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Abstract: Progenitor cells derived from the oral mucosa lamina propria (OMLP-PCs) demonstrate an ability to differentiate into tissue lineages removed from their anatomical origin. This clonally derived population of neural-crest cells have demonstrated potential to differentiate along mesenchymal and neuronal cell lineages.
Objective: Significant efforts are being made to generate functioning muscle constructs for use in research and clinical tissue engineering. In this study we aimed to determine the myogenic properties of clonal populations of expanded OMLP-PCs.
Design: PCs were subject to several in vitro culture conditions in an attempt to drive myogenic conversion. Methodologies include use of demethylation gene-modifying reagents, mechanical conditioning of tissue culture substrates, tuneable polyacrylamide gels and a 3-dimensional construct as well as published myogenic media compositions. PCR and immunostaining for the muscle cell markers Desmin and MyoD1 were used to assess muscle differentiation.
Results: The clones tested did not intrinsically express myogenic lineage markers. Despite use of two and 3-dimensional pre-published in vitro culture protocols OMLP clones could not be differentiated down a myogenic lineage.
Conclusions: Within the confines of these experimental parameters it was not possible to generate identifiable muscle using the clonal populations. When reviewing the previously successful reports of myogenic conversion, cells utilised have either been derived from tissues that are already 'primed' with the requisite myogenic genetic potential or have undergone specific genetic reprogramming to enhance the myogenic conversion rate. This, along with as yet unidentified stromal interplay, may therefore be required for positive myogenic differentiation to be realised.

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26th January 2015

Re: Archives of Oral Biology Journal paper submission.

Dear Editors

Please find an electronic submission of our manuscript, "Oral Mucosal Progenitor Cells are Resistant to Myogenic Differentiation" for consideration for publication as a research article in the Archives of Oral Biology Journal.

As a continuance of our Institution's work investigating the biological potentials of oral mucosa derived progenitor cells we present an *in vitro* laboratory study that examines the ability of such cell types to undergo myogenic conversion. This work was conducted utilising previously derived progenitor cell populations and animal muscle cell lines subjected to two- and three-dimensional constructs. Previous work through our group had identified the oral progenitors to have similar properties to those of mesenchymal stem cells. Despite the favourable embryological derivation of our oral mucosal progenitors we were not able to differentiate cells through to a myogenic lineage despite use of widespread published protocols.

We confirm that this manuscript has not been published elsewhere and is not under consideration by another journal. All authors have approved the manuscript and agree with its submission to your Journal, there are no conflicts of interest. Please address all correspondence to the contact details above.

We thank you in advance for your attentions.

Yours sincerely

Dr Matthew Locke

***Conflict of Interest Form**

Journal: Archives of Oral Biology	**Author name**: Dr Matthew Locke

Declarations

The following additional information is required for submission. Please note that failure to respond to these questions/statements will mean your submission will be returned to you. If you have nothing to declare in any of these categories then this should be stated.

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There are no conflicts of interest

Please state any sources of funding for your research

Royal College of Surgeons of England Small Grants Scheme, 2011

Please state whether Ethical Approval was given, by whom and the relevant Judgement's reference number

N/A, no ethical approval required

If you are submitting a Randomized Controlled Trial, please state the International Standard Randomised Controlled Trial Number **(ISRCTN)**

N/A

Oral Mucosal Progenitor Cell Clones Resist *In Vitro* Myogenic Differentiation

Highlights

- Oral mucosal lamina propria progenitor cells are a distinct progenitor cell population
- They are of neural crest origin and express many putative stem cell markers
- Have shown to be potently immunosuppressive
- They were subjected to published myogenic tissue culture protocols
- Proved resistant to muscle differentiation

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1 **Title**

2 Oral Mucosal Progenitor Cell Clones Resist *In Vitro* Myogenic Differentiation

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Abstract

Progenitor cells derived from the oral mucosa lamina propria (OMLP-PCs) demonstrate an ability to differentiate into tissue lineages removed from their anatomical origin. This clonally derived population of neural-crest cells have demonstrated potential to differentiate along mesenchymal and neuronal cell lineages.

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Results: The clones tested did not intrinsically express myogenic lineage markers. Despite use of two and 3-dimensional pre-published *in vitro* culture protocols OMLP clones could not be differentiated down a myogenic lineage.

