Silk fibroin scaffolds with muscle-like elasticity support \textit{in vitro} differentiation of human skeletal muscle cells

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\textbf{Abstract}

Human adult skeletal muscle has a limited ability to regenerate after injury and therapeutic options for volumetric muscle loss are few. Technologies to enhance regeneration of tissues generally rely upon bioscaffolds to mimic aspects of the tissue extracellular matrix (ECM). In the present study, silk fibroins from four Lepidoptera (silkworm) species engineered into three-dimensional scaffolds were examined for their ability to support the differentiation of primary human skeletal muscle myoblasts. Human skeletal muscle myoblasts (HSMMS) adhered, spread and deposited extensive ECM on all the scaffolds, but immunofluorescence and quantitative polymerase chain reaction analysis of gene expression revealed that myotube formation occurred differently on the various scaffolds. Bombyx mori fibroin scaffolds supported formation of long, well-aligned myotubes, whereas on Antheraea mylitta fibroin scaffolds the myotubes were thicker and shorter. Myotubes were oriented in two perpendicular layers on Antheraea assamensis scaffolds, and scaffolds of Philosamia/Samia ricini (S. ricini) fibroin poorly supported myotube formation. These differences were not caused by fibroin composition per se, as HSMMs adhered to, proliferated on and formed striated myotubes on all four fibroins presented as two-dimensional fibroin films. The Young’s modulus of A. mylitta and B. mori scaffolds mimicked that of normal skeletal muscle, but A. assamensis and S. ricini scaffolds were more flexible. The present study demonstrates that although myoblasts deposit matrix onto fibroin scaffolds and create a permissive environment for cell proliferation, a scaffold elasticity resembling that of normal muscle is required for optimal myotube length, alignment, and maturation. © 2016 The Authors Journal of Tissue Engineering and Regenerative Medicine Published by John Wiley & Sons Ltd.

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Keywords silk fibroin; extracellular matrix; primary human myoblasts; myotubes; skeletal muscle tissue engineering; biomaterials; elasticity

1. Introduction

When skeletal muscle is damaged and undergoes myofibre necrosis, the satellite (progenitor) cells of skeletal muscle are activated to proliferate, differentiate and fuse to form mature myotubes. However, when large amounts of muscle become necrotic or are lost, such as in battlefield injuries or car accidents, this classic regenerative response is not sufficient to repair the defect (Grogan et al., 2011). Current therapies such as muscle grafts to replace lost muscle mass are not ideal because of donor site morbidities, and strategies to enhance endogenous repair of muscle also have limitations (reviewed in Grounds, 2014). Recently, the efficacy of acellular bioscaffolds to stimulate new muscle formation \textit{in vivo}, or the \textit{ex vivo} formation of muscle-like structures for subsequent implantation have been explored (Wang et al., 2014). At present, one of the most successful bioscaffolds reported in clinical skeletal muscle repair is decellularized porcine extracellular matrix (ECM) derived from small intestine or bladder. These scaffolds have been implanted in load-bearing limb muscles and, in combination with physical therapy, have resulted in functional improvement in human patients, albeit in small studies (Mase et al., 2010; Gentile et al., 2014; Sicari et al., 2014). Although numerous studies have examined different bioscaffolds for their compatibility with muscle cells \textit{in vitro}, these have generally used the murine myoblast cell line C2C12. The use of primary human skeletal muscle myoblasts (HSMMs) to test the cytocompatibility of different scaffolds is an essential first step to assess their viability for human muscle bioengineering.

Silk is a biopolymer produced by the silk glands of arthropods with the main source being the larvae of various Lepidoptera silkworms. Two major groups of silkworm are of commercial importance: Bombyxidae (mulberry) and Saturniidae (non-mulberry) (Mahendran et al., 2006). The domesticated mulberry species Bombyx mori is widely distributed, whereas the commercially available non-
mulberry varieties *Antheraea mylitta* (Tropical Tasar), *Antheraea assamensis/assama* (Muga) and *Philosamia/ Samia ricini* (Eri) are all from the Indian subcontinent.

There are two types of silk proteins obtained from the cocoons of *B. mori*: a water-soluble protein called sericin and a fibrous hydrophobic protein called fibroin. Fibroin is the key component of silk from *B. mori* and consists of a heavy (H) chain (390 kDa) and a light (L) chain (26 kDa) connected by a disulphide bond (Zhou et al., 2000). A 30 kDa glycoprotein (P25) is non-covalently linked to the H–L chain complex through hydrophobic interactions (Tanaka et al., 1999). The H chain comprises a highly repetitive glycine-rich core, flanked by non-repetitive sequences. These glycine motifs form highly ordered β-sheets which gives rise to the strength and toughness of silk, while the non-crystalline regions contribute flexibility and elasticity (Fu et al., 2009).

Fibroin proteins from non-mulberry silkworm species have slightly different structures. *Antheraea mylitta* is a homodimer containing 197 kDa subunits, *A. assamensis* fibroin is a heterodimer comprising chains of 220 kDa and 20 kDa, and fibroin from *S. ricini* consists of a heterodimer of chains of approximately 245 kDa and 210 kDa (Kundu et al., 2012b; Pal et al., 2013). Non-mulberry silk also contains poly-alanine rather than poly-glycine repeats and is more hydrophobic than *B. mori* silk (Kundu et al., 2012a). In addition, fibroin from *A. mylitta* contains the integrin binding motif arginine-glycine-aspartic acid (RGD), whereas the others do not (Morgan et al., 2008).

