Novel Engineering of Masseter Muscle Tissue Using Hydrogel Scaffolds with Cod Skin Particulate

2025 Research Aid Awards (RAA)

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FollowUp Form

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Title of Project:*
Novel Engineering of Masseter Muscle Tissue Using Hydrogel Scaffolds with Cod Skin Particulate

Award Type
Research Aid Award (RAA)

Period of AAOF Support
July 1, 2025 through June 30, 2026

Institution
Trustees of Indiana University

Names of principal advisor(s) / mentor(s), co-investigator(s) and consultant(s)
Rishma Shah, Lester Smith

Amount of Funding
$6,000.00

Abstract

(add specific directions for each type here)

See attached document.

Respond to the following questions:

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TERM-S-26-00414-compressed.pdf
Please see attached file for the PDF of the manuscript that was submitted to Tissue Engineering and Regenerative Medicine.

Were the original, specific aims of the proposal realized?*

Specific Aim 1 was realized, while Specific Aim 2 was not; we were able to investigate the two-dimensional biocompatibility of craniofacial muscle fiber formation, but the three-dimensional investigation was not.

Were the results published?*

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The manuscript has been submitted for publication in Tissue Engineering and Regenerative Medicine.

Presented

Please list titles, author or co-authors of these presentation/s, year and locations:*

Skeletal Muscle Engineering Utilizing a Novel Cod Skin Scaffold
G. Chaffin, C. Matias, R. Shah
Indiana University School of Dentistry Research Day, April 2026

Was AAOF support acknowledged?

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Yes, the AAOF support was acknowledged in the poster presentation and manuscript.

Internal Review

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Tissue Engineering and Regenerative Medicine

Skeletal Muscle Engineering Utilizing a Novel Cod Skin Scaffold

--Manuscript Draft--

Manuscript Number:	
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Abstract:	Background: Volumetric muscle defects create functional and aesthetic problems, and are challenging to treat. Current techniques are associated with limitations and skeletal muscle tissue engineering offers an alternative approach using cells and scaffolds. Unfortunately, there is no ideal scaffold. Marine-derived biomaterials may bridge this gap. Therefore, this study aimed to C2C12 mouse muscle cell biocompatibility and differentiation on FDA-approved cod skin scaffolds. Methods: C2C12 cells were expanded under standard culture conditions and seeded at 2 x 10^3 cells/cm^2 onto Kerecis SurgiClose® cod skin scaffolds. Control cells were cultured on gelatin-coated tissue culture plastic. Samples (n=3/timepoint) were processed on days 3, 7, 10, and 14 for western blotting and qPCR. The alamarBlue cell viability assay determined cell biocompatibility. Mixed-model ANOVA was used to test for differences between the groups and over time. Results: The alamarBlue assay showed increased cell viability in both groups over time, with greater expression on the cod skin scaffolds at later timepoints ($p<0.05$). There was expression of the MYOD1, MYOG, MYH3, and MYH8 genes, and MyoD1 and myogenin proteins in both groups over time indicating myogenic differentiation and early myofiber maturation. Compared to the cod skin scaffolds, control cells demonstrated greater gene expression in addition to MyoD1 protein expression at all timepoints ($p<0.05$). Myogenin protein expression was greater on cod skin scaffolds after day 10, albeit non-significant ($p>0.05$). Conclusion: FDA-approved cod skin scaffolds are biocompatible and can support myoblast differentiation and early myofiber maturation. Further studies are needed to investigate how scaffold modification can best support the myogenic process.

Suggested Reviewers:	

Title: Skeletal Muscle Engineering Utilizing a Novel Cod Skin Scaffold

Running Title: Skeletal Muscle Engineering Using Cod Skin Scaffolds

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Abstract

Background: Volumetric muscle defects create functional and aesthetic problems, and are challenging to treat. Current techniques are associated with limitations and skeletal muscle tissue engineering offers an alternative approach using cells and scaffolds. Unfortunately, there is no ideal scaffold. Marine-derived biomaterials may bridge this gap. Therefore, this study aimed to C2C12 mouse muscle cell biocompatibility and differentiation on FDA-approved cod skin scaffolds.

Methods: C2C12 cells were expanded under standard culture conditions and seeded at 2 x 10^3 cells/cm^2 onto Kerecis SurgiClose® cod skin scaffolds. Control cells were cultured on gelatin-coated tissue culture plastic. Samples (n=3/timepoint) were processed on days 3, 7, 10, and 14 for western blotting and qPCR. The alamarBlue cell viability assay determined cell biocompatibility. Mixed-model ANOVA was used to test for differences between the groups and over time.

Results: The alamarBlue assay showed increased cell viability in both groups over time, with greater expression on the cod skin scaffolds at later timepoints ($p<0.05$). There was expression of the *MYOD1*, *MYOG*, *MYH3*, and *MYH8* genes, and MyoD1 and myogenin proteins in both groups over time indicating myogenic differentiation and early myofiber maturation. Compared to the cod skin scaffolds, control cells demonstrated greater gene expression in addition to MyoD1 protein expression at all timepoints ($p<0.05$). Myogenin protein expression was greater on cod skin scaffolds after day 10, albeit non-significant ($p>0.05$).

Conclusion: FDA-approved cod skin scaffolds are biocompatible and can support myoblast differentiation and early myofiber maturation. Further studies are needed to investigate how scaffold modification can best support the myogenic process.

