 Bovine primary skeletal muscle cell isolation

Bovine primary skeletal muscle cells were isolated as described (Gaster et al., 2001a, 2001b) with minor modifications. In brief, small muscle pieces (~1 g) from *Longissimus thoracis* were digested for 1 h with 70 rpm shaking in 10 ml DMEM with 0.72 mg/ml collagenase. Cells were further dissociated from the tissue with 0.05% trypsin/EDTA for 25 min before addition of 10% FBS to stop trypsinization. This step was repeated three times, and the harvested cells were pooled. For the removal of fast-adhering fibroblast cells from the primary cell cultures, the cells were placed in uncoated cell flasks for 1 h at 37 °C. This allowed the fibroblast cells to adhere to the plastic. The non-adhering cells were then collected and further seeded onto 25 cm² coated culture flasks coated with ECL as described below. The isolated cells were proliferated, split into 75 cm² coated culture flasks, and then stored in DMSO in liquid nitrogen until further use. All experiments were performed in 2nd ~~nd~~ or 3rd ~~rd~~ passage.

2.3 GAG preparation

Previously characterized GAG extracts were prepared as described (Tingboe et al., 2005). Briefly, 75 g muscle from cod fillets was incubated for 4 days at 37 °C in 0.05 M ~~Tris-HCl~~ ~~Tris-HCl~~ pH 7.4 with 0.005 M ~~cysteine-HCl~~ ~~cysteine-HCl~~ and 25 mg papain (Papainase EC 3.4.22.2 from papaya latex, Sigma-Aldrich Chemie GmbH, Steinheim, Germany). Sample to liquid ratio was 3:2 and more papain was added after 48 h to a total of 50 mg. After enzymatic digestion samples were centrifuged and subjected to DEAE ion-exchange chromatography. PolyPrep columns (Bio-Rad Laboratories, Hercules, CA, USA) were packed with 3 ml DEAE Sephacel ion-exchange medium (Amersham Pharmacia Biotech, Uppsala, Sweden). Columns were equilibrated using 4 M urea in 0.05 M ~~Tris-HCl~~ ~~Tris-HCl~~ pH 8 and washed using 12 ml 4 M urea added 0.01 M NaCl in 0.05 M ~~Tris-HCl~~ ~~Tris-HCl~~ pH 8. GAG-containing fractions were eluted using 1.5 M NaCl in 0.05 M ~~Tris-HCl~~ ~~Tris-HCl~~ pH 8. GAGs were precipitated in 70% ethanol ON at ~~-20-20~~ °C, followed by centrifugation. The pellets were resuspended in distilled water and the precipitation was repeated twice. The GAG composition was determined by enzymatic degradation and ion-exchange chromatography as described (Tingboe et al., 2005). The results showed that the pellet contained 25% chondroitine ~~sulfate/dermatan sulfate~~ ~~sulphate/dermatan sulphate~~, 3% keratan ~~sulfate~~ ~~sulphate~~ and 43% heparan ~~sulfate~~ ~~suphate~~. This implies that 71% of the isolated GAG is composed of ~~sulfated~~ ~~sulphated~~ GAG chains while 29% of the dry-matter could not be identified.

2.4 Cell culture and treatment

Tissue culture coverslips (Menzel-Gläser, Braunschweig, Germany), 6- and 24-well plates (BD Falcon, Franklin Lakes, NJ, USA), and cell culture flasks (VWR, West Chester, PA, USA) were coated with 1 μ l/cm² of the following coating solutions:

1 mg/ml laminin (~~Mouse laminin-nidogen~~ (~~Mouse laminin-nidogen~~-complex, Millipore), 3 mg/ml collagen (Type I collagen from bovine skin, Sigma-Aldrich), 2 mg/ml GAGs, 1 mg/ml ~~entactin-laminin-collagen~~ ~~Entactin-Laminin-Collagen~~ (ECL) (~~Millipore~~), ~~Millipore~~), a mix of ECL (0.5 mg/ml)+GAGs (1 mg/ml), or a mix of ECL (0.5 mg/ml)+heparan ~~sulfate~~ ~~sulphate~~ (1 mg/ml) (~~heparan sulfate~~ (~~Heparan-sulphate~~ from bovine kidney, ~~Sigma-Aldrich~~ ~~Sigma-Aldrich~~), ~~Sigma-Aldrich~~) for a minimum of 4 h at room temperature. Subsequently the coating solutions were removed and the coated surfaces were washed twice with PBS. The early experiments performed in this study ~~were was~~ performed on laminin coatings, but ~~were was~~ replaced with ECL during the later experiments as a standard. The primary cells were grown in Dulbecco's modified Eagles's medium (DMEM) with ~~L-glutamine~~ ~~L-glutamine~~ (2 mM), 2% FBS, 2% Ultrosor G, P/S (~~10.000~~ ~~10.000~~ units/ml), and Amphotericin B (250 µg/ml) until ~~70-80%~~ ~~70-80%~~ confluence (the cells underwent proliferation for 3 days). Cell proliferation was quantified using a Countess™ automated cell counter (Invitrogen, Carlsbad, CA, USA). The cells were then washed with PBS and placed in differentiation medium (DMEM, 2% FBS, P/S, Amphotericin B, and 25 pmol Insulin) to induce myogenesis. The proliferation and differentiation media ~~were was~~ changed every ~~2-3~~ ~~2-3~~ days during cell isolation and the experiments. To examine the Golgi complex, proliferating cells were transduced with 3 µl ~~CellLight~~ ~~CellLight~~ Golgi[®] Golgi marker, and differentiated for ~~3~~ ~~three~~ days before immunocytochemistry.

2.5 Western blotting

Proliferating and differentiating cell cultures were washed twice with PBS, before addition of lysis buffer (10 mM Tris, pH 6.8, 5 mM EDTA, 50 mM NaF, 30 mM sodium pyrophosphate, 2% (w/v) sodium dodecyl sulfate (SDS), containing AEBSF and phosphatase cocktail inhibitor II). Cell debris was removed by centrifugation at 13,000×g for 10 min, and the cleared lysate was subjected to SDS-Page gel electrophoresis. Following electrophoresis, the proteins were transferred onto nitrocellulose membranes using an iBlot Gel Transfer Device (Invitrogen). All membranes were blocked with 2% Enhanced Chemiluminescence Advanced blocking agent (GE Healthcare) in TBS-tween for 1 h at RT. Primary and secondary antibodies were diluted in 0.5% blocking agent and incubated for 1.5 h at RT (or ON at 4 °C) with gentle shaking. Membranes were washed 3×10 min with TBS-tween after both incubations. Proteins were scanned and visualized using Ettan DIGE Imager (GE Healthcare), and the images were analyzed by ImageQuant TL software (GE Healthcare).