Conclusions: Within the confines of these experimental parameters it was not possible to generate identifiable muscle using the clonal populations. When reviewing the previously successful reports of myogenic conversion, cells utilised have either been derived from tissues that are already 'primed' with the requisite myogenic genetic potential or have undergone specific genetic reprogramming to enhance the myogenic conversion rate. This, along with as yet unidentified stromal interplay, may therefore be required for positive myogenic differentiation to be realised.

Abbreviations:

ADSC - Adipose Derived Stem Cells

BM-MSC - Bone Marrow-Derived Mesenchymal Stem Cells

ESC - Embryonic Stem Cell

OMLP-PCs - Oral Mucosal Lamina Propria Progenitor Cells

5-Aza - 5-azacitidine

MYOD1 – Myogenic differentiation 1 gene

Key Words:

Oral progenitor, oral mucosa, myogenic, differentiation, pluripotential

Introduction

Oral mucosal lamina propria progenitor cells (OMLP-PCs) are a distinct progenitor cell (PC) population that has recently been reported [1]. These oral PCs are of neural crest origin, express many putative stem cell markers and are potently immunosuppressive [2]. Furthermore, these OMLP-PCs, like bone marrow-derived mesenchymal stem cells (BM-MSCs), adipose-derived stem cells (ADSCs) and gingiva-derived progenitor cells can be driven down osteoblastic, adipogenic, chondrogenic and neuronal lineages [1,3,4,5].

Myogenesis is characterized by a period of myoblast proliferation, followed by the expression of muscle-specific proteins and then fusion to form multinucleated myotubes. It is a developmental cascade that principally involves the regulatory MYOD gene family controlling the transition of multipotential mesodermal stem/PCs into the myogenic lineage [6].

Both human and animal studies have sought to reproduce the muscle phenotype from embryologically distinct tissue types. Early studies by Guan *et al.,* (1999) [7] developed protocols for cultivating embryonic stem cells (ESCs) through 'hanging drop/embryoid body' stages prior to their differentiation to skeletal muscle. Later animal studies by Zheng *et al.,* (2006) [8] demonstrated that human ESC-derived precursors could incorporate into host muscle efficiently and become part of regenerating muscle fibres. Adult MSCs, most commonly BM-MSCs, have been reported to form skeletal muscle under defined *in vitro* conditions [9,10]. *In vivo* studies have demonstrated that BM-MSCs differentiate into myofibres and contribute to the replenishment of the muscle satellite cell compartment and thus to muscle regeneration [11].

Recent work has reported successful derivation of myogenic cell-types with *in vitro* manipulation of cells from a variety of tissue sources. Gang and co-workers (2004) [12] demonstrated that umbilical cord blood-derived MSCs, when incubated in pro-myogenic conditions expressed myogenic markers in accordance with the myogenic differentiation pattern. Murine ADSCs were reported to convert to the myogenic phenotype when in co-culture with primary myoblasts [13] and enhanced conversion rates were realised when utilising stiffened 'muscle-mimicking' extracellular matrices that more closely imitate the *in vivo* situation [14].

Further investigations have utilised more invasive methods of cellular and genetic manipulation in order to achieve differentiation. Work by Taylor and Jones (1979) [15] (replicated by Wakitani *et al*., 1995 [16]), on use of the DNA methylation inhibitor 5-azacitidine (5-Aza), reported that subpopulations of mouse and rat cell lines undergo transformation to the myotube muscle phenotype when treated. Nakatsuka *et al*. (2010) [17] demonstrated 5-Aza demethylation resulted in skeletal muscle differentiation by mouse dental pulp stem cells. Authors have also demonstrated similar *in vitro* muscle conversion on utilisation of the ß-galactoside-binding lectin Galectin-1. Goldring *et al*. (2002) [18] reported that human dermal fibroblasts expressed the myogenic marker, Desmin, and Chan *et al.,* (2006) [19] presented data that suggest human foetal-MSCs readily undergo muscle differentiation in response to Galectin-1. The purpose of this study was specifically not to undertake such invasive molecular manipulation techniques but rather to investigate any innate myogenic potential in order to be able to exploit this via pre-published myogenic maintenance/differentiation tissue culture protocols which may lend themselves towards future translatability.