Properties such as high tensile strength, elasticity, thermal stability, aqueous preparation, slow degradability and biocompatibility make silk a useful biomaterial. Silk proteins are used in implantable biomaterials, drug delivery systems, and as bioscaffolds for skeletal muscle formation and repair. All reagents and chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA), unless otherwise stated.

### Table 1. Previous in vitro studies of myoblast growth and differentiation using silk protein matrices

<table>
<thead>
<tr>
<th>Silk matrix type</th>
<th>Cell type</th>
<th>Experiment</th>
<th>Outcome</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibroin–polyurethane blended films</td>
<td>C2C12 mouse myoblasts</td>
<td>Compatibility and differentiation</td>
<td>Silk fibroin improved the cell compatibility of polyurethane films</td>
<td>Park et al. (2013b)</td>
</tr>
<tr>
<td>Fibroin immobilized on polyurethane membranes</td>
<td>Cells from skeletal muscle biopsy (primary hypopharynx myoblasts)</td>
<td>Compatibility and differentiation</td>
<td>Cell proliferation, alignment and myotube differentiation achieved in aligned microchannels</td>
<td>Shen et al. (2013)</td>
</tr>
<tr>
<td>Fibroin–tropoelastin films</td>
<td>C2C12 cells and human bone marrow stem cells (hMSCs)</td>
<td>Cell attachment, proliferation and myogenic lineage differentiation</td>
<td>Roughness &amp; stiffness favouring proliferation and differentiation of C2C12 cells or hMSCs determined</td>
<td>Hu et al. (2011)</td>
</tr>
<tr>
<td>Recombinant spider silk protein sADf4(16) film</td>
<td>C2C12 mouse myoblasts</td>
<td>Cell adhesion and orientation</td>
<td>Myoblasts adhered to and proliferated best on structured, not unstructured films</td>
<td>Bauer et al. (2013)</td>
</tr>
<tr>
<td>Sericin supplemented serum-free medium</td>
<td>C2C12 mouse myoblasts</td>
<td>Myoblast differentiation</td>
<td>Myotubes formed with sarcomeres</td>
<td>Fujita et al., 2010</td>
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*Silk protein obtained from *Bombyx mori*, a mulberry silkworm species.
2.1. Collection of mulberry and non-mulberry silkworm species

*Bombyx mori* cocoons were collected from Debra Sericulture Farm, West Midnapore, West Bengal, India. *Antheraea mylitta* fifth instar mature larvae were collected from the Indian Institute of Technology (IIT) Kharagpur Farm. Both *A. assamensis* and *S. ricini* fifth instar larvae were collected from Coochbehar, West Bengal, India.

2.2. Processing of silk protein fibroin into 3D scaffolds

The methods used to prepare the regenerated fibroin solutions are outlined in Figure 1. Briefly, fibroin was isolated from *B. mori* cocoons using a protocol described elsewhere (Sofia et al., 2001). Fibroins from the non-mulberry species were isolated by squeezing the glands of fifth instar larvae immediately prior to them spinning their cocoons following the steps described elsewhere (Datta et al., 2001a; Kar et al., 2013; Pal et al., 2013). Fibroin solutions (2% w/vol) were used to fabricate 3D porous sponges/scaffolds using the freeze drying technique described in Nazarov et al. (2004). The scaffolds (14 mm in diameter and 4 mm high) were fixed with 90% ethanol for 1 h and then, for 30 min each, with 70% and 50% ethanol. Scaffolds were washed twice in phosphate-buffered saline (PBS) for 30 min, placed under ultraviolet (UV) light for 20 min and then into the wells of a 24-well plate and preconditioned in Skeletal Muscle Growth Medium-2 (SkGM-2; Lonza, Basel, Switzerland) for 2 h at 37°C.

2.3. Solubilization of the silk fibroin from freeze dried scaffolds for 2D culture

All silk fibroin scaffolds were solubilized in PBS. Scaffolds were placed in 1.5 ml tubes, 500 μl of PBS was added and incubation proceeded at 42°C for 30 min with rocking. Supernatants collected after centrifugation at
9000 rpm for 10 min at 4°C were centrifuged again and their protein content measured using a BCA protein assay kit (Pierce Biotechnology, Rockford, IL, USA). The solubilized silk was stored at 4°C. Routinely, 10 μg/cm² silk fibroin was used to coat tissue culture plastic or etched glass surfaces.

2.4. Antibodies

Rabbit polyclonal antibodies against fibronectin and glucose transporter 4 (GLUT4) were purchased from Abcam (Cambridge, UK). The anti-perlecan rabbit polyclonal antibody was a gift from Prof. John Whitelock (University of NSW, Sydney, Australia). Anti-myosin [slow skeletal heavy chain (MyHCB), clone NOQ7.5.4D] mouse monoclonal antibody (mAb) was purchased from Millipore (Temecula, CA, USA). Secondary antibodies were goat anti-rabbit Alexa Fluor 546 (AF546), goat anti-mouse Alexa Fluor 488 (AF488) and goat anti-rabbit AF488 (all Molecular Probes, Life Technologies, Eugene, OR, USA). Primary antibodies were used at concentrations of 2–4 μg per test. The control antibodies were rabbit IgG and mouse IgG (Zymed; Life Technologies).

2.5. Cell culture

The HSMMs (derived from a single donor) were purchased from Lonza (catalogue number CC-2580) and maintained in a humidified 37°C incubator equilibrated at 5% CO₂ in SkGM-2 (Lonza) containing human epidermal growth factor (EGF), dexamethasone, L-glutamine (all Molecular Probes, Life Technologies, Eugene, OR, USA). The HSMMs were passaged for a further 10 or 11 days with medium replacement every third day, then differentiated. Cells were cultured for a further 10 or 11 days with medium replacement every third day, then fixed, permeabilized and stained with anti-MyHCB mAb as described previously.