Keywords: Skeletal muscle, bioengineering, tissue engineering, tissue regeneration, craniofacial abnormalities, masseter muscle

Introduction

Volumetric muscle defects are associated with functional, psychosocial, and aesthetic problems leading to a reduced quality of life [1]. Restoring form and function of the hard tissues, such as bone, is reliable and predictable, but restoration of soft tissue defects, specifically volumetric muscle loss, is challenging [2, 3]. Postnatal muscle has an intrinsic capacity to repair and regenerate due to the presence of resident satellite cells [4], however, larger defects often surpass the capability for repair and regeneration because of the impaired migration of satellite cells to the site of injury [5]. Current approaches to manage volumetric muscle defects, such as following traumatic injury or resection for cancer, include fat tissue transfer or the use of complex autologous muscle grafting techniques [2, 6]. Unfortunately, limitations include donor site morbidity, lack of available tissue, and tissue mismatch leading to continued poor function and quality of life [2, 6]. There is an urgent need to identify novel techniques to overcome the limitations of current soft tissue reconstruction modalities.

Tissue engineering is a realistic alternative to current reconstructive approaches [1, 2, 6]. Accordingly, skeletal muscle tissue engineering has gained increasing interest as a potential approach for restoring both form and function in craniofacial soft tissue defects. Skeletal muscle form is fundamental to function with multinucleated muscle fibers aligned in a parallel fashion and within an extracellular matrix (ECM) composed of mainly Type I and Type III collagen [7]. Engineering approaches often utilize scaffolds to provide a structural framework for cell adhesion, proliferation, orientation, and differentiation during early growth and development [6]. The addition of mechanical strain can also help cells assume a parallel configuration [8]. Various

organic and inorganic materials have been used to create biocompatible scaffolds of differing architecture to support the muscle regenerative process prior to implantation into sites of defect. However, these materials are all associated with multiple advantages and limitations.

Collagen, inherent to muscle fibers, provides an excellent substrate for cell growth and has been used in a hydrogel form for skeletal muscle regeneration [8-11]. Type I collagen (bovine, rat tail, and porcine) is most commonly used, because it is the major structural protein of the skeletal muscle ECM [11-14]. However, hydrogels are prone to mechanical weakness and lack muscle conductive properties [13, 15, 16]. It has been shown that increasing the stiffness of hydrogels can improve mechanical strength, but this is at the detriment of the desired cell behavior and diffusion of metabolites thus limiting the size of tissue that can be produced [8].

Other scaffolds used for skeletal muscle engineering purposes include non-mammalian organic materials, such as alginate, fibrin, and chitosan in film, hydrogel, or sponge form [15, 17]. Synthetic polymers can be produced to degrade at the rate of tissue formation and have specific stiffness and porosity to allow nutrient exchange, however, they lack bioactive cues inherent to natural materials [17]. Common synthetic polymers investigated for skeletal muscle tissue engineering include polyvinyl alcohol (PVA), polycaprolactone (PCL), polyglycolic acid (PGA), polylactic acid (PLA), and their copolymer, poly(lactic acid)-co-(glycolic acid) (PLGA) [15, 17]. PCL has become one of the most widely utilized synthetic polymers due to its flexibility, slow degradation rate, and suitability for electrospinning into aligned nanofibrous constructs [9, 17]. PLA and PLGA scaffolds similarly provide favorable structural support and customizable degradation profiles, while polyglycolic acid (PGA) contributes high porosity and rapid

biodegradation [14, 17]. Aligned electrospun synthetic polymer scaffolds were shown to promote C2C12 alignment, elongation, and multinucleated myotube formation to represent the structure of native skeletal muscle [18, 19]. However, the degradation byproducts from PLA-, PGA-, and PLGA-based systems may locally alter pH and negatively influence cell behavior, while use of overly stiff scaffolds will not mimic the compliant mechanical environment of native skeletal muscle tissue [14, 17, 19]. Moreover, the overall bioinert and hydrophobic nature of synthetic polymers can limit cell adhesion and myogenic signaling [14, 19].

To mitigate their limitations, synthetic scaffolds are frequently functionalized or blended with bioactive materials such as collagen, gelatin, laminin, or extracellular matrix-derived coatings to improve cellular attachment, proliferation, and differentiation [13, 14, 16]. Such hybrid scaffolds include polydimethylsiloxane (PDMS) and fibrin, polyethylene glycol (PEG) and fibrin, PLGA and collagen, PCL and collagen, and PCL and silk fibroin [14, 19]. Recent skeletal muscle tissue engineering strategies have increasingly employed hybrid scaffolds that combine the mechanical advantages of synthetic polymers with the biologic activity of natural extracellular matrix-derived biomaterials [20-22]. Despite the combined advantages offered by hybrid scaffolds, challenges still include incomplete vascularization, limited innervation, insufficient force generation, inadequate nutrient diffusion within larger constructs, and difficulties in reproducing the complex hierarchical architecture of native skeletal muscle. These are significant hurdles to successful skeletal muscle tissue engineering [14, 17, 19].