In addition, there are reports that suggest conversion rates for functioning muscle may be exaggerated. Di Castro *et al*. (2008) [20] cited conflicting evidence of myogenic differentiation from human haematopoietic-derived stem cells. They demonstrated that human peripheral blood-derived stem cells labelled with Green Fluorescent Protein (GFP) and co-cultured with mouse C2C12 myoblast cell line demonstrated transference of GFP markers to muscle cells but without the signs of myogenic stem cell differentiation. It appears that the method of stem cell and muscle fibre fusion is not clear and it is debated as to whether this represents true cellular fusion, transdifferentiation or the effects of circulating tissue-specific precursors.

Given the multipotent nature of OMLP-PCs and the ease with which these can be isolated from individuals (via a simple non-scarring buccal mucosal biopsy) the aim of this investigation was to determine their potential to form identifiable muscle subunits when subjected to previously published myogenic tissue culture approaches.

Materials and Methods

Isolation and expansion of the OMLP-PCs

Unless otherwise stated all laboratory reagents were obtained from Invitrogen, UK. Normal, disease-free buccal mucosa biopsies were obtained from written consented patients undergoing dental procedures at the School of Dentistry, Cardiff University. Local ethical committee approval had been previously obtained (South East Wales Research Ethics Committee; 09/WSE03/18). OMLP PCs were obtained by differential adhesion to fibronectin as previously reported [1]. In brief, biopsy tissue was separated into epithelial and lamina propria component. LP tissue was then disaggregated and PCs were separated by subsequent differential adhesion to fibronectin. Briefly, OMLP single-cell suspensions in basal culture media (Dulbecco's modified Eagle's medium [DMEM] supplemented with 2 mM l-glutamine, 100 U/mL penicillin G, 100 µg/mL streptomycin sulfate, 0.25 µg/mL amphotericin B) were seeded onto fibronectin (10 µg/mL overnight pre-coating; Sigma-Aldrich Company Ltd) coated 6-well plates and incubated for 20 min at 37°C. Non-adherent cells within this time frame were discarded and adhered cells were allowed to form single-cell colonies. A total of nine clones were isolated and expanded from n=3 patients (i.e. 3 clones per patient) and cells utilized in experiments at 22-35 population doublings. Previous work has concluded that PC populations passaged through basal medium were characterized by a high initial rate of proliferation (on average >4 PDs/week), before establishing a constant level of 1 PD/week and reaching cellular senescence at ~50–60 PDs. These growth kinetics were representative of all clones utilised in these investigations.

The control culture utilised was the murine myoblast muscle cell line C2C12, originally obtained by Yaffe and Saxel (1977) [21] through serial passage of myoblasts cultured from the thigh muscle of C3H mice after crush injury.

Myogenic Cell Culture Variables

Media composition

In an attempt to drive myogenic conversion of our PCs they were subjected to media conditions previously published as being successful in driving the muscle phenotype in committed muscle cell culture [22,23,24,25]. Media formulations were based on DMEM basal media (as above) with the addition of varying horse serum combinations and the inclusion of insulin-like growth factor-1 (10 ng/ml) and the corticosteroids dexamethasone (0.1 µM) and hydrocortisone (50 µM). Specifically, PCs were subject to an initial high (10%) horse serum concentration to induce differentiation (Differentiating Medium [DM]), levels were then reduced to 2% in order to stimulate cellular fusion (Fusion Medium [FM]) (media formulations described by Gang *et al.,* 2004) [12].

Gene modifying reagents

A further methodology involved the use of the demethylation gene-modifying reagent 5-Aza (Sigma, A3656) and the lectin Galectin-1 (Sigma, G4720). Clones were seeded and cultured in in T75 flasks and standard DMEM/foetal calf serum growth medium at 37°C until established and the then media changed to DM containing 0.3 µM 5-Aza for 3 days, or 200 ng/ml Galectin-1 for 14 days (media being replenished every three days). After these time periods medium was changed to fresh DM for two further days then to FM for four weeks.