2.7. Immunofluorescence of matrix proteins

The HSMMs in SkGM-2 medium were seeded on 3D silk scaffolds (7.5 × 10⁴ cells/cm²) and cultured for 3 days. The scaffolds were washed and fixed with 4% paraformaldehyde (PFA)/PBS, then incubated in blocking solution for 1 h at room temperature (RT). Following washing with PBS they were incubated for 2 h at RT in a polyclonal antibody recognizing either fibronectin or perlecan, then washed with PBS and incubated in secondary antibody (anti-rabbit AF488). All antibodies were diluted in blocking solution. The scaffolds were washed and mounted in Vectashield (Vector Laboratories, Burlingame, CA, USA). The images were captured with an A1+ confocal microscope (Nikon, Tokyo, Japan) using NIS-Elements AR analysis version 4.10 software (Nikon, Tokyo, Japan). All images were acquired at the same gain and exposure settings.

2.8. Confocal laser scanning microscopy of scaffolds

To generate a single image, 15–25 z-stack images (2, 5 or 10 μm stacks) of cell-laden scaffolds were taken using a Nikon A1+ confocal microscope and merged. Six images/scaffold were captured at different locations and representative images are presented. To image large areas of the scaffold 50 z-stack images (2.5 μm stack) were taken using an UltraVIEW VoX Spinning Disc Confocal Microscope (PerkinElmer, Waltham, MA, USA) and the images were stitched together using the Volocity software (PerkinElmer). Myotube lengths were measured using the line tool measurement facility.

2.9. Scanning electron microscopy of scaffolds

The silk scaffolds (± HSMMs) were fixed in 4% PFA/PBS for 30 min at RT and then dehydrated in ethanol solutions of increasing concentration (30–100%) for 20 min per concentration, freeze dried using a ScanVac (LaboGene, Lyngen, Denmark), mounted on aluminium stubs and sputter coated with platinum (5 nm) using a 208HR sputter coater (Cressington, Redding, CA, USA). The data were collected using either a Neon 40EsB scanning electron microscope (PerkinElmer, Waltham, MA, USA) or MIRA SEM (TESCAN, Brno, Czech Republic).

2.10. Rheological measurements of 3D silk scaffolds

Sample measurements were conducted using a HAAKE MARS III Modular Advanced Rheometer (Thermo Fisher Scientific, Waltham, MA, USA) with 35 mm diameter parallel plate geometry. Silk scaffolds (14 mm in diameter and 4 mm high) were fixed as described then hydrated in PBS. The gap height for the parallel plate arrangement was determined by monitoring the normal force, which was maintained at 0.3 ± 0.1 N during all measurements. The linear viscoelastic region, determined by performing stress sweep measurements, is the area where the moduli...
values were independent of the applied stress or strain. The moduli values: elastic modulus (G') and the viscous modulus (G''), were determined in the linear viscoelastic region by strain sweep measurements. The stress sweep was performed from 1 to 70 Pa, the strain sweep was performed from 0.01–3% strain. Both sweeps were done at a frequency of 1 Hz and a temperature of 37°C. The data was analysed using HAAKE RheoWin software (version 3.61). The stress and strain values measured during the strain sweep (which correspond to the linear viscoelastic region for the scaffolds) were used to calculate Young's modulus. The stress was plotted against the strain and a linear regression was applied to the data. The gradient of the regression curve is equal to Young's modulus; the average value of four replicates was plotted as mean ± SD.

2.11. Alamar Blue assay for HSMM proliferation

HSMMs were cultured in the wells of a 48-well plate (Corning Inc., Corning, NY, USA) at a seeding density of 1 × 10⁵ cells/well in SkGM-2 medium/10% FBS (500 μl). After 2, 5, 8 and 11 days of proliferation on 2D substrates of a silk fibroin (10 μg/cm²), or collagen I or fibronectin (10 μg/cm²); or tissue culture plastic, 50 μl of Alamar Blue reagent (Life Technologies, Carlsbad, CA, USA) was added. The plates were incubated in a CO₂ incubator for 4 h and the fluorescence intensity measured using excitation and emission wavelengths of 560 nm and 590 nm, respectively, on an EnSpire Multimode plate reader (Perkin Elmer). Blank medium was the negative control and fluorescence units (RFU) relative to the control were calculated. Previous experiments indicated that the RFUs were in the linear range of an Alamar Blue standard curve obtained by titrating cell numbers.

2.12. Etching of glass coverslips

coverslips (13 mm diameter; ProScitech, Thuringowa, Australia) that had been stored in 100% ethanol were treated with etch solution [6.0 g NaOH dissolved in 24 ml double distilled (dd) H₂O and the volume made up to 60 ml with 95% ethanol] for 30 min at RT. Coverslips were then washed with ddH₂O, dried at RT and sterilized by UV light.

2.13. Immunofluorescent staining of myoblasts in 2D culture

The HSMMS in SkGM-2 medium were seeded (15 × 10²/cm²) on etched glass coverslips coated with fibroin, collagen type I or fibronectin (coating concentration 10 μg/cm²) and cultured for 3 days. The medium was changed to differentiation medium (DMEM/F12/2% HS). After day 7 or 10 of differentiation cells were fixed in 4% PFA/PBS, permeabilized with 0.1% Triton X-100/PBS at RT and blocked using 10% FBS/1% BSA/PBS (blocking solution) for 1 h. Cells were incubated with the anti-MyHC mAb and the GLUT4 antibody diluted in blocking solution for 2 h at RT, washed with PBS and incubated for 1 h in AF488-conjugated goat anti-mouse antibody and AF546-conjugated goat anti-rabbit antibody diluted in blocking solution. The coverslips were washed and mounted in 4',6-diamidino-2-phenylindole (DAPI)-containing Vectashield. Fluorescent images were captured using a Axioskop microscope (Carl Zeiss) with a ×40 objective and Spot Advanced software (SPOT imaging solutions, Sterling Heights, MI, USA). Nuclei and myotubes were counted in six random fields of view (>250 nuclei) and fusion index (FI) was calculated according to the formula: FI = (number of nuclei within myotubes containing ≥2 nuclei/total number of nuclei) × 100. Two independent experiments were performed and representative data are shown.