2.6 RNA isolation and real-time PCR

Proliferating and differentiating cell cultures were washed twice with PBS, and subsequently placed in Trizol reagent (Life Technologies, Carlsbad, CA, USA), and further purified by RNeasy mini kit including a DNase treatment (Qiagen, Hilden, Germany). RNA concentration and purity was measured using a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, Waltham, MA, USA). cDNA was generated from ~200 ng mRNA using TaqMan[®] Reverse Transcription Reagents (Invitrogen) according to the manufacturer's protocol. The cDNA was subjected to real-time PCR analysis using an ABI Prism 7700 Sequence Detection system (Applied Biosystems, Carlsbad, CA, USA). The real-time PCR reaction volume of 25 µl contained 1 µl template cDNA, 0.2 µM of each primer, 0.1 µM probe, 1.25 units Taq DNA polymerase (AmpliTaqGold, Applied Biosystems), 0.3 units uracil *N*-glycosylase (AmpErase UNG, Applied Biosystems), 0.2 mM dATP, dCTP, dGTP (Applied Biosystems) and 0.4 mM dUTP (Applied Biosystems), 5 mM MgCl₂, and 1 × TBA buffer (Applied Biosystems). The cycling profile was as follows: an initial decontamination step for 2 min at 50 °C to allow optimal UNG enzymatic activity, followed by a denaturation step of 10 min at 95 °C, followed by 40 repeats of 15 s denaturation at 95 °C and 60 s synthesis at 60 °C. The sequences of primers and probes, the amplicon length and sequence accession numbers used are listed in Table 1. Gene expression of the samples was normalized against β-actin and TATA, and ΔCt was calculated. The results using TATA and β-actin were similar (Fig. S1), and therefore only β-actin was chosen for further analyses. PCR efficiency and melting point analysis were performed on all targets. Comparison of the relative gene expression between proliferating (day 0) and differentiating cells (day 3) was derived by using the comparative Ct method. In short, values are generated by subtracting ΔCt values between two samples and this gives a ΔΔCt value. The relative gene expression is then calculated by the formula $2^{-\Delta\Delta Ct}$. The efficiency of each set of primers was always higher than 96%. The real-time PCR was performed in technical triplicates on at least three independent experiments seeded out in duplicates.

Table 1 List of primers and probes used for quantitative real-time PCR.^a

Primers and probes	Sequence	Amplicon size	GeneBank acc. no.
<i>Reference genes</i>			
β-Actin forward	CTGCGGCATTACGAAACTA	83 bp	NM_173979
β-Actin reverse	GCACCGTGTGGCGTAGAG		
β-Actin probe	ATTCCATCATGAAGTGTGACGTCGACATCC		
TATA forward	CGTTTTGCTGCTGTAATCATGAG	73 bp	NM_001075742
TATA reverse	CCATCTTCCCAGAAGTGAATATCA		
TATA probe	ATAAGAGAGCCCCGCACCACTGCA		
<i>Myogenic markers</i>			

MyoD forward	CCCAAAGATTGCGCTTAAGTG	87 bp	NM_001040478
MyoD reverse	AGTTCCTTCGCCTCTCCTACCT		
MyoD probe	ACCACTCTCCTCCCAACAGCGCTTTAAA		
Desmin forward	GCTGAAAGAAGAAGCGGAGAAC	68 bp ^b	NM_001081575
Desmin reverse	GAGCTAGAGTGGCTGCATCCA		
Desmin probe	ATTTGGCTGCCTTCCGAGCCG		
Myogenin forward	CCCTACAGACGCCACAATC	70 bp	EF636458
Myogenin reverse	AGCGACATCCTCCACTGTGAT		
Myogenin probe	CACTCCCTCACCTCCATCGTGGACA		

^a All sequences were examined for specificity using BLAST.

^b An intron is present in the genomic desmin [sequence](#).

2.7 Immunocytochemistry and fluorescence microscopy

Cells were grown on coated coverslips (Assistant, Sondheim/Rhön, Germany), washed in PBS and fixed in 2% PFA (Reidel-de Haën, Seelze, Germany) for 15 min. The cells were washed three times in PBS, permeabilized using 0.1% Triton X-100 in PBS and incubated with 5% milk for 30 min before incubation with the primary antibody for 1 h. Subsequent incubation with secondary antibodies was performed for 30 min before mounting using Dako fluorescent mounting medium (Glostrup, Denmark). The cells were examined by [fluorescence](#) microscopy analysis (ZEISS Axio Observer Z1 microscope), and images were processed using Adobe Photoshop CS3. To quantify [NCAM⁺NCAM⁻](#) and TE7 [+](#) myoblasts, immunostained cells were counted manually. The pictures were chosen randomly from at least three regions from each well in order to ensure a non-biased quantification. Fusion index was calculated based on scoring of at least four randomly chosen regions with nuclei and myotubes stained. For each region the number of nuclei incorporated in myotubes and the total number of nuclei was scored. The fusion index (FI) was calculated as the percentage of total nuclei incorporated in myotubes.

2.8 Statistical analysis

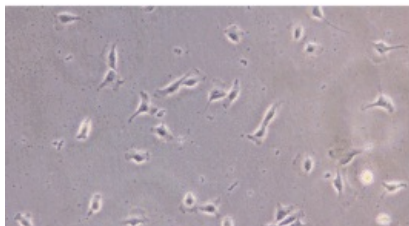
Statistical analysis was performed using a two-tailed, unpaired Student's *t*-test. *P*-values ≤ 0.05 were considered statistically significant and are indicated in each figure.

3 Results

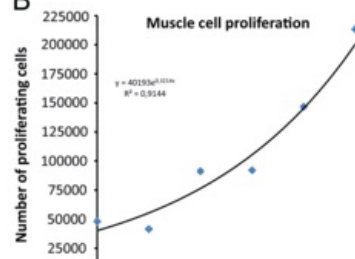
3.1 Isolation and characterization of bovine primary muscle cells isolated from *Longissimus thoracis*

Bovine primary skeletal muscle cells isolated from [Longissimus thoracis](#) demonstrated a typical morphology (triangle shaped) after 5 days, growing as an even layer of single cells (Fig. 1A). After 7 days the cells grew denser, aligned to each other, and changed into larger square-shaped cells. The muscle cells underwent proliferation with a doubling time of approximately 2.2 days (Fig. 1B). When the cells reached a density of 80–90% confluence, the cells started to fuse into multinucleated myotubes (Fig. 1C). A small portion of the isolated cells displayed a different morphology, growing in clusters with a spindle-shaped fibroblastic morphology (data not shown). We therefore investigated the presence of fibroblasts using the fibroblast marker TE7. Immunofluorescence microscopy experiments in myoblast cell cultures showed a small proportion (approximately 10%) positive for TE7 (Fig. 1D). These findings were confirmed with immunofluorescence analyses of the myogenic satellite cell marker, NCAM, which displayed that a small portion of the cells were negative for NCAM (approximately 10%).