Culture substrate conditioning

This technique involved the mechanical conditioning of tissue culture substrates utilising either (a) gelatin, (b) rat tail type I collagen (Life Technologies, A1048301), (c) Matrigel™ (BD Biosciences; Grefte *et al.,* (2012) [26]) or (d) tuneable polyacrylamide gels (Engler *et al.,* (2004) [27]). Briefly, 0.2% w/v gelatin or 0.2 mg/ml type I collagen were adhered to

tissue culture plastic overnight at 37°C. Matrigel™ at 1 mg/ml was used to coat 24-well plates at 37°C overnight.

3D Cell Culture

Based on work conducted by Smith *et al*., (2012) [28] an *in vitro* 3-dimensional construct was used to support tissues during the differentiation protocols. Wire supports were constructed to hold in position a 2 mm pore size nylon mesh suspended between the walls of a single well (9.4 cm^2) chamber slide (Lab-Tek®, UK). Cells were suspended in 3 ml of neutralised type I collagen (0.1% w/v). The cell/collagen matrix was then lifted to release it from the floor of the chamber and DM was replaced daily throughout 7 days of culture.

Culture substrate conditioning

Solutions of acrylamide (6% w/v) and bis-acrylamide (0.14% w/v) were polymerized by ammonium persulfate (1:100 v/v) and tetramethylethylenediamine (1:1000 v/v). These hydrogels were then cast between a glass coverslip activated with 3-aminopropyltrimethoxysilane and a glass slide activated with dichlorodimethylsiloxane. The hydrogel surface layer was then activated with application of sulfo-SANPAH solution and exposure to 365 nm UV light. Fresh type I collagen solution (0.2 mg/ml) was utilised to cover the gel surface at 37°C overnight. The coated coverslips were UV sterilised before being utilised in cell culture experiments.

RT-PCR

Total RNA was purified with Trizol® reagent and treated with DNA-Free DNase I treatment (Ambion, Austin Texas, USA) to remove genomic DNA contamination. Two micrograms of total RNA was used for the reverse-transcription reaction with random hexamer primers

(Promega, UK). PCR was performed utilising the Invitrogen supermix and products visualised on a 10% agarose gel. Published primers were for β-actin [F 5'-CCA CAC TGT GCC CAT CTA CGA GGG GT-3' and R 5' AGG GCA GTG ATC TCC TTC TGC ATC CT [1] and MyoD1 [F 5' AAG CGA CCT CTC TTG AGG TA 3' and R 5' GCG CCT TTA TTT TGA TCA CC 3' [25]. A Desmin primer [F 5' GAC CAC GCG CAC CAA CGA GA 3' and R 5' CCG CTC GGA AGG CAG CCA AA 3']) was designed using Blast primer design (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). cDNAs created from Total Human Skeletal Muscle (Agilent, Stratagene Products, UK) and C2C12 myoblast cell line RNs were used as positive controls.

Immunostaining

Cells were fixed with 4% (w/v) paraformaldehyde for 10 mins and washed in tris-buffered saline three times. Samples were blocked with rabbit serum at 1:50 for 30 mins and then incubated with primary Desmin antibody (Dako, UK; 0.02 mg/ml) for 1 hour at room temperature with agitation. After washing, secondary FITC-conjugated rabbit anti-mouse (Dako, UK; 1 µg/ml) in 2% non-fat milk was added and incubated for 1 hour. Nuclei were counter-stained with 1 µg/ml Hoechst33342 (Invitrogen, Carlsbad, CA, USA) and slides mounted in Crystalmount™ before imaging by epi-fluorescent microscopy.

Results

Myogenic Lineage potential of OMLP-PCs: Previous work by our group [1] has demonstrated that all OMLP-PC clones derived express the stem cell marker series CD44, CD90, CD105, CD166 and were negative for the hematopoietic and fibrocyte markers CD34 and CD45. These studies have also demonstrated the multipotency of OMLP-PCs by differentiation of the cells down mesenchymal (chondrogenic, osteoblastic, and adipogenic) and neuronal cell lineages (1). To characterise the baseline myogenic lineage potential, initial assessment of the cells maintained either within myogenic culture media alone or in conjunction with 5-Aza was undertaken. End-point RT-PCR analysis revealed the expanded OMLP-PCs to be negative for both Desmin and MyoD1 when untreated and after attempted myogenic conversion (Fig. 1 & Table 1). The C2C12 control was positive for both markers.