Marine-derived biomaterials have gained attention as biocompatible alternatives to mammalian-derived collagen scaffolds, because they offer the advantages of structural preservation due to

minimal processing and the reduced risk of any viral disease transfer [23, 24]. Taking advantage of these characteristics, Kerecis® has developed an intact cod skin product that is FDA-approved for the management of skin and mucosal trauma with widespread adoption for the treatment of chronic wounds, burns, trauma, and surgical defects [25]. The technology has generated a substantial body of clinical literature [26-32] and achieved sufficient market penetration to support a $1.3 billion acquisition by Coloplast in 2023, reflecting growing acceptance of cod-skin-derived products. Studies have shown that Kerecis acellular cod skin grafts support angiogenesis and accelerated wound closure cell and vascular ingrowth while supporting fibroblast infiltration and vascularized tissue ingrowth within the preserved ECM scaffold [32, 35, 36]. Marine-derived collagen biomaterials are composed predominantly of Type I collagen, the principal structural collagen of skeletal muscle connective tissue and major component of the perimysium and epimysium [37, 38]. Despite these congruencies, to our knowledge, cod skin has not been investigated in skeletal muscle approaches. Therefore, we hypothesized that cod skin scaffolds have the potential to support skeletal muscle regeneration, and to test our hypothesis, we investigated the behavior of the C2C12 mouse muscle cell line on cod skin scaffolds.

Materials and Methods

Preparation of Cod Skin Scaffolds

Commercially available, sterile acellular cod skin sheets (Omega3 SurgiClose™, Kerecis, Ísafjörður, Iceland) were donated by Kerecis and used without further chemical modification. The scaffolds were prepared in a Class II biosafety cabinet. Circular discs with a diameter of 20

mm were cut to fit within the wells of 12-well plates (Costar®, Corning Life Sciences). In order to secure the scaffolds to the base of the wells, a silicone layer was applied in each well as per the technique of Robert Dennis' group [39]. Briefly, polydimethylsiloxane (PDMS) (DOW SYLGARD™ 184 Silicone Elastomer, Midland, MI, USA) was prepared by chilling the base liquid at 4°C for 30 minutes to improve handling characteristics, and then mixing the base and curing agents at a 10:1 (v/v) ratio. 1 mL was dispensed into each well of a 12-well plate and cooled at -20°C freezer for 30 minutes to allow for any bubbles to dissipate, followed by incubation at 37°C for 24-hours to set the solution. The silicone-coated plates were stored at a minimum of 2 weeks at 4°C to minimize cell toxicity. Cod skin scaffolds were secured to the silicone base using two shortened 27-gauge (Monoject, REF 8881250362, LOT23A026) needles (BD PrecisionGlide, REF 305193, LOT 4003307) at the edge of each scaffold. The base of the control wells was coated with 500 μL 0.2% (w/v) gelatin solution (Type B; Sigma-Aldrich, G9391) for 30 minutes prior to cell seeding. Any excess solution was syphoned off without completely drying the gelatin coating.

Cell Culture and Myogenic Differentiation

Cryogenically frozen C2C12 murine myoblasts were used to study myogenic behavior on the cod skin scaffolds. Cells were thawed and cultured in standard growth medium (SGM) consisting of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Corning, REF 35-016-CV, LOT 11125001) and 1% penicillin-streptomycin (Corning, REF 30-002-CI, LOT 30002403). Cells from passages 3 to 5 were used in all experiments at a seeding density of $2x10^3$ cells/cm². Following direct seeding, the cod skin scaffolds and control

cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2 over a period of 14-days. The culture medium was changed daily during the experimental period. Upon reaching approximately 70–80% confluency, SGM was replaced with differentiation medium (DM) consisting of DMEM supplemented with 2% horse serum (Corning, REF 35-030-CV, LOT 03724001) and 1x penicillin-streptomycin to induce myogenic differentiation.

Cell Biocompatibility

The alamarBlue® cell viability assay (Bio-Rad Laboratories, Hercules, CA, USA) was used to determine biocompatibility of the cells with the cod skin scaffolds. Cells were evaluated in triplicate per condition on days 1, 3, 7, 10, and 14. At each time point, culture medium was removed, and the wells were washed once with warm phosphate-buffered saline (PBS). A working solution consisting of 10% alamarBlue reagent diluted in culture medium (1:9 ratio) was then added to each well, and cells were incubated for 2 hours at 37°C in a humidified atmosphere containing 5% CO_2. Following incubation, 100 µL of the solution from each well (n = 3) were transferred to a black 96-well plate. Fluorescence was measured using a SpectraMax i3 microplate reader (Molecular Devices, San Jose, CA, USA) with SoftMax Pro 6.4 software at an excitation wavelength of 530 nm and an emission wavelength of 590 nm. Blank wells containing the alamarBlue working solution without cells were included for background subtraction. Fluorescence values were averaged across technical replicates and reported as relative fluorescence units (RFU) as an indicator of cellular metabolic activity.

RNA Isolation and Quantitative PCR

Total RNA was isolated from cod skin scaffolds (n = 3) and control cells (n = 3) on days 3, 7, 10, and 14 using TRIzol™ reagent (Thermo Fisher Scientific) according to the manufacturers' instructions. Briefly, samples were homogenized in TRIzol. The cod skin scaffolds were subjected to mechanical homogenization by mincing with dissection scissors and further disrupted using sterilized plastic pestles prior to RNA isolation. Following homogenization, chloroform was added to induce phase separation, and samples were centrifuged to separate the aqueous RNA-containing phase from the interphase and organic phase. Glycogen was added as a carrier during the RNA precipitation step to enhance RNA recovery. RNA was precipitated from the aqueous phase using isopropanol, washed with 75% ethanol, and subsequently resuspended in RNase-free water. RNA concentration and purity were assessed spectrophotometrically prior to downstream applications. Equal amounts of RNA were used as input for reverse transcription to ensure consistency across samples.