A



B



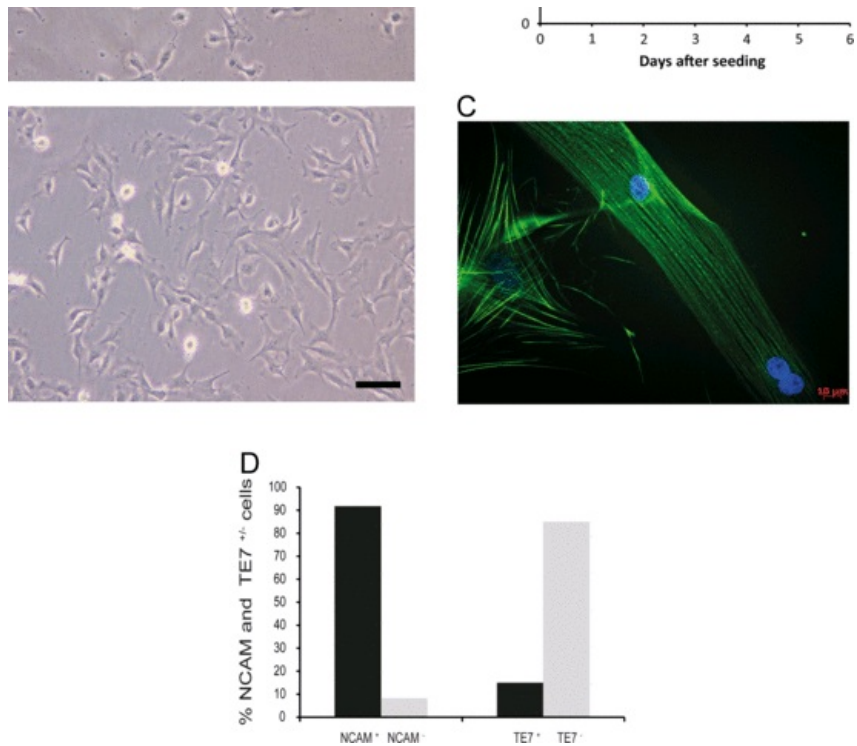
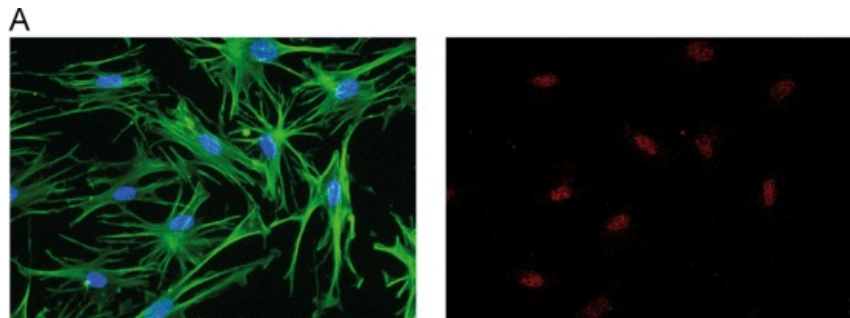


Fig. 1 Proliferating and differentiating myoblast cells. **(A)** Proliferating cells five and seven days after seeding. Scale bar 10 μ M. **(B)** Quantification of cell proliferation from 0 to 5-6 days after seeding. Values presented are the mean total cell count for two biological replicates. The solid line is a logarithmic fit for the total number of cells. **(C)** Differentiated cells were fixed with 2% PFA and incubated with Alexa 488-Phalloidin for staining of Actin (green) before fluorescence microscopy. Nuclei were stained with DAPI (blue). Scale bar 10 μ M. **(D)** Quantification of NCAM^{+/+} and TE7^{+/+} myoblasts. Immunostained cells were counted manually. The NCAM^{+/+} and TE7^{+/+} cells are presented as percentage of the total number of counted cells. A total of 197 cells were counted for NCAM, and a total of 30 cells were counted for TE7. (For interpretation of the references to color in this figure caption, the reader is referred to the web version of this article.)

MRFs such as MyoD and myogenin, as well as filament proteins such as desmin, are important for proliferation and differentiation. Immunofluorescence staining showed that almost all the proliferating cells expressed MyoD (Fig. 2A). During the first day of differentiation, myogenic cells showed increased myogenin expression, which is an early myogenic regulator (Fig. 2B), indicating that differentiation was taking place. The expression level of desmin was detectable, but low in proliferating cells, and increased in differentiated cells (Fig. 2C). We investigated the protein level of MyoD, myogenin and desmin in proliferating cells (day 0) and differentiating cells (day 3) (Fig. 2D). The expression of MyoD persisted during the differentiation. The protein expression of desmin, on the other hand, increased significantly after 3-4 days. These findings were consistent with the relative mRNA expression, showing an increase in markers throughout the differentiation process (Figure S2-S4). We were unable to detect myogenin protein expression by western blotting although we tried two different antibodies that previously had worked well on immunofluorescence.



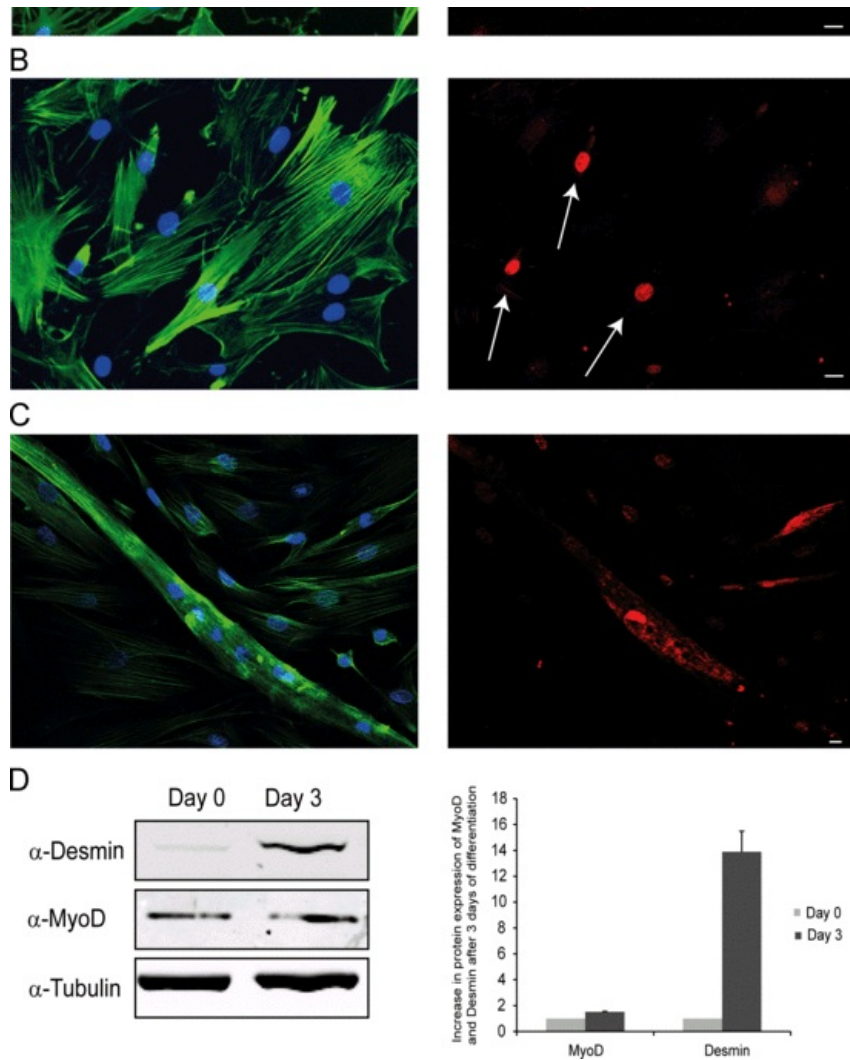


Fig. 2 Localization and expression of myogenic regulatory transcription factors (MRFs) during proliferation and differentiation. (A) Proliferating cells were fixed with 2% PFA, immunostained with Alexa 488-Phalloidin (green), and rabbit anti-MyoD, followed by DyLight 649-conjugated donkey anti-rabbit (red) before fluorescence microscopy analysis. Nuclei were stained with DAPI (blue). Scale bar 10 μ M. (B) Cells differentiated for 3 days were fixed with 2% PFA, immunostained with Alexa 488-Phalloidin (green), and goat anti-Myogenin, followed by DyLight 649-conjugated donkey anti-goat (red) before fluorescence microscopy analysis. Nuclei were stained with DAPI (blue). Arrows indicate Myogenin positive cells. Scale bar 10 μ M. (C) Differentiating cells were fixed with 2% PFA, immunostained with Alexa 488-Phalloidin (green) and rabbit anti-Desmin, followed by DyLight 549-conjugated mouse anti-rabbit (red) before fluorescence microscopy analysis. Nuclei were stained with DAPI (blue). Scale bar 10 μ M. (D) *Left*: a representative western blot showing the expression level of MyoD and Desmin after 0 and 3 days of differentiation. Cell lysates were subjected to western blotting using antibodies to MyoD, Desmin, and Tubulin (loading control). *Right*: quantification of Western blots. Bars show the expression of Desmin and MyoD, relative to tubulin, after 0 and 3 days of differentiation. The graph represents the average of three experiments with SD. (For interpretation of the references to color in this figure caption, the reader is referred to the web version of this article.)