2.14. Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) of muscle differentiation genes

Total mRNA was isolated using TRI Reagent (Sigma-Aldrich) as per the manufacturer’s instructions from HSMMS cultured on B. mori and A. mylitta scaffolds (seeded at 5 × 10⁵ cells/scaffold) in SkGM-2 media and differentiated in DMEM/F12/HS as described. mRNA concentration and purity was assessed using a Nanodrop spectrophotometer (Thermo Fisher Scientific). All mRNA samples had an A260/A280 ratio of >1.8. Reverse transcription was performed on 500 ng of mRNA using the Tetro CDNA synthesis kit (Bioline, Alexandria, Australia) as instructed by the manufacturer. A qRT-PCR was performed on cDNA prepared from differentiation day 0 (day 4 of proliferation, immediately before triggering differentiation) and differentiation day 4, with gene

<table>
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<th>Table 2. Primer Information</th>
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<tr>
<td><strong>Gene</strong></td>
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<tr>
<td>SDHA</td>
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<tr>
<td>ACTA1B</td>
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<tr>
<td>TBP</td>
</tr>
<tr>
<td>MYOD1</td>
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<td>MYH5</td>
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<tr>
<td>MYH7</td>
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<tr>
<td>TBP</td>
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F, forward; R, reverse.
expression levels normalized to the baseline expression at day 0, and from cDNA prepared on differentiation days 2 and 10, with gene expression on day 10 normalized to expression on day 2. The qRT-PCR reactions were performed using SensiFAST SYBR Lo-Rox kit (Bioline), with triplicate reactions containing 5 μl SYBR Green Lo-RoX Mix, 2 μl template cDNA, 1 μl forward/reverse primer (25 ng/μl) and 2 μl RNase free H2O. The reactions were performed on a ViiA™ 7 Real-Time PCR system (Applied Biosystems, Life Technologies) with fast 96-well block using the cycling conditions: denaturation at 95°C for 2 min, 40 cycles of denaturation at 95°C for 5 s and annealing and extension together in one step at 60°C for 20 s followed by a melt step ranging from 55 to 95°C. Primers (Table 2) were selected for three reference genes [succinate dehydrogenase complex, subunit A (SDHA), beta actin (ACTAB), TATA-binding protein (TBP)] and five genes of interest for muscle transcription factors [myogenic factor 5 (Myf5), myogenic determinant protein-1 (MyoD1), heavy chain myosin (MYH1 and MYH7) and skeletal muscle alpha actin (ACTA1)] using the PrimerBank database (Spandidos et al., 2010) and purchased from Geneworks (Hindmarsh, Australia). Of the reference genes, TBP had the most stable expression according to Normfinder (Andersen et al., 2004) and Bestkeeper (Pfaff et al., 2004) software and was used as the reference gene. TBP is a central regulatory eukaryotic transcription factor, used by cellular RNA polymerases and is a stable reference gene for HSMMs under differentiation conditions (Stern-Straeter et al., 2009). The expression levels for MyoD1, Myf5, MYH1, MYH7 and ACTA1 were normalized to TBP expression values and fold-change determined using the 2-delta delta Ct method with baseline expression at differentiation day 0 set at 1. The mean ± standard error of four replicates are provided.

2.15. Statistical analysis
The data from three to four independent replicates per data point were collected from duplicate experiments and represented as means ± standard deviations. Analysis of cell proliferation experiments and rheology measurements was performed using one-way analysis of variance (ANOVA) followed by Tukey’s post-hoc test, after

![Figure 2](image_url)

Human skeletal muscle myoblasts (HSMMs) adhere and secrete extracellular matrix (ECM) proteins on three-dimensional (3D) silk scaffolds. The HSMMs were cultured on 3D silk scaffolds for 2 days in skeletal muscle growth medium-2 proliferation medium, then fixed and stained with rhodamine-phalloidin (F-actin staining; b,f,j,n), or antibodies recognizing either fibronectin (c,g,k,o), or perlecan (d,h,l,p). 4′,6-diamidino-2-phenylindole (DAPI) stained the silk scaffolds (all panels). Images were captured using a Nikon A1 confocal laser scanning microscope. The merged images of several z-stack images (5 μm each stack, 150–200 μm deep into the scaffold) are presented. A representative image of six fields of view is shown. Bar: 50 μm
the data were confirmed to have a normal distribution using the Shapiro–Wilk test. Statistical analyses of qRT-PCR data were performed using the non-parametric Mann–Whitney U-test, because these data have a non-normal distribution.