Following, complementary DNA (cDNA) synthesis using the Applied Biosystems™ High-Capacity RNA-to-cDNA Kit (REF 4387406, Thermo Fisher Scientific) according to the manufacturer's instructions, quantitative PCR (qPCR) was performed using singleplex TaqMan Gene Expression Assays and the QuantStudio 3 real-time PCR system (Thermo Fisher Scientific). The genes of interest were *MYOD1* (TaqMan Assay ID Mm00440387_m1), *MYOG* (TaqMan Assay ID Mm00446195_g1), *MYH3* (TaqMan Assay ID Mm01332463_m1), and *MYH8* (TaqMan Assay ID Mm01329492_g1). These genes were selected to represent distinct stages during muscle regeneration. *MYOD1* is a master myogenic regulatory factor (MRF) that

controls myoblast commitment and early myogenic determination. *MYOG* is required for terminal differentiation and fusion of myoblasts into multinucleated myotubes. *MYH3* and *MYH8* encode for the embryonic and neonatal myosin heavy chain isoforms, respectively, as markers of early and developmental myofiber formation during skeletal muscle maturation. All samples were investigated using three technical repeats, and *GAPDH* (TaqMan Assay ID Mm99999915_g1) served as the endogenous reference with no template controls (NTCs) serving as negative controls. Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

Protein Extraction and Western Blot Analysis

On days 3, 7, 10, and 14, samples were washed twice with 1-2 mL of cold PBS to remove residual serum proteins. Samples and lysis buffers were kept on ice during processing. Protein was extracted from mechanically homogenized cod skin samples and gelatin-coated control cells using Radio Immunoprecipitation Assay (RIPA) lysis buffer (50 mM Tris-HCl, pH 7.4; 150 mM NaCl; 1% NP-40; 0.5% sodium deoxycholate; 0.1% SDS) supplemented with Pierce™ Protease Inhibitor Mini (Thermo Scientific, Rockford, IL, USA; REF A32955, LOT ZI4431702). Samples were turned end-to-end at 4 degrees Celsius for at least 30 minutes prior to centrifugation at 14,000 RPM at 4° Celsius for 30 minutes. The resulting supernatant was transferred to 1.5mL Eppendorf tubes and stored in a -20° Celsius freezer until further analysis.

At the time of western blot analysis, samples were thawed on ice and protein concentration was determined using a bicinchoninic acid assay (BCA) as per the manufacturer's instructions. Equal concentrations of protein (10 μg/well) were resolved by 10% SDS–PAGE and transferred to

polyvinylidene difluoride (PVDF) membranes with a BIO RAD wet transfer system at 120V for 70 minutes; stain-free gels were activated according to BIO RAD protocols. After transfer and blot activation, blocking of membranes was performed in LICOR boxes with 5% bovine serum albumin (BSA) in tris-buffered saline (TBS) on a rocker for one hour at 65 RPM. After blocking, the buffer was removed and PVDF membranes were incubated overnight at 4 °C with with either anti-MyoD1 antibodies raised in mouse (MilliporeSigma, MAB3878, Clone 5.2F) or anti-myogenin antibodies raised in mouse (MilliporeSigma, MAB3876) at a 1:1000 dilution in TBS Tween in 2% BSA. This was followed with a triple wash for 5 minutes each with TBS on a rocker at 65 RPM. A one-hour incubation with mouse-derived secondary IgG antibodies was carried out at a 1:20,000 ratio in TBS+Tween and 5% BSA in TBS, and then the PVDF membranes were rinsed with TBS prior to another triple wash for 5 minutes each with TBS + Tween. Membranes were developed with 1mL of Immobilon® Western Chemiluminescent HRP Substrate (MilliporeSigma, Burlington, MA, USA) according to the manufacturer's instructions. Chemiluminescent signals were captured and total protein visualized using a ChemiDoc MP Imaging System (Bio-Rad Laboratories, Hercules, CA, USA). Band intensities were quantified and normalized to total lane protein prior to comparison between conditions.

Statistical Analysis

Mixed-model ANOVA was used to test for differences in gene and protein expression between the two groups and over time. The Tukey post-hoc test was performed when appropriate. A two-sided significance level of $\alpha = 0.05$ was used for all tests.

Sample Size Calculation

A 1-fold change in gene expression was considered biologically and clinically significant. Based on prior data from non-loaded muscle cell-seeded scaffolds, effect sizes for detecting a 1-fold change were estimated at 2.0 for *MYOD1* [40]. With n = 3 biological replicates per treatment condition, the study achieved at least 80% statistical power at a two-sided 5% significance level.

Results and Discussion

Handling of the Cod Skin Scaffolds

The Kerecis cod skin scaffolds are FDA-approved and have become popular adjuncts in the clinical management of traumatic wounds. The skin is dehydrated and fairly tough, however, it can be trimmed to size. In this study, scaffolds were cut to fit within 12-well plates and pinned to prevent them from floating in the wells. Some of the scaffolds rolled with time, however, the most noticeable change over time was the softer consistency of the scaffolds. This was seen as advantageous, because the ideal scaffold would eventually degrade as the desired tissue develops and grows in size.

Use of the C2C12 Myoblast Cell Line

The C2C12 mouse myoblast cell line was selected for this investigation because of its extensive use in skeletal muscle research, ease of culture, reproducibility, and suitability for controlled

mechanistic studies prior to advancing to more complex models [41]. The C2C12 cells are an immortalized myogenic cell line derived from the skeletal muscle of C3H mice [42] and can proliferate in growth media and differentiating into multinucleated myotubes under appropriate differentiation conditions [43] (Figure 1). These cells express key myogenic regulatory factors (MRFs), including MyoD and myogenin [44], which were evaluated in the present study.