To investigate the organization of the Golgi complex in bovine muscle cells we transduced proliferating cells with GFP-tagged [CellLightCellLight®](#) Golgi marker. We observed that in proliferating cells the Golgi complex displayed a classic organization, with a stack of flattened cisternae next to the nucleus (Fig. 3A). During differentiation, however, the complex transformed to a highly fragmented network of small stacks of cisternae positioned throughout the myotubes as well as around the nucleus (Fig. 3B).

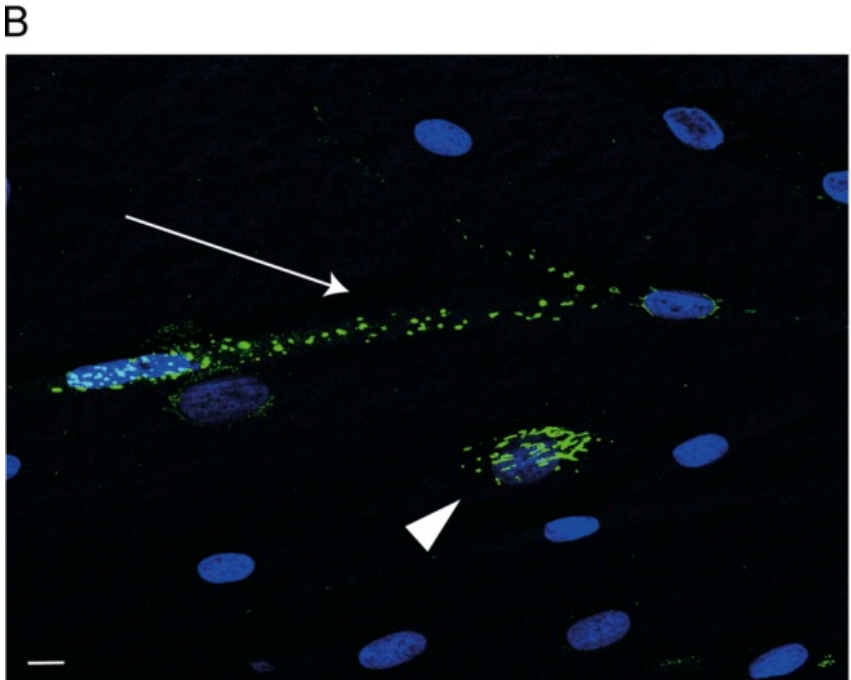
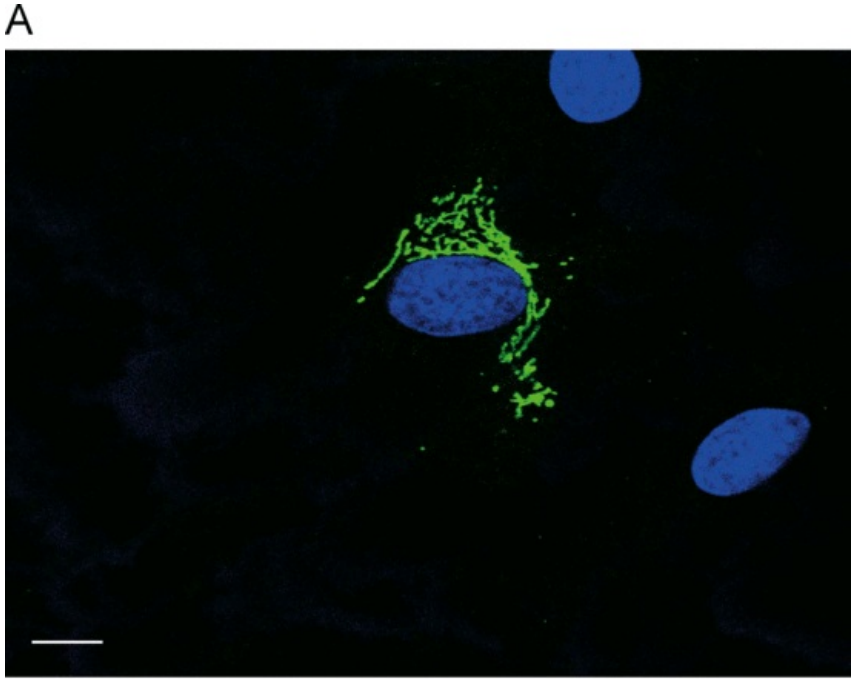


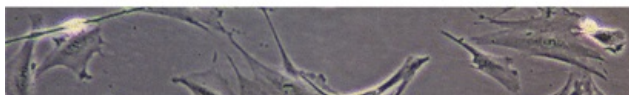
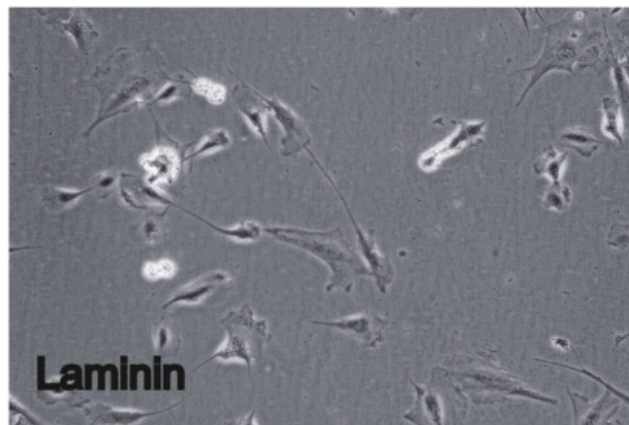
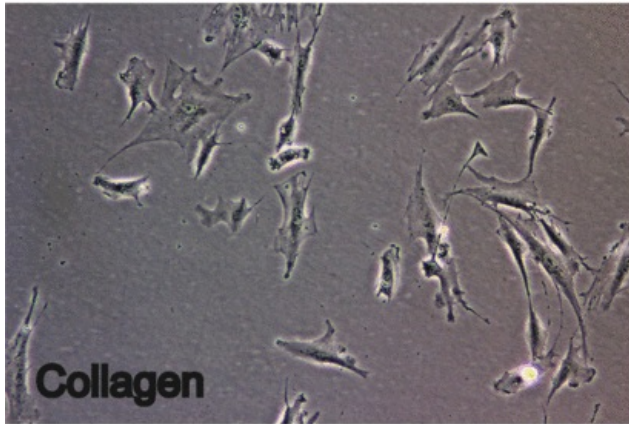
Fig. 3 ~~Re-organization~~ ~~Re-organisation~~ of the Golgi complex during differentiation. Proliferating cells were transduced with a GFP-tagged ~~CellLight~~ ~~CellLight~~[®] ~~Golgi~~ ~~Golgi~~-marker (green), differentiated for ~~3~~ ~~three~~ days and fixed with 2% PFA before nuclei were stained with DAPI (blue) ~~and~~