3. Results

3.1. The interaction of HSMMs with the silk 3D scaffolds

At 2 days after seeding HSMMs in proliferation medium onto the scaffolds, rhodamine–phalloidin staining of polymerized actin fibres revealed that these cells had adhered to all four scaffold types and were covering the scaffold surfaces (Figure 2). The F-actin fibre arrangement indicated the cells were well spread on all scaffolds. On A. mylitta, S. ricini or A. assamensis scaffolds the cells appeared to be oriented around the scaffold pores (Figure 2f,j,n), whereas on B. mori scaffolds a different F-actin arrangement was apparent, with actin fibres oriented in parallel and, in places, crossing over the pores (Figure 2b). Staining of unseeded scaffolds revealed their porosity (Figure 2a,e,i,m). Immunostaining of HSMMs on 3D scaffolds revealed deposition of both fibronectin and perlecan. The staining intensities suggested comparable deposition of these molecules on all four scaffolds (Figure 2). The organization of the matrix proteins largely mirrored the F-actin fibre arrangement, although this is less apparent on A. assamensis scaffolds. A comparison of the fibronectin staining on the different scaffolds revealed fibronectin was deposited as parallel fibres on B. mori scaffolds (Figure 2c), whereas on A. mylitta and S. ricini scaffolds the fibronectin fibres were arranged in a circular pattern around the pores (Figure 2g,k).

3.2. HSMMs differentiation on 3D silk scaffolds

When stimulated with differentiation medium the HSMMs fused to form myotubes. Immunostaining with a mAb recognizing slow muscle myosin (MyHCB) revealed that multinucleated myotubes formed on all scaffolds (Figure 3), although there were markedly fewer myotubes on S. ricini scaffolds. Confocal imaging indicated that the myotubes were not necessarily confined to the same focal plane, suggesting they were formed by the fusion of cells at different depths in the scaffolds. The parallel alignment of the MyHCB-positive myotubes on B. mori, A. mylitta and A. assamensis scaffolds was striking. On A. assamensis scaffolds there appeared to be two layers of myotubes, one at a 90° orientation to the other. Confocal microscopy revealed that the area covered by the myotubes on B. mori

Figure 3. Immunostained images of differentiated human skeletal muscle myoblasts (HSMMs) on three-dimensional (3D) silk scaffolds. The HSMMs were cultured on the silk scaffolds for 4 days in proliferation medium and a further 4 days in differentiation medium. After differentiation, the scaffolds were stained with anti-myosin monoclonal antibody (NOQ7.5.4D) and goat anti-mouse IgG-AF488. The nuclei are stained with 4',6-diamidino-2-phenylindole (DAPI, blue). Images were captured using a Nikon A1 confocal microscope. The 3D merged images of several z-stack images (5 μm each stack, 150–200 μm deep into scaffold) are represented. Bar: 100 μm
scaffolds was greater than that seen with the other scaffolds at identical cell seeding densities. In addition, as the longest myotubes formed on *B. mori* scaffolds, the length of these myotubes and the range in their lengths were determined. Measurements of 25 myotubes indicated an average length of 347 ± 7.2 μm and a range of 175–515 μm (Figure 4).

3.3. Ultrastructure and elastic properties of the 3D scaffolds

To determine if the ultrastructure of the scaffolds could have contributed to the differences in myotube formation, SEM was performed. All scaffolds had interconnecting pores, with diameters ranging from 50–120 μm on *A. mylitta*, 30–110 μm on *B. mori* and 30–90 μm on *S. ricini* and *A. assamensis* scaffolds, although *S. ricini* had a larger number of smaller pores than *A. assamensis* (Figure 5A;a, c,e,g). Despite these minor differences in pore size the HSMMs formed a continuous layer on all scaffolds, virtually covering the scaffold after 5 days (Figure 5A;b,d,f,h). On *A. assamensis* scaffolds, this appeared to consist of multiple cell layers (Figure 5A;h). Scanning electron microscopy of HSMMs on an *A. mylitta* scaffold revealed the cells were well spread, flat and elongated, with many contact points with the scaffold visible at 2 days after seeding (Figure 5B;b). The deposition of fibrous material, probably ECM proteins, onto the scaffold surface was also evident (Figure 5B;c).

Rheology measurements revealed the elastic properties of the silk scaffolds were quite different (Figure 6). The *B. mori* silk scaffolds had the highest Young’s modulus with a mean value of 16.53 kPa followed by *A. mylitta* scaffolds with a mean value of 10.67 kPa, both of which were higher than those of *S. ricini* and
A. assamensis scaffolds, which had similar Young’s moduli (3.74 kPa and 3.73 kPa, respectively). A similar pattern was observed in the viscous modulus of the scaffolds with B. mori scaffolds having the highest viscous modulus and S. ricini scaffolds the lowest modulus (Figure 6).

3.4. Growth and differentiation of HSMMs on 2D silk fibroin substrates

To clarify whether amino acid composition differences in the fibroins could have affected the behaviour of the HSMMs, the proliferation and differentiation of these...
cells on 2D fibroin substrates was examined and compared with substrates of fibronectin and type I collagen. The 2D format was chosen to eliminate any confounding effects from scaffold structure differences. The growth and viability of HSMMs cultured for 11 days in SkGM-2 proliferation medium on the 2D substrates were measured using the Alamar Blue assay, which detects metabolic activity. The time-points chosen were days 2, 5, 8 and 11 as the doubling time of HSMMs was more than 42 h (data not shown). The HSMMs cultured on the silk fibroin and matrix protein substrates exhibited similar growth when subconfluent, at up to 8 days of culture (Figure 7). However, when the cells reached confluence (day 11), the assay measurements for HSMMs cultured on A. mylitta fibroin were significantly higher than those obtained from similarly cultured cells on the other substrates.

Immunostaining with an anti-MyHCB mAb of cells differentiated on 2D silk fibroin substrates revealed multinucleated myotubes with elongated nuclei (Figure 8).