Cell Viability

The alamarBlue reagent is a non-toxic, resazurin-based solution that has been widely used to investigate cell proliferation, cytotoxicity, and cellular metabolic activity in various applications [45]. Resazurin is converted to fluorescent resorufin by harnessing the natural reducing power of living cells, and the advantage is the ability to investigate cell viability in the same samples over time. In our study, scaffolds and control wells demonstrated similar metabolic activity up to day 3. On day 7, metabolic activity increased markedly on cod skin scaffolds (1.9-fold vs control), peaking at day 10 with a 3.6-fold increase. By day 14, activity declined in both groups but remained 2.6-fold higher on the cod skin scaffolds (Figure 2). Our data showed that the cod skin scaffolds were highly compatible with the cod skin scaffolds.

The Myogenic Regulatory Factors

The myogenic regulatory factors (MRFs) are important in the process of myogenesis. There is temporal expression with the early expressed Mrf-5 and MyoD1 functioning as factors determining myogenic lineage [44, 46]. Myogenin and MRF4 are mediators of terminal

differentiation whereby mononuclear myoblasts exit the cell cycle and fuse to form multinucleated myofibers. MyoD1 and myogenin were investigated as early and late markers in the myogenic process. Gene and protein expression confirmed upregulation of both MRFs in the control cells and the cod skin scaffolds (Figure 3). When comparing the two conditions, it was noted there was significantly greater expression of the *MYOD1* and *MYOG* genes in the control cells. MyoD1 protein expression also followed a similar pattern. However, myogenin protein expression was noted to peak by day 7 in the control cells but continued to persist on the cod skin scaffolds throughout the experimental period. Mechanosensitive muscle cells require highly compliant surfaces in order to align, fuse, and have the appropriate contractile function [47, 48]. Therefore, it is possible that a rigid surface such as tissue culture plastic does not support long-term maintenance of myofibers, whereas the softer cod skin can.

The Embryonic and Perinatal Myosin Heavy Chains

Terminal differentiation is marked by the appearance of the myosin heavy chain (MyHCs) isoforms, which determine the contractile properties of myofibers [49]. Early nascent myofibers express the embryonic and perinatal MyHCs, which are eventually replaced by the fast and slow adult MyHC isoforms [50]. In this study, it was noted there was upregulation of the *MYH3* and *MYH8* encoding for the embryonic and perinatal MyHCs respectively at all timepoints in the control cells (Figure 4). *MYH3* and *MYH8* gene expression was only demonstrated from day 10 on the cod skin scaffolds. It is possible that this delay may be a result of the cells needing to adapt to the scaffold, which includes extracellular matrix remodeling, before differentiating and

exhibiting signs of maturation [51]. Increasing the experimental period may show a switch adult MyHC isoforms.

Conclusion

Cod skin scaffolds support myoblast differentiation and early maturation. However, the scaffolds are inferior when compared to a conventional gelatin-coated substrate. The cod skin is likely to need further modification, such as initial hydration, to better support myoblast attachment and growth. In addition, it will be important to investigate response of primary muscle cells rather than the immortalized C2C12 mouse muscle cell line.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflicts of interest

None to disclose.

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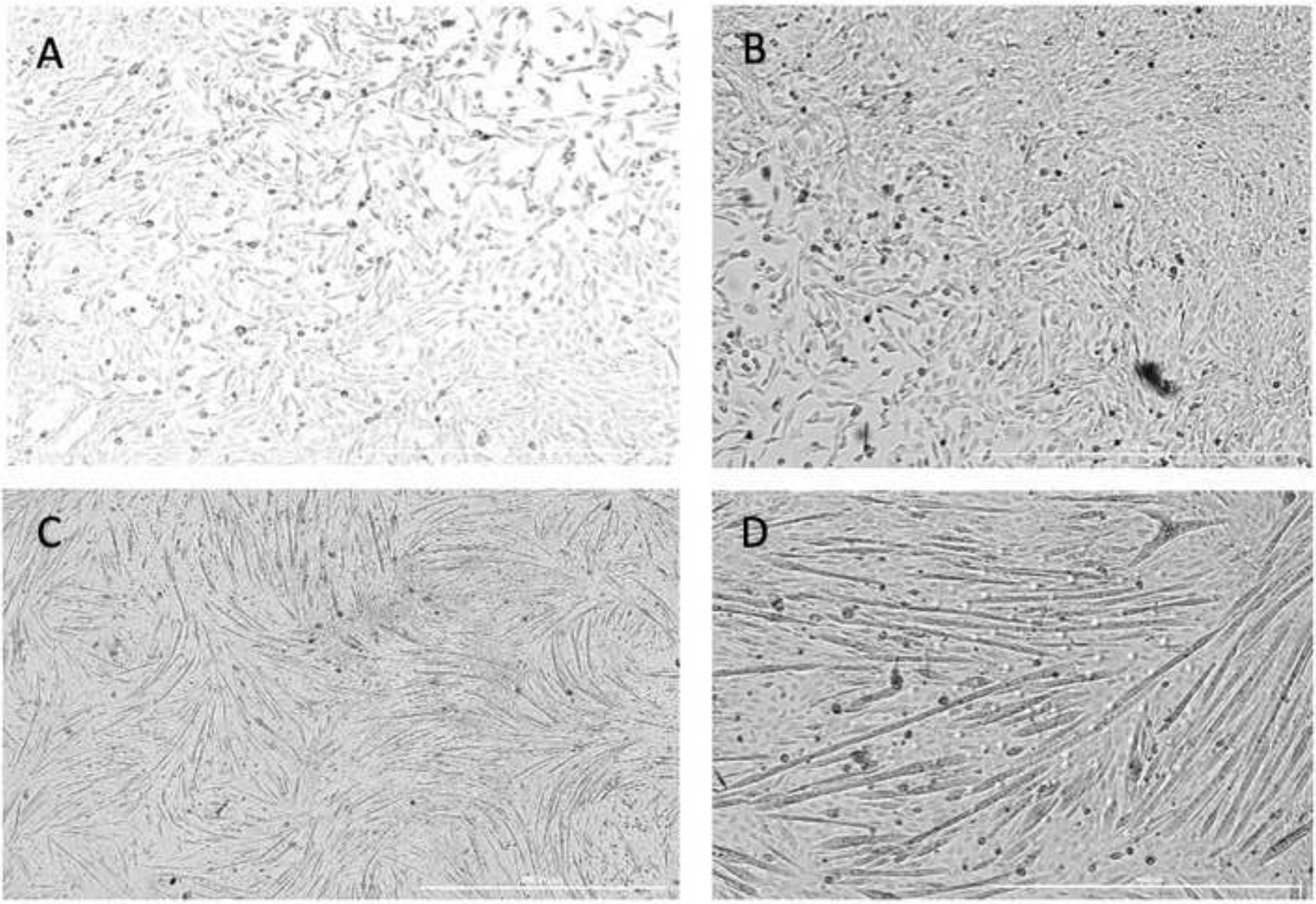
Figures and Tables