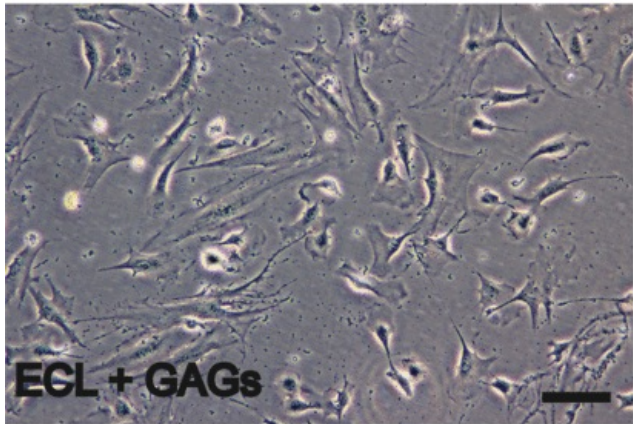
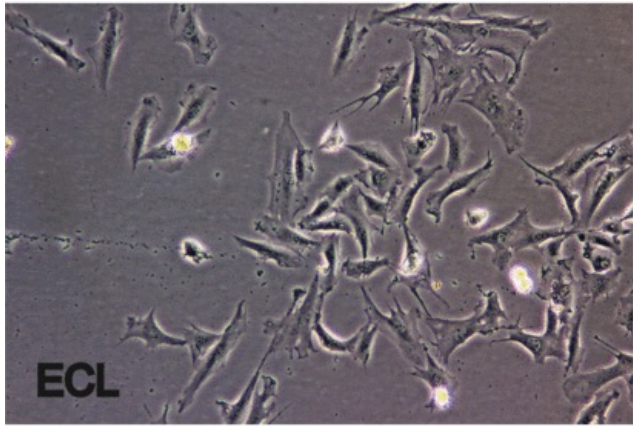
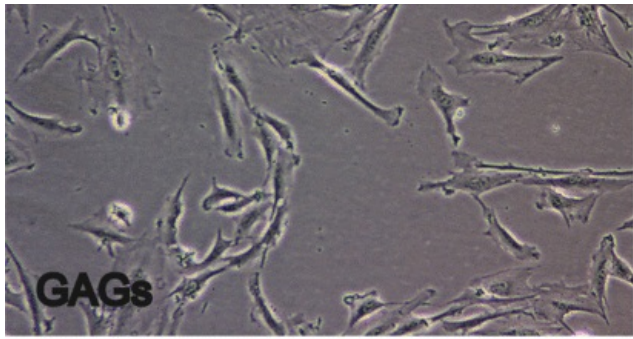
analysed by fluorescence microscopy analysis, and analysed by fluorescence microscopy. (A) Proliferating cells. (B) Differentiated cells. Arrow indicates a differentiated myotube and myotube, arrowhead indicates a proliferating cell. Scale bar 10 μM. (For interpretation of the references to color in this figure caption, the reader is referred to the web version of this article.)

3.2 Glycosaminoglycans in combination with extracellular matrix proteins influence proliferation and differentiation

To investigate the effects different combinations of ECM proteins have on bovine skeletal muscle cells, we cultured the isolated cells on various surfaces coated either with GAGs, single protein coatings (laminin or collagen), combined protein coating (ECL) or complex ECM surface coating (ECL+GAGs). We observed a distinct proliferation pattern between the different surface coatings during the first day after seeding. The muscle cells cultured on ECL and ECL+GAGs clearly grew faster compared to cells cultured on single-protein coatings (Fig. 4A and B). After ~~2-3~~ days of cultivations the cells had reached ~~80-90%~~ confluence, and underwent differentiation. Complex, multinucleated myotubes were observed after ~~3~~ days in differentiation media for cells grown on composite ECL coatings (Fig. 5A). The process was faster in cells grown on complex surface coatings as the fusion index is significantly higher after ~~3~~ days (Fig. 5B), and the myotubes observed after ~~3~~ days were more complex and displayed a more branched morphology compared with cells grown on single protein coatings or GAGs. After ~~5~~ days we observed similar complex, branched myotubes also in cells cultured on single protein surface coatings and GAGs (data not shown), and no differences were observed in the fusion index between the surface coatings.

A





B

120000
ells

*

▲ Collagen

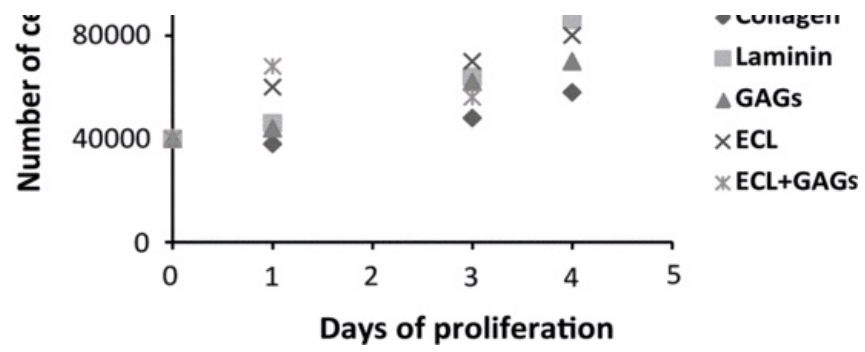
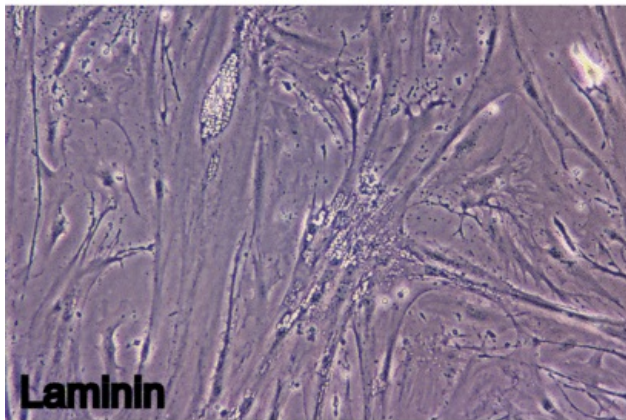
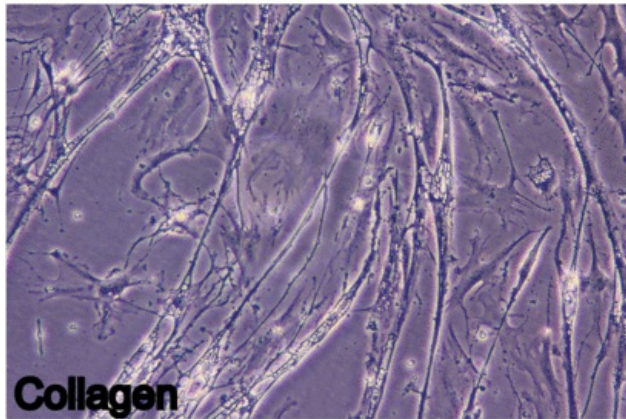
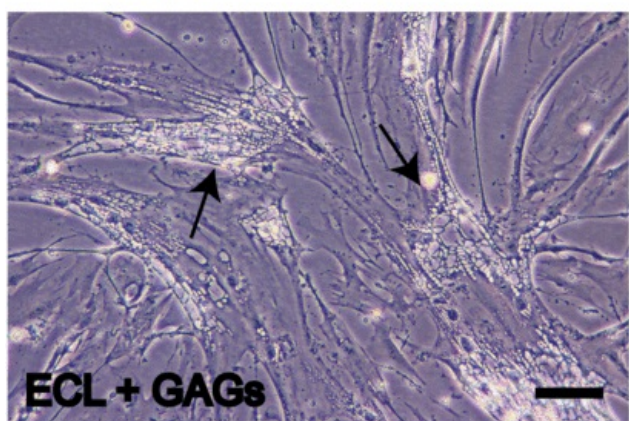
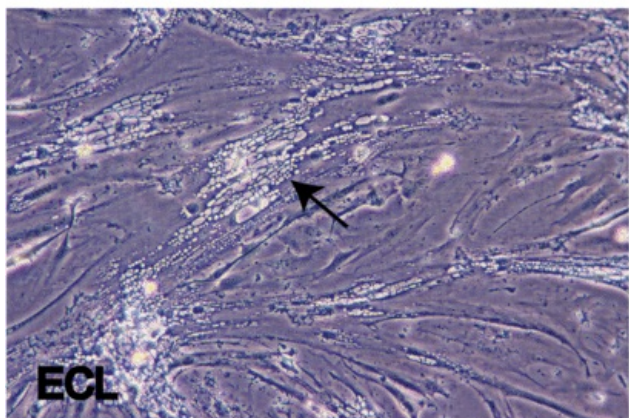
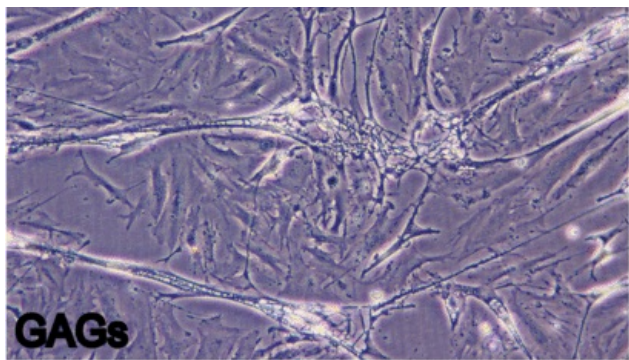