The effect of defined 'myogenic' media on OMLP-PC myo-differentiation: In culture there were no discernible morphological changes between non-treated cultures and those subjected to myogenic media formulations (Figs. 2A&B). Clonal OMLP-PC populations failed to demonstrate positive immuno-staining for Desmin even after exposure to the different myogenic cell culture protocols (Figs. 2C&D). This was in distinct contrast to the C2C12 cell line that continued to undergo growth, proliferation and eventual fusion to the myotube phenotype which demonstrated Desmin positivity (Figs. 2E-H).

DNA manipulation and myogenic conversion: OMLP-PCs were individually exposed to 5-Aza and Galectin-1. After treatment with 5-Aza there was evidence of the cells becoming larger, more flattened and forming numerous filopodia however, this was not characteristic of myotube formation (Fig. 3A). After exposure to Galectin-1 there were no discernible

morphological changes to the cells in culture (Fig. 3B). There continued to be a lack of Desmin antibody staining following treatment with both reagents (*data not shown*).

The effect of culture substrate on OMLP-PC myo-differentiation: Although all substrates were permissive for normal OMLP-PC growth (Fig. 4A), myogenic differentiation was once again only observed for the control C2C12 cells and not for the OMLP-PCs (Fig. 4B).

The effect of substrate stiffness on OMLP-PC myo-differentiation: A growing body of work has demonstrated that *in vitro* culture substrate stiffness can influence the direction of differentiation of 'progenitor' cells. Engler and co-workers (2004) [27] have previously demonstrated that a specific Young's modulus (E) of ≈10KPa can result in myogenic differentiation of human MSCs. Following this methodology we produced tuneable polyacrylamide gels that were tested for stiffness using rheology. Three gel thicknesses (0.4, 0.6 and 1.0 mm) were developed with a range of stiffness from 8.8 to 9.2 KPa. OMLP-PCs when cultured on these polyacrylamide 'stiff' substrates responded to the morphological cues by flattening out, forming numerous processes and elongating (Figs. 5A-D). However, this change of appearance was not indicative of a myotube-like phenotype when compared to the response of the C2C12 control cell line. This was further confirmed through staining for Desmin in the C2C12 controls whilst there was no Desmin positivity in the OMLP-PCs (Figs. 5E-F).

The effect of 3-dimensional culture on OMLP-PC myo-differentiation: Reorganisation of a 3D extracellular matrix environment was demonstrated for the OMLP-PCs but macroscopically this was substantially less than for the control C2C12 cells which resulted in detachment of the collagen lattice from the surrounding support structures (Figs. 6A&B).

Microscopic examination of the cell-seeded lattices revealed distinct differences between OMLP-PC and C2C12 systems in that, unlike the C2C12 cells the oral progenitor cells did not take on the appearance of myotubes or stain positively for Desmin (Figs. 6C-F).

Discussion

The innate ability of embryologically distinct tissue types to differentiate to muscle has been attributed to a small percentage of pre-existing cells that already express myogenic genes such as Myf5, MYOD, Myogenin and Desmin [29]. However, all such reports to date have resulted in disappointingly small numbers of muscle differentiating cells. This may suggest that tissue 'stem'/'progenitor' cells are in a dynamic state, constantly changing in response to the microenvironment/stromal cell counterparts/cell density and can maintain their myogenic phenotype only under the effect of numerous specific cues. Replication of these conditions in *in vitro* culture is unfortunately, far from completely understood.

It was clear for the described investigations that OMLP-PC clones could not take on a myogenic phenotype. This inability to demonstrate myotube-like structures and the expression of Desmin in OMLP clonal populations, despite the use of multiple published myogenic culture protocols, indicates that modified cell culture environments alone are not able to drive these expanded clonal oral progenitors down a myogenic pathway. As with all *in vitro* investigations some consideration must be given to the fact that the clones had to be culture expanded and therefore there may be some potential alteration of their phenotype from that of a native, tissue-resident OMLP-PC. It is also possible of course that mixed (non-clonal) populations of OMLP-PCs could harbour sub-populations from the neural crest-derived lamina propria that may actually be needed to drive progenitor cell myogenic differentiation.