Figure 1: C2C12 cells grown over 14-days (A = day 3, B = day 7, C = day 10, D= day 14). The cells became confluent over time and fused into multinucleated myofibers. Images were taken with the Lionheart microscope and stitched together.

Figure 2: Relative fluorescence units demonstrating cell viability in control cells and cod skin scaffolds over time. Error bars represent one standard deviation (+SD).

Figure 3: Relative *MYOD1* and *MYOG* gene expression in control cells and on the cod skin scaffolds. Data are expressed as fold-change ($2^{-\Delta\Delta Ct}$) relative to the baseline expression on day 3. MyoD1 and myogenin western blot images are also shown in addition to the fold-change relative to the baseline expression on day 03 Error bars represent one standard deviation (+SD).

Figure 4: Relative *MYH3* and *MYH8* gene expression in engineered 3D masseter muscle constructs. Data are presented as fold-change ($2^{-\Delta\Delta Ct}$) relative to the baseline expression on day 3. Error bars represent one standard deviation (+SD).



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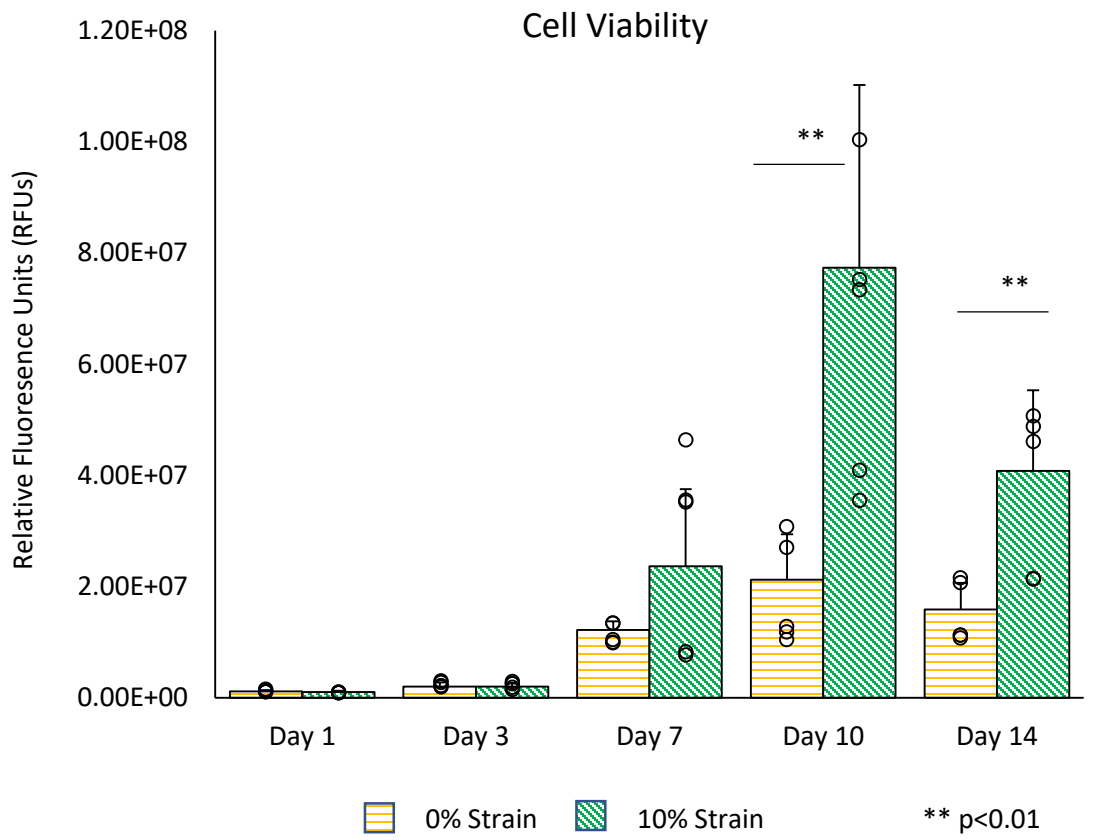
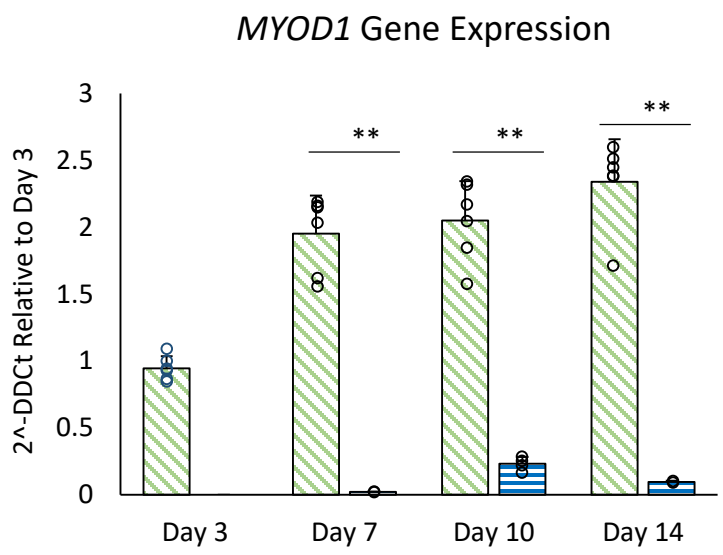
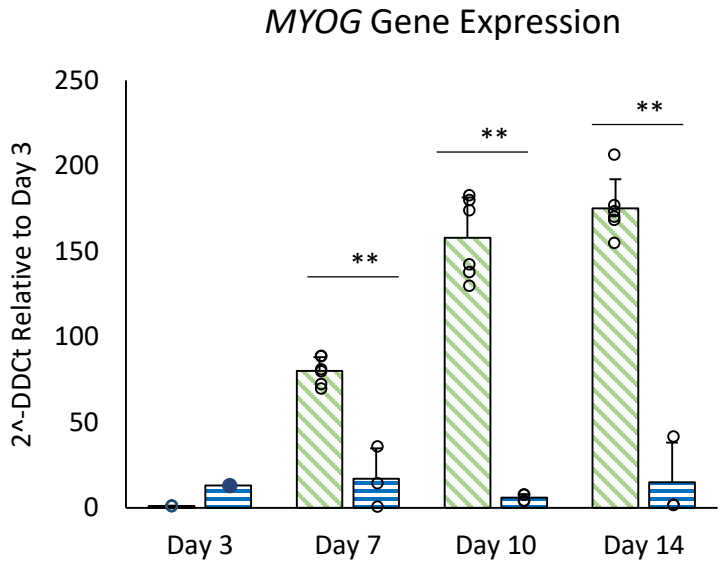
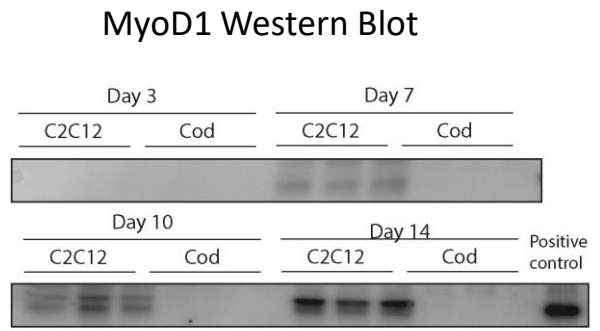
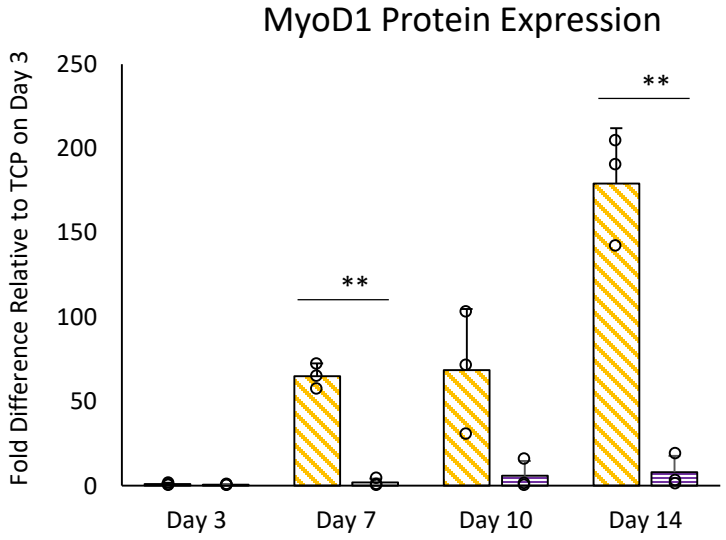

Cell Viability
Relative Fluoresence Units (RFUs)
1.20E+08
1.00E+08
8.00E+07
6.00E+07
4.00E+07
2.00E+07
0.00E+00
**
**
Day 1
Day 3
Day 7
Day 10
Day 14
0% Strain
10% Strain
** p<0.01