3.5. Gene expression of myogenic markers in HSMMs on B. mori and A. mylitta 3D scaffolds

As the HSMMs readily formed myotubes on B. mori and A. mylitta fibroins in both the 3D scaffold and 2D film formats, cells grown on 3D scaffolds of these silk fibroins were selected for gene expression studies. The mRNA levels for muscle transcription factors (Myf5 and MyoD1), heavy chain myosins (MYH1 and MYH7), skeletal muscle alpha actin (ACTA1) and the reference gene (TBP) were determined by qRT-PCR. Gene expression by HSMMs in differentiation medium was examined at two sets of time-points to capture gene expression changes both early and late in myotube formation. Early gene expression was assessed at differentiation days 0 and 4, with day 4 data presented relative to expression levels at day 0 (Figure 9). On day 4, Myf5 mRNA levels decreased and MyoD1 expression increased on both scaffolds as the cells proceeded towards fusion and myotube formation (Figure 9a,b). A time-dependent increase was observed in ACTA1, MYH1 and MYH7 levels, indicative of maturing myotubes. The expression of the ACTA1 gene was much higher on A. mylitta scaffolds than on B. mori scaffolds. The expression of these genes was also examined at differentiation day 10 relative to differentiation day 2, thereby detecting late gene expression changes. In this latter data set ACTA1 expression in HSMMs at day 10 on A. mylitta scaffolds was significantly lower than that at day 2, whereas for the other genes, expression remained stable at day 2 and day 10. In contrast, on B. mori scaffolds the expression of ACTA1 and MYH1 was higher at day 10 than at day 2 (Figure 9c,d) and Myf5 and MyoD1 expression levels had decreased from day 10 to that at day 2. Collectively, these data suggest that HSMMs began to differentiate earlier on A. mylitta scaffolds than on B. mori scaffolds.

These myotubes formed parallel to each other on all substrates by day 7 of differentiation and striations in the myotubes were visible by day 7 and remained at day 10. The staining pattern of an antibody to GLUT4, a glucose transporter protein and a marker of functional myotubes (Guillet-Deniau et al., 1994) revealed that GLUT4 was present along the length of the myotubes, although some perinuclear accumulation also occurred on all silk substrates (Figure 8e–h). To quantify differentiation, fusion indices of MyHCB-positive myotubes were determined (see the Supplementary material online, Figure A). The results show a similar percentage of nuclei within the myotubes, (77–81%) on collagen I, fibronectin and fibroins from B. mori, A. mylitta and S. ricini. However, on A. assamensis fibroin, the percentage of nuclei within the myotubes was significantly lower (57%) than that of HSMMs on the other substrates. The number of myotubes formed per field of view was also similar on collagen I, fibronectin and the fibroins from B. mori, A. mylitta and S. ricini, but on A. assamensis fibroin, fewer myotubes formed (see the Supplementary material online, Figure B).
scaffolds, but the mature myotube gene expression pattern was lost at day 10, whereas on *B. mori* scaffolds it was maintained.

4. Discussion

The present study used four silk fibroins originating from different silkworm species to investigate what features are likely to be important in a 3D biomaterial for use in human skeletal muscle formation during regeneration. Those scaffolds that more closely resembled muscle in their elasticity (approximately 12–16 kPa) were best able to support myotube formation and alignment. Scaffolds of fibroin from *A. mylitta*, which has an RGD motif, did not enhance HSMM fusion and myotube formation over that seen with scaffolds of *B. mori* fibroin, which does not have an RGD sequence. Rather, the HSMMs adhered to, and deposited extensive ECMs on all of the 3D

Figure 8. Human skeletal muscle myoblasts (HSMMs) differentiate to form myotubes on silk fibroin substrates. HSMMs were seeded on etched glass coated with the silk fibroins (10 μg/cm²) and cultured for 4 days in skeletal muscle growth medium-2 proliferation medium and a further 7 or 10 days in differentiation medium. After 7 days of differentiation myotubes were stained with a mouse anti-myosin heavy chain monoclonal antibody (mAb) (NOQ7.5.4D) and goat anti-mouse IgG-AF488 (a–d) (MyHCB, mAb recognizing slow muscle myosin); after 10 days, myotubes were stained with both the anti-myosin mAb/goat anti-mouse IgG-AF488 combination and with rabbit anti-glucose transporter 4 (GLUT4) antibody followed by goat anti-rabbit IgG-AF546 (e–h). The nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). Myotubes were imaged using a Zeiss Axioskop fluorescent microscope. Bar: 50 μm (a–d), 25 μm (e–h)

Figure 9. Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) analysis reveals muscle differentiation on *Bombyx mori* and *Antheraea mylitta* three-dimensional (3D) silk scaffolds. qRT-PCR was used to determine the expression of five differentiation markers (Table 2) in HSMMs grown in skeletal muscle growth medium-2 on *Bombyx mori* (a,c) and *A mylitta* (b,d) scaffolds at day 4 (a,b) and day 10 (c,d) of differentiation. Relative expression levels for *MYF5, MYOD1, MYH1, MYH7* and *ACTA1* are normalized to the Ct value of the reference gene (*TBP*) and fold-change was determined using the using 2-delta delta Ct method. Day 4 and day 10 differentiation expression levels were normalized to day 0 and day 2 differentiation, respectively. The mean ± standard error of four biological replicates are presented. The Mann–Whitney U-test was performed and significance (*p*) values shown at each time-point for each gene using *p* < 0.05
scaffolds regardless of their fibroin composition. Nevertheless, the scaffolds did not equally support myotube formation and alignment, even though striated myotubes, expressing a functional marker, formed 2D films of all fibroin types. When the length, alignment and gene expression pattern of the myotubes are considered, it is likely that scaffolds of *B. mori* fibroin will be the most useful for clinical applications involving skeletal muscle.