Published work has demonstrated that the DNA methylating agent 5-Aza can induce differentiation of PCs down a myogenic lineage as evidenced by the production of myotubes from mouse and rat BM-MSCs [15,16] and mouse dental pulp stem cells [17]. However, as 5-

Aza is a highly potent DNA methylating agent and therefore alters the epigenetic control of gene expression, it is unknown whether these cells are indeed pluripotent or whether pluripotency/skeletal muscle differentiation is being induced via methylation alone. Such alteration in gene expression was not intrinsic to this particular study however, it was demonstrated that 5-Aza could not drive the myogenic differentiation of the tested OMLP-PCs. This result differed to studies by Gornostaeva *et al*., (2006) [30] where myogenesis was observed in prenatal-MSCs derived from the main sites of haemopoiesis (bone marrow, thymus, liver). Interesting however, this study failed to identify myogenic conversion when using splenic MSCs. This suggests a difference in differentiation potential of MSCs from haemopoietic organs dependent upon the source of cells. Interestingly, Nakatsuka *et al*., (2010) [17] utilised dental pulp stem cells in their myogenic differentiation investigations. Exposure to 5-Aza drove myotube formation significantly however, this first required transfection with the MYOD1 gene and (in line with the findings with OMLP-PCs) no myotube-like structures were observed in the control dental pulp cells despite use of myogenic medium.

Experimentation by Engler *et al*. (2004) [27] has demonstrated myogenic conversion of human MSCs when cultured upon polyacrylamide gel substrates of known stiffness. They report that the physical nature of a cell's microenvironment, specifically the elasticity of the surrounding tissues, exerted influence upon morphology, cytoskeleton and gene expression. *In vitro* work had demonstrated that committed muscle cells developed skeletal muscle sarcomeric striations only when the cells were grown on a compliant gel that closely matched the passive compliance of skeletal muscle. Later work revealed that, with the same gels, MSCs maximally express myogenic genes, even in the absence of tailored soluble factors. Our replication of such systems certainly produced alterations in cellular morphology and

indeed there were variations in both C2C12 and progenitor cell turnover and appearance however, true multinucleate cells were not identified in the OMLP-PC clones which also did not stain positively for Desmin.

To promote the greatest levels of cellular maturation and functionality in an *in vitro* culture it is generally accepted that a 3D extracellular matrix is required which more accurately models *in vivo* situation [31]. Utilising a 3D protocol devised by Smith *et al.,* (2012) [28] when type I collagen gel systems were seeded with either C2C12 or the OMLP-PCs both cell sources were able to reorganise the matrix and undergo replication within their respective 3D environments. Some contraction of the matrix by the OMLP-PCs was expected due to passive remodelling of the matrix but this was noticeably less when compared to the control muscle cell line. This differential result correlated with a lack of acquisition of a morphological or immunohistochemically-identified myogenic phenotype in the OMLP-PC cohort.

In summary, neural crest derived OMLP-PC clones do not intrinsically express markers of the myogenic lineage. Despite experimentation with several (published) *in vitro* myogenic culture protocols that have included specific culture media components and culture substrate conditioning we were unable to drive the OMLP-PCs down a myogenic lineage. Nor were the OMLP-PC populations able to be driven into a muscle phenotype when stimulated by a 3D collagenous environment. In support of our findings, current data in the field of deriving muscle from progenitor cells which have a potential to 'convert' to a myogenic lineage is still limited. Indeed, the majority of successful *in vitro* muscle studies have utilised cells derived from tissues that are already 'primed' with the requisite myogenic genetic potential to convert. Significant gaps in our knowledge still exist with respect to the muscle

microenvironment and the cell-cell, cell-stromal interactions required to convert to and maintain an *in vitro* muscle cell system.

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The authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article

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Figure Captions

Fig. 1: Myogenic Polymerase Chain Reaction

RT-PCR analysis demonstrating positive identification of myogenic markers Desmin and MYOD1 within positive control C2C12 muscle cell line but a lack of expression in the OMLP-PC clones.

Fig. 2: Comparisons of OMLP clones and control under myogenic media conditions

Unchanged morphology of OMLP clones (a) before and (b) after culture under myogenic media conditions. OMLP-PC failure to demonstrate positive Desmin immuno-staining both (c) before and (d) after exposure to myogenic culture. C2C12 cells (e) before and (f) after culture under myogenic media conditions demonstrating multinucleate myotube formation. C2C12 cells demonstrating positivity for Desmin both (g) before and (h) after culture under myogenic media conditions.