Fig. 4 Surface coatings influence the proliferation of myoblast cells. (A) Cell morphology and confluence of proliferating cells after 1 day of proliferation, grown on different surface coatings as indicated. Scale bar 5 μ M. (B) Cell proliferation presented as the total cell count value. Each data point represents the average cell number in a single well of a 6-well plate. All wells were counted twice.

A





B

80

70

n.s

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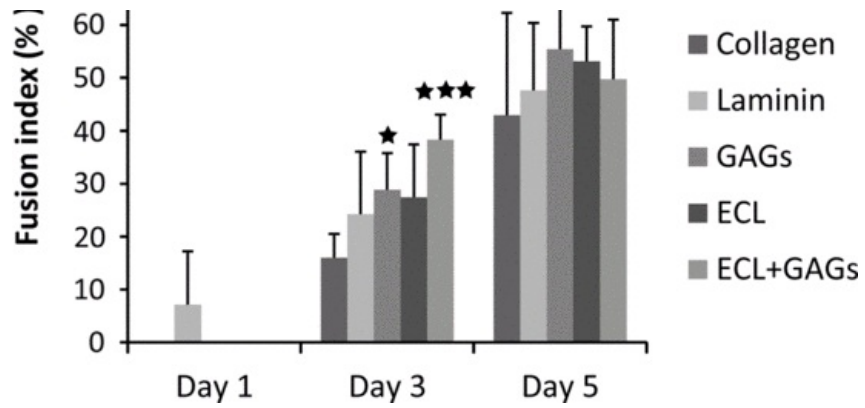
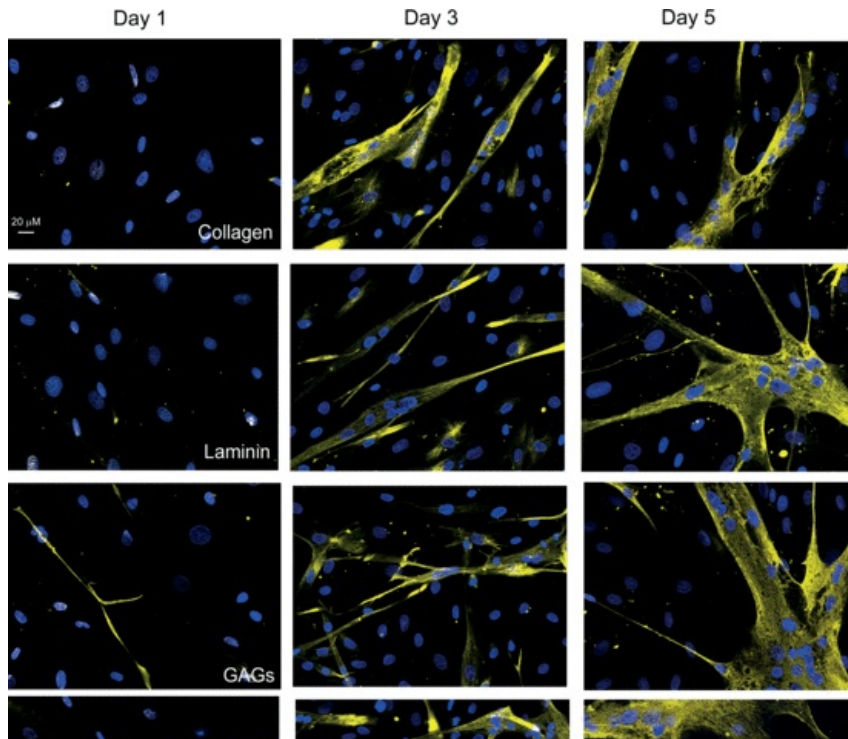


Fig. 5 Surface coatings influence the early differentiation of myoblast cells. (A) Cell morphology and confluence of muscle cells after 3 days in differentiation media on different surface coatings as indicated. Arrows indicate complex multinucleated myotubes. Scale bar 5 μ M. (B) Fusion index was calculated based on scoring of at least four randomly chosen regions with nuclei and myotubes stained. For each region the number of nuclei incorporated in myotubes and the total number of nuclei were scored. The fusion index (FI) was calculated as the percentage of total nuclei incorporated in myotubes. Asterisks denote significant differences between surface coatings compared with collagen (* p <0.05, *** p <0.001). n.s. means not significant.

The morphology of differentiating cells at **1, 3** and **5** days was determined by immunocytochemical **labeling** of desmin followed by fluorescence microscopy analysis (Fig. 6). Desmin displayed an intracellular localization pattern, visible in early and late differentiating cells, but was barely detectable in proliferating cells. The differentiation was rapid for cells grown on complex ECM coatings, and the formation of large, multinuclear myotubes was evident already at day three. For cells grown on laminin and collagen on the other hand, the myotubes observed were smaller and thinner. The differences between the surface coatings were prominent in early phases of differentiation, while they were not as apparent in late phases as cells grown on single layer coatings apparently caught up with the cells grown on complex ECM.