Other *in vitro* studies of silk as a biomaterial for skeletal muscle tissue engineering have only used fibroin from *B. mori* (Table 1) and most have used the immobilized murine myoblast cell line, C2C12 (Table 1). The present study used primary human myoblasts and four different silk fibroins and indicated that all the fibroins, when displayed in either a 2D or a 3D format, were compatible with HSMMs in tissue culture. On 3D silk scaffolds the HSMMs adhered to and spread on all scaffolds to form a cell sheet by day 5 in culture (Figures 2 and 5). Immunofluorescence analyses indicated that the HSMMs deposited ECM molecules on all of the silk scaffolds, a result supported by the SEM data of HSMMs on an *A. mylitta* scaffold (Figure 5B). These endogenous matrix proteins will interact with integrins on the cell surface of HSMMs to facilitate spreading and adhesion. Myoblasts express both the RGD binding integrins, α5β1, αvβ3 and αvβ5, as well as other non-RGD binding integrins including α3β1, α4β1, α4β7, α6β1 and α7β1 (García et al., 1999; Mayer, 2003). The rapid secretion of matrix proteins probably compensates for the absence of RGD peptides in some of the silk fibroins (Datta et al., 2001b), particularly as stable interactions mediated by α5β1 require another site in fibronectin, located adjacent to RGD (minimal sequence: PHSRN), and the αv integrins also bind to another site in fibronectin in addition to RGD (Leiss et al., 2008). As fibronectin-assembles into fibrils at the cell surface bound to α5β1 or the αv integrins (Leiss et al., 2008), it is likely all of the silk fibroins provide a surface to which fibronectin fibrils can adsorb and this is what stabilizes cell adhesion and myotube formation on the different silk scaffolds. Others have similarly concluded that what is required for cell-self organization is a surface that facilitates cells to deposit and assemble their own, endogenous, adhesive ECM (Cerchiarì et al., 2015).

These secreted ECM proteins are likely to have a role in myotube formation. A number of studies have reported a role for integrins in C2C12 cell myotube formation (Garcia et al., 1999) and in an earlier study it was shown C2C12 cells very rapidly secreted fibronectin and perlecan under conditions of serum-free culture and formed myotubes (Chaturvedi et al., 2015). Data from *in vivo* animal models of muscle injury and repair indicate that fibronectin levels increase in regenerating areas of damaged muscle and there is increased production of collagen IV and the laminin α2 chain as the muscle myotubes mature (Grounds, 2008). Recent reviews also highlight the importance of fibronectin and perlecan (with its glycosaminoglycan chains), for muscle satellite cell self-renewal and skeletal muscle morphogenesis (Goody et al., 2015; Thomas et al., 2014). In a different system, silk/chitosan scaffolds modified by fibronectin and laminin deposited by Schwann cells initiated better repair of sciatic nerve injuries than non-coated scaffolds (Gu et al., 2014). Hence, biomaterials that trigger the resident cells to synthesize and deposit their own matrix may better support tissue repair.

Although there are differences in the capabilities of the fibroins to support myotube formation and alignment, this seemed to be because of the way the fibroins were presented to the cells rather than the composition of the fibroins *per se*. When the fibroins were used as a coating on tissue culture plastic, they all supported HSMM proliferation and differentiation, with only slight differences being evident. For example, the rapidly increased metabolic activity measured by the Alamar Blue assay in cells plated on *A. mylitta* fibroin suggested that these cells may have differentiated earlier than cells on the other three silk substrates (Figure 7), and the fusion indices and myotube numbers indicated that myotubes formed less well on *A. assamensis* films, but similarly on *B. mori*, *A. mylitta* and *S. ricini* fibroin films (Figure 8, and see the Supplementary material online, Figure S1). Interestingly, cell proliferation and myotube formation on the fibroin films was comparable to that seen on fibronectin and collagen I used at the same concentration and coating conditions (Figure 7 and Figure S1).

In contrast to the 2D format, the different fibroin 3D scaffolds supported HSMM myotube formation to varying degrees and this was quite apparent following immunostaining with an anti-MyH CB antibody (Figure 3). On *B. mori* scaffolds, HSMMs formed extremely long, well-aligned myotubes, whereas the myotubes that formed on *A. mylitta* scaffolds were thicker and shorter. The parallel alignment of HSMMs on *B. mori* scaffolds occurred by day 2 of proliferation, as revealed by the similar orientation of most of the F-actin fibres and by the orientation of the fibronectin fibrils (Figure 2). In contrast, on the other scaffolds the F-actin fibres tended to follow the edges of the scaffold pores, indicating that HSMMs on these scaffolds were not spanning the pores. Myotubes on *A. assamensis* fibroin scaffolds were predominately oriented in two perpendicular layers in an arrangement that will not favour a functioning skeletal muscle. The SEM images suggested multiple layers of cells on *A. assamensis* (Figure 5), whereas the cell layer was thinner on *A. mylitta* and *B. mori* scaffolds. Myosin expression was much lower in HSMMs seeded on the *S. ricini* fibroin scaffolds (Figure 3), suggesting that the architecture of these scaffolds was not ideal for myotube maturation.

The degree to which the HSMMs differentiated on the different scaffolds was examined by comparing the expression patterns of genes associated with myogenesis. *Myf5* is the first muscle-specific regulatory factor to be expressed during mouse embryogenesis while *MyoD1* promotes myoblasts cycle withdrawal and the induction of differentiation (Bentzinger et al., 2013). *MYH1* and *MYH7* are adult heavy chain myosin isoforms expressed in myotubes and myofibres, and *ACTA1* is a muscle actin isoform (Stern-Straeter et al., 2011). In the present study,
TBP was the most stable reference gene of those tested, which is in agreement with the study by Stern-Straeter et al. (2009).