Fig. 3: OMLP-PCs after DNA manipulation culture conditions

OMLP at 3 days exposure to (a) 5-Aza with evidence of the cells becoming larger, more flattened and forming numerous filopodia and (b) Galectin-1. Neither condition resulted in myotube formation.

Fig. 4a: Collagen tissue culture substrates

(a) OMLP-PCs cultured upon type I collagen; (i) cell morphology and (ii) negative Desmin staining. OMLP-PC cell morphology after culture upon type I collagen and with the addition of 5-Aza (iii) and subsequent negative Desmin staining (iv). C2C12 cells cultured upon type I collagen; (v) cell morphology and (vi) positive Desmin IHC staining. C2C12 cell

morphology after culture upon type I collagen and with the addition of 5-Aza (vii) and subsequent positive Desmin staining (viii).

Fig. 4b: Matrigel tissue culture substrates

(b) OMLP-PCs cultured upon Matrigel; (i) cell morphology and (ii) negative Desmin staining. OMLP-PC morphology after culture upon Matrigel and with the addition of 5-Aza (iii) and subsequent negative Desmin staining (iv). C2C12 cells cultured upon Matrigel; (v) cell morphology and (vi) positive Desmin IHC staining. C2C12 cell morphology after culture upon Matrigel and with the addition of 5-Aza (vii) and subsequent positive Desmin staining (viii).

Fig. 5: The effect of substrate stiffness on OMLP-PC myo-differentiation

(a) OMLP-PCs and (b) C2C12 cells cultured upon polyacrylamide substrates in the presence of myogenic medium. (c) OMLP-PCs and (d) C2C12 cells cultured upon polyacrylamide substrates in the presence of myogenic medium and the addition of 5-Aza. (e) OMLP-PCs cultured upon polyacrylamide substrates demonstrating a lack of Desmin staining. (f) C2C12 cells cultured upon polyacrylamide substrates demonstrating Desmin positivity.

Fig. 6: The effect of 3-dimensional culture on OMLP-PC myo-differentiation

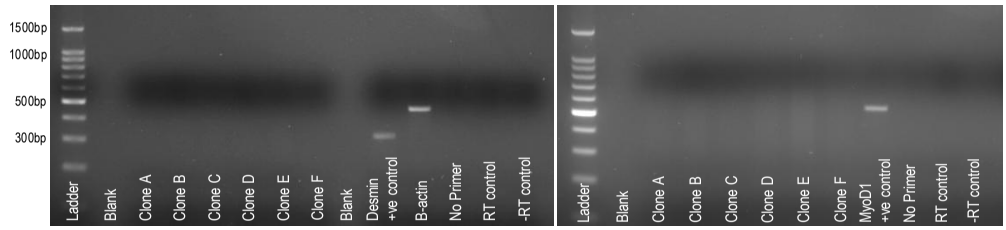
(a) Reorganisation of the 3D extracellular matrix environment by the OMLP-PCs. (b) Reorganisation of the 3D extracellular matrix environment by C2C12 cells demonstrating increased contraction (and subsequent detachment) compared to the OMLP-PCs. The appearance of the (c) OMLP-PCs and (d) C2C12 cells within the 3D collagen lattices. (e) Negative Desmin staining of OMLP-PCs within 3D collagen lattice as opposed to (f) positive Desmin staining of the C2C12 cells.

Table 1: Myogenic polymerase chain reaction results

Undifferentiated OMLP-PCs did not express myogenic markers nor could they be stimulated to do so by alteration of the culture conditions. The positive control C2C12 muscle cell line demonstrated expression of myogenic markers

		OMLP-PC Clones (combined cell data)				
PCR Product		**Untreated Cells**	**Myogenic Media Exposure**	**5-Aza Exposure**	**+ve Control**	**-ve Control**
Muscle	Desmin (335bp)	-	-	-	+ C2C12 myoblast RNA	- H_2O
	MyoD1 (515bp)	-	-	-	+ C2C12 myoblast RNA	- H_2O
Control	Actin (480bp)	+	+	+	+ total Human Skeletal Muscle RNA	- H_2O

Figure 1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RT control
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Ladder
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