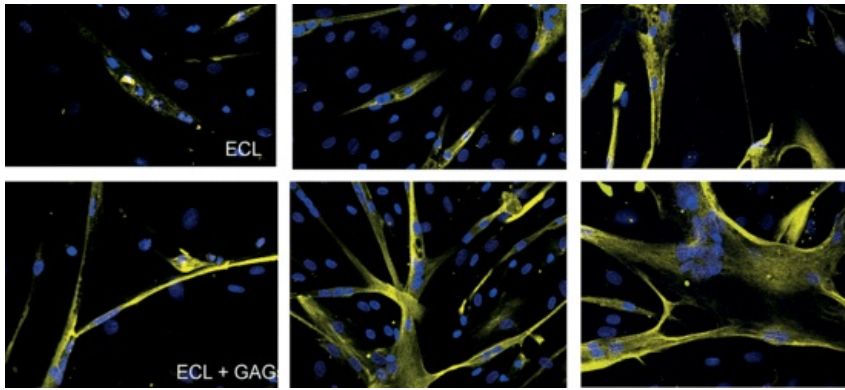


Fig. 6 Desmin staining of myotubes on different surface coatings after ~~1, 3, one, three~~ or ~~5, five~~ days in differentiation medium. Differentiating cells were fixed with 2% PFA and immunostained with rabbit anti-Desmin, followed by DyLight 549-conjugated mouse anti-rabbit (yellow) before fluorescence microscopy analysis. Nuclei were stained with DAPI (blue). Scale bar 20 μ M. (For interpretation of the references to color in this figure caption, the reader is referred to the web version of this article.)

Microscopy analyses and quantitative assays showed that cells proliferated faster, and differentiated earlier on complex surface coatings (i.e. results from Figs. 4–6). We therefore investigated the gene expression of MRFs and desmin in cells grown on various surface coatings at 0 and 3 days of differentiation (Fig. 6). Although not significant, the general trend is an increase in gene expression of all the myogenic markers during differentiation. More interestingly is that the expression levels were highly sensitive to the surface coating. The gene expression of all markers was highest for the complex surface coating containing *both* GAGs and proteins (ECL+GAGs), particularly for myogenin (Fig. 7). A similar trend was observed with a combination of ECL and heparan sulfate (Fig. S3).

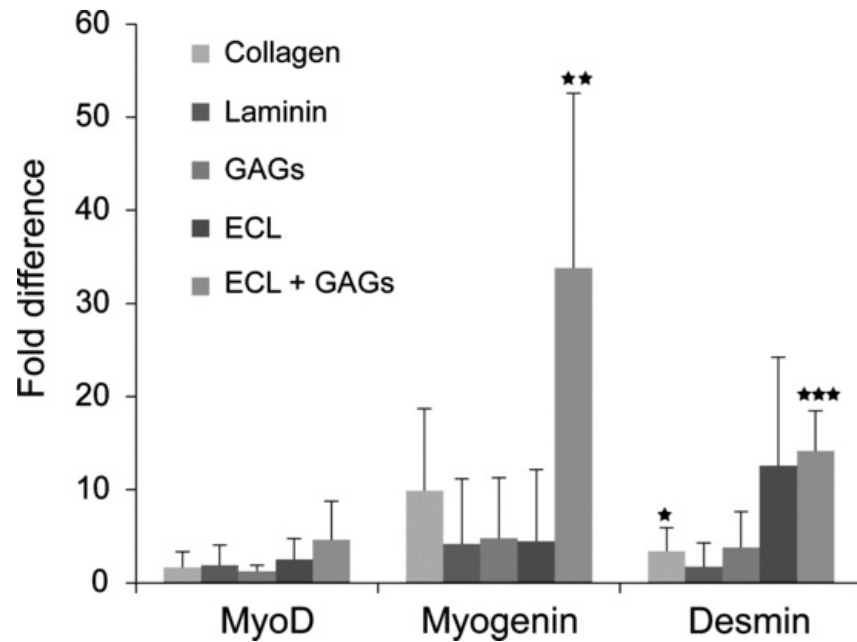


Fig. 7 The relative gene expression of myogenic markers during differentiation varied among the surface coatings. Bars show the relative mRNA expression of myogenic markers in differentiating cells compared to proliferating cells. The data are presented as the average mean of at least three independent experiments performed in technical triplicates, \pm SD. Asterisks denote significant differences between surface coatings compared with proliferating cells grown on collagen coating (* p <0.05, ** p <0.01, *** p <0.001).

4 Discussion

We have described a primary muscle cell model system from bovine *Longissimus thoracis* muscle and demonstrated that proliferation and differentiation of these primary cells in culture appear to be regulated in a similar way as cells from humans and other model organisms. This is a readily available and attractive **in-vitro** in vitro model system to study bovine muscle. The system may also be used as a supplement in human medical research, as cattle are evolutionary closer to humans than rodents and the bovine genome is fully sequenced (Tellam et al., 2009). For some purposes it is desirable to perform many analyses on the same set of cells. Then bovine primary muscle cells could be a good choice, as the muscles are large and thus it is easy to obtain a large stock of cells. Differences between donors are a major concern when using human primary muscle cell cultures (McIntyre et al., 2004; Henry et al., 1995). We did not observe differences between samples collected from different animals; however all our cell cultures are isolated from animals of the same age, gender and breed.

When cells are seeded onto the flat surfaces in 2D cultures, cells adhere to multiple attachment points and this may result in fusion of randomly aligned myoblasts, which in turn leads to the formation of branching myotubes. This is a feature uncharacteristic of **in-vivo** in vivo muscle fibers, and has been observed by us and others (such as Allen et al., 1997; Langelaan et al., 2011; Velleman et al., 2000). Experiments with porcine primary cells showed that the morphology of the myoblasts during the first days of culturing differed according to the surface coating (Allen et al., 1997). We did not observe any differences in morphology of proliferating cells between the different surface coatings. The proliferation rate, however, differed among the various surface coatings. We observed in our cell model system significant differences between the surface coatings, and interestingly the complex ECM coating consisting of *both* proteins and GAGs (ECL+GAGs) was the surface coating with highest proliferation and differentiation rate. The cells grew faster and differentiated earlier. The differences between the surface coatings were prominent in early phases of differentiation, while they were not as apparent in late phases when cells grown on single component coatings apparently caught up with the cells grown on complex ECM. Reported effects on how protein surface coatings influence proliferation rates varies. The proliferation rate in primary muscle cells from mice was higher for cells grown on surfaces that resembled the physiological elasticity of muscle cells. Proliferation was, on the other hand, not influenced by protein coating in these experiments (Langelaan et al., 2011). Proliferation can be stimulated by different laminin isoforms, and this can be cell-type dependent (i.e. skeletal and smooth muscle cells respond differently) (Lu et al., 2001). Macfelda and colleagues discovered that different double-coatings gave different results, either increased or decreased proliferation rate (Lu et al., 2001). The reason for these discrepancies regarding proliferation and surface coating is not clear.