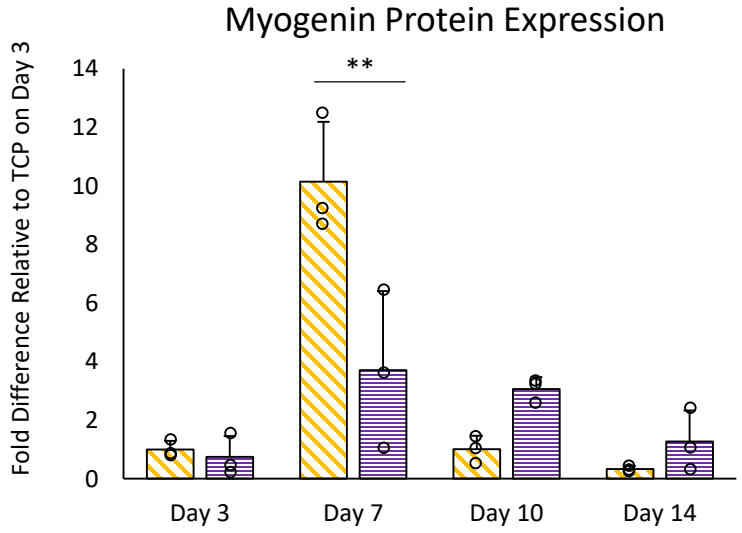
Title Page

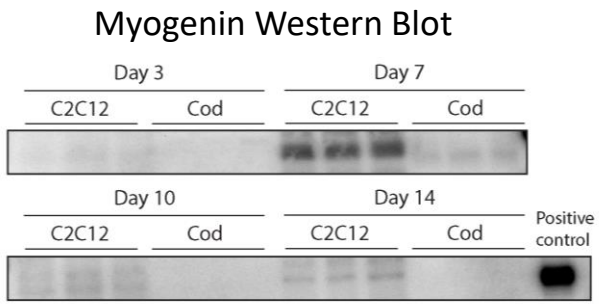






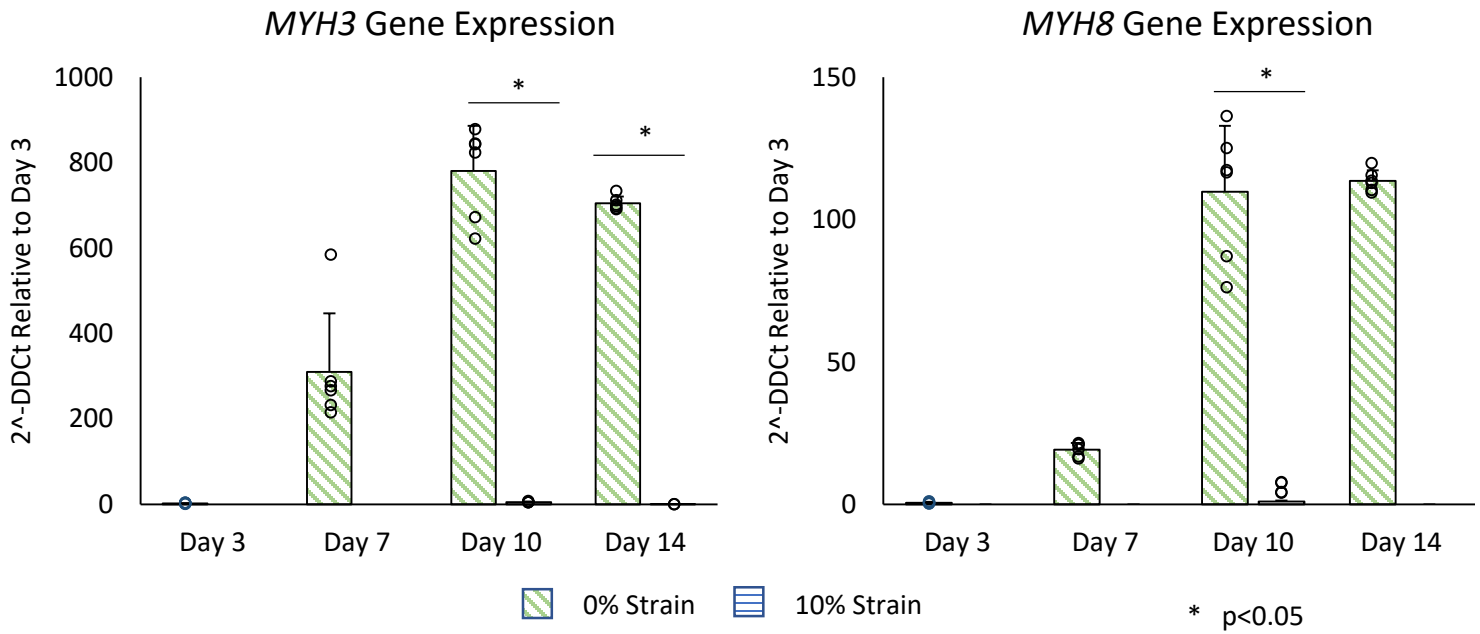






* p<0.05
** p<0.01

Title Page


MYH3 Gene Expression
2^-DDCt Relative to Day 3
1000
800
600
400
200
0
Day 3
Day 7
Day 10
Day 14
*
*
MYH8 Gene Expression
2^-DDCt Relative to Day 3
150
100
50
0
Day 3
Day 7
Day 10
Day 14
*
0% Strain
10% Strain
* p<0.05

TERM
TISSUE ENGINEERING AND REGENERATIVE MEDICINE

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