The qRT-PCR data were consistent with the immunostaining data and indicated that HSMMs differentiated well on A. mylitta and B. mori scaffolds. On both scaffolds, a decrease in Myf5 mRNA levels and an increase in Myod1 levels indicated that most myoblasts had begun to differentiate after 4 days in differentiation medium. The levels of Myh7 mRNA were 8- to 10-fold higher at day 4, compared with day 0, which correlated with the strong myosin immunostaining of myotubes on both scaffolds (Figure 9). In contrast, Acta1 levels were much higher on A. mylitta than on B. mori at day 4 in differentiation medium, but by day 10 Acta1 mRNA levels in cells on A. mylitta scaffolds decreased relative to the levels at day 2, while the levels of expression of the other genes were similar to at both day 2 and day 10 (Figure 9). These data were quite different from those of cells on B. mori scaffolds and from the gene expression studies of human myoblast differentiation conducted by Stern-Straeter et al. (2011). The expression levels of all the genes examined at both the early and late differentiation stages on B. mori scaffolds were as expected from the earlier study (Stern-Straeter et al., 2011). Collectively these data suggest that although HSMMs started to differentiate more quickly on A. mylitta than on B. mori scaffolds, it appeared the myotubes that formed on A. mylitta scaffolds did not maintain their phenotype at the latter time-point. Possibly, the formation of long, well-aligned myotubes on B. mori scaffolds (Figure 8), occurred because on this scaffold, the gene expression pattern was characteristic of mature human muscle.

Pore size and the mechanical properties of scaffolds have been described as factors regulating cell proliferation and differentiation. Zhang et al. (2010) reported that human bone marrow mesenchymal stromal cells seeded on silk fibroin scaffolds showed greatest proliferation on scaffolds, with a pore size of 100–300 μm (Zhang et al., 2010). All the scaffolds used here have similar porosities, but the SEM indicated that the pore size of S. ricini (Figure 5A) was smaller than the other three scaffolds. Substrate stiffness or elasticity can also determine cell fate by altering mechanical signal transduction pathways (Engler et al., 2004, 2006). Possibly the HSMMs differentiated best on B. mori and A. mylitta scaffolds as these two scaffolds had a Young’s modulus (E) of around 10–16 kPa, which is very close to the elastic modulus of human skeletal muscles (gastrocnemius and soleus) at rest (16.5 kPa and 14.5 kPa, respectively) (Shinohara et al., 2010) (Figure 6). There are no data on the Young’s modulus of cultured HSMMs but for C2C12 myoblasts the Young’s modulus is between 12 and 15 kPa (Collinson et al., 2002). The findings of the present study are consistent with those of Engler et al. (2004) who investigated the elastic properties of C2C12 myoblasts cultured on different substrates of varying stiffness (Engler et al., 2004). They found that although the myoblasts fused into myotubes irrespective of substrate flexibility, actin/myosin striations appeared only in myotubes cultured on gels with a stiffness close to normal muscle (Young’s modulus: E ~ 12 kPa), indicating that substrate stiffness is a critical factor regulating myotube maturation (Engler et al., 2004). Later, the same group demonstrated the significance of matrix elasticity on stem cell fate (Engler et al., 2006). When mesenchymal stem cells (MSCs) were cultured on collagen-coated gels of varying elasticity that mimicked brain, muscle and bone matrix in their stiffness, they differentiated into myogenic cells on medium stiffness gels (E ~ 12 kPa), osteocytes on high-stiffness gels, and displayed neuronal morphology when cultured on matrices mimicking brain elasticity (Engler et al., 2006). Here it was shown that the viscous moduli of the two scaffolds that best supported myotube formation were similar and quite different from that of scaffolds from S. ricini and A. assamensis. These last two scaffolds either did not support differentiation or the myotubes that formed were not aligned in a single direction.

For some adult human skeletal muscles, individual myofibres may extend up to lengths of 35 cm, in parallel with the longitudinal orientation of the ECM (Paul, 2001; Harris et al., 2005). To form a functional new muscle in vivo, the implanted regenerated myofibres on the bioscaffold should fuse with the existing myofibres and become innervated. The in vitro data of the present study demonstrate that 3D fibroin scaffolds from B. mori supported HSMM differentiation into long, well-aligned myotubes, and are encouraging in this context. In contrast, short, thick myotubes formed on A. mylitta scaffolds suggesting that rapid differentiation may not produce the best myotubes.

5. Conclusion

Silk fibroins from four different silkworm species, under 2D and 3D culture conditions, effectively supported HSMM proliferation, myotube formation and maturation, although their efficacy differed. All the scaffolds stimulated the HSMMs to deposit an endogenous ECM and so the presence of an RGD motif in A. mylitta fibroin did not confer an advantage. Of greater importance for myotube formation and maturation was a scaffold elasticity that resembled that of normal muscle. This comparative study suggested that silk fibroin from the domesticated mulberry species, B. mori, when processed into a scaffold with elasticity similar to skeletal muscle, could be a useful biomaterial for skeletal muscle bioengineering, although fibroin from A. mylitta similarly prepared may also be useful. Bombyx mori silk fibroin materials are widely used and well tolerated in clinical situations, and this demonstration of the suitability of B. mori fibroin bioscaffolds, is a necessary first step for potential bioengineering of human skeletal muscle cells for future clinical applications.
Conflicts of interest

The authors have declared that there is no conflict of interest.

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