Our experiments demonstrated improved proliferation and early differentiation for cells grown on complex surface coatings containing a mixture of GAGs and fibrous proteins (ECL). Normal myogenesis is dependent on the presence of ECM, both through the direct interactions of ECM molecules and membrane proteins, but also through the modulation of growth factors. Proteoglycans (PGs) are macromolecules with GAG chains attached to the core. The PGs, especially the heparan sulfate PGs, are important in muscle for maintaining muscle structure, regenerating, growth and development (Cornelison et al., 2004). Inhibition of PG synthesis inhibits the process of skeletal muscle differentiation. It has also been shown that the GAG composition changes in myoblasts from proliferation to differentiation. The expression and importance of GAGs and PGs in myogenesis are known, yet their biological function with regard to muscle development is not fully understood. It has been demonstrated that several growth factor activities are upregulated by PGs (Villena and Brandan, 2004). Conversely, some GAG–growth factor interactions can also downregulate their activity. (Fthenou et al., 2008). Increase in calcium concentrations might be another possible explanation to why GAGs improve bovine cell myoblasts proliferation and differentiation. Artificial GAGs applied to pre-fusing myoblasts induced transient increase in calcium concentrations, and any mechanism that increases intracellular calcium is expected to trigger myoblast differentiation (Martelly et al., 2010). An interesting observation in our study is that neither GAGs alone, nor ECL alone, did influence early myogenesis significantly. The combination of GAGs with fibrous proteins, however, improved proliferation and early differentiation. Differential binding and presentation of the serum-contained growth factors to the complex coating in the mixture compared to GAGs alone may affect proliferation and differentiation. We have not investigated this possibility in the described experiments. Early studies of heparan sulfate and dermatan sulfate show that these GAGs have high affinities for laminin, and that heparin modulates laminin polymerization **in-vitro** in vitro (Parthasarathy et al., 1998; Yurchenco et al., 1990). Similarly, PGs interact with collagen in specific locations in order to maintain the structure and organization of the ECM (Gillies and Lieber, 2011). Hence the binding of GAGs to fibrous proteins such as laminin and collagen contributes to the formation of a functional ECM meshwork. We have not measured the binding efficiency of GAGs to the surface of coverslips and plastic plates. Thus we cannot rule out whether the results with GAG alone do not resemble the effect of uncoated surfaces. In addition, we have used very high concentrations of GAGs in our experiments, and so our artificial ECM does not resemble the physiological **in-vivo** in vivo ECM composition. Still, our experiments suggest that GAGs and fibrous proteins mixed together into a composite biomaterial potentially can mimic a natural ECM with natural GAG–ECM interactions, and this could potentially improve cell proliferation and differentiation in **in-vitro** in vitro cell cultures. Muscle-derived fibroblasts are the principle cell type that may contaminate myoblast preparations, and can potentially dominate the cell cultures (Allen et al., 1997). We took advantage of the more rapid attachment of fibroblasts in order to enrich for myoblasts (Allen et al., 1997), and managed to obtain a high purity of myoblasts in the isolated cell cultures (i.e. more than 90% of the cells were negative for TE7 which is a marker for fibroblasts **in-vitro** in vitro). The components of the ECM influence the fibroblasts and myoblasts differently, and we used either laminin or a composite surface coating containing entactin, laminin and collagen IV. This helps to enrich the myoblast fraction as myoblasts show a higher affinity for collagen IV/laminin-coated surfaces than for collagen I/fibronectin-coated surfaces, the latter is preferred by fibroblasts (Kuhl et al., 1986). NCAM was the first marker used to identify human muscle satellite cells, and this marker is expressed in satellite cells, myoblasts, myotubes and muscle fibers during development (Boldrin et al., 2010). This marker is expressed in proliferating human and rat muscle cells, but is not a reliable marker in mice muscle cells; mice muscle cells express NCAM only when they have become committed to differentiation (Capkovic et al., 2008). We observed that NCAM is expressed in proliferating cells from bovine muscle. The fraction of NCAM positive and TE7 negative populations was in sum approximately 100% suggesting that the expression of these proteins was mutually exclusive. Desmin is another well used marker for the identification of myoblasts and myotubes. Desmin is abundantly expressed in the cytoplasm of skeletal muscle cells, and interconnects and anchors mature fibers to the plasma membrane (Yablonka-Reuveni and Day, 2011). Desmin is expressed in both rat and porcine myoblasts and myotubes, and is often used as a myoblast marker (Allen et al., 1991; Wilschut et al., 2010).

The expression of desmin in myoblasts, however, seems to vary among different species. We observed in our experiments that desmin expression was barely detectable by immunostaining in myoblasts, while the expression increased dramatically during differentiation (more than tenfold). This is in line with previous observations where 98% of rat myoblasts were desmin-positive, while only 14% of bovine myoblasts were desmin-positive (Allen et al., 1991). We believe that desmin is more suitable as a marker for differentiation rather than myoblast proliferation in bovine cell cultures. Another characteristic feature that we observed during the skeletal muscle differentiation was a reorganization of the Golgi complex. The Golgi complex was situated around the microtubule organizing center in proliferating cells, in series of closely associated flattened membrane stacks. During differentiation the Golgi complex transformed to a highly fragmented formation network of small stacks of cisternae positioned throughout the myotubes as well as around the nucleus/ER. Many structural reorganization events take place during myogenesis, including reorganization of centrosomes, microtubules, the Golgi complex and ER exit sites (Lu et al., 2001; Zaal et al., 2011). However, the mechanisms of these changes and their functional implications are still poorly understood. The reorganization of the Golgi complex observed in the bovine cells is in line with previous observations performed in rodents, and could provide an interesting model system for further studies.

The activation, proliferation and differentiation of muscle satellite cells correlate with the distinct expression profiles of the MRFs. The MRFs have different roles during myogenesis, and their expression levels differ. MyoD and Myf5 are myogenic determinators, and are expressed during the myoblast stage. In porcine primary cells the expression of both Myf5 and MyoD decreased significantly during differentiation (Wilschut et al., 2010). This is in contrast to the observations in chicken primary cells, where Myf5 expression declined during differentiation while MyoD expression continued well into the differentiation step (Day et al., 2009). The latter is in line with our results. We observed MyoD expression in proliferating cells and this expression persisted during differentiation. The secondary MRFs, myogenin and MRF4, are required downstream of MyoD as differentiation factors, essential for terminal differentiation in myoblasts committed to differentiation. We observed that myogenin expression was increased in cells committed to differentiation, and myogenin expression was activated before myotube formation, indicating that differentiation was in progress.

In conclusion the highest proliferation and differentiation rate in bovine primary muscle cells was obtained using a complex ECM coating consisting of *both* proteins and GAGs. We suggest that GAGs and fibrous proteins mixed together into a composite biomaterial can mimic a natural ECM, and this could improve myogenesis for *in-vitro* in vitro cell cultures. ~~Uncited reference(Zhu et al., (Chen))~~

Acknowledgments

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.diff.2013.06.006](https://doi.org/10.1016/j.diff.2013.06.006).

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Appendix A. Supporting information

Fig. S1 The relative gene expression of myogenic markers after 2, 4 and 6 days of differentiation. Bars show the relative mRNA expression of myogenic markers in differentiating cells compared to proliferating cells. The data are presented as the average mean of at least three independent experiments performed in technical triplicates, \pm SD. Asterisks denote significant differences between differentiated cells compared to proliferating cells ($*p < 0.05$).

[Multimedia Component 1](#)

Fig. S2 Comparison of the relative gene expression of myogenic markers calculated with either β -actin (A) or TATA (B). Bars show the relative mRNA expression of myogenic markers in differentiating cells compared to proliferating cells, the single protein coating Laminin and the complex surface coating ECL+GAGs. The reference genes TATA and β -actin are both acceptable for normalization. The data are presented as the average mean of at least two independent experiments performed in technical triplicates, \pm SD.

[Multimedia Component 2](#)

Fig. S3 The relative gene expression of myogenic markers during differentiation was higher on cells grown on culture plates coated with ECL in combination with commercial heparan sulfate (HS) compared to ECL alone. Bars show the relative mRNA expression of myogenic markers in differentiating cells compared to proliferating cells. The data are presented as the average mean of at least three independent experiments performed in technical triplicates, \pm SD.

[Multimedia Component 3](#)

Highlights

- Glycosaminoglycans in combination with extracellular matrix proteins improved cell proliferation and early differentiation.
- The Golgi complex was re-organized during differentiation.
- The level of MyoD persisted during differentiation, while the level of [myogenin](#) **Myogenin** and [desmin](#) **Desmin** increased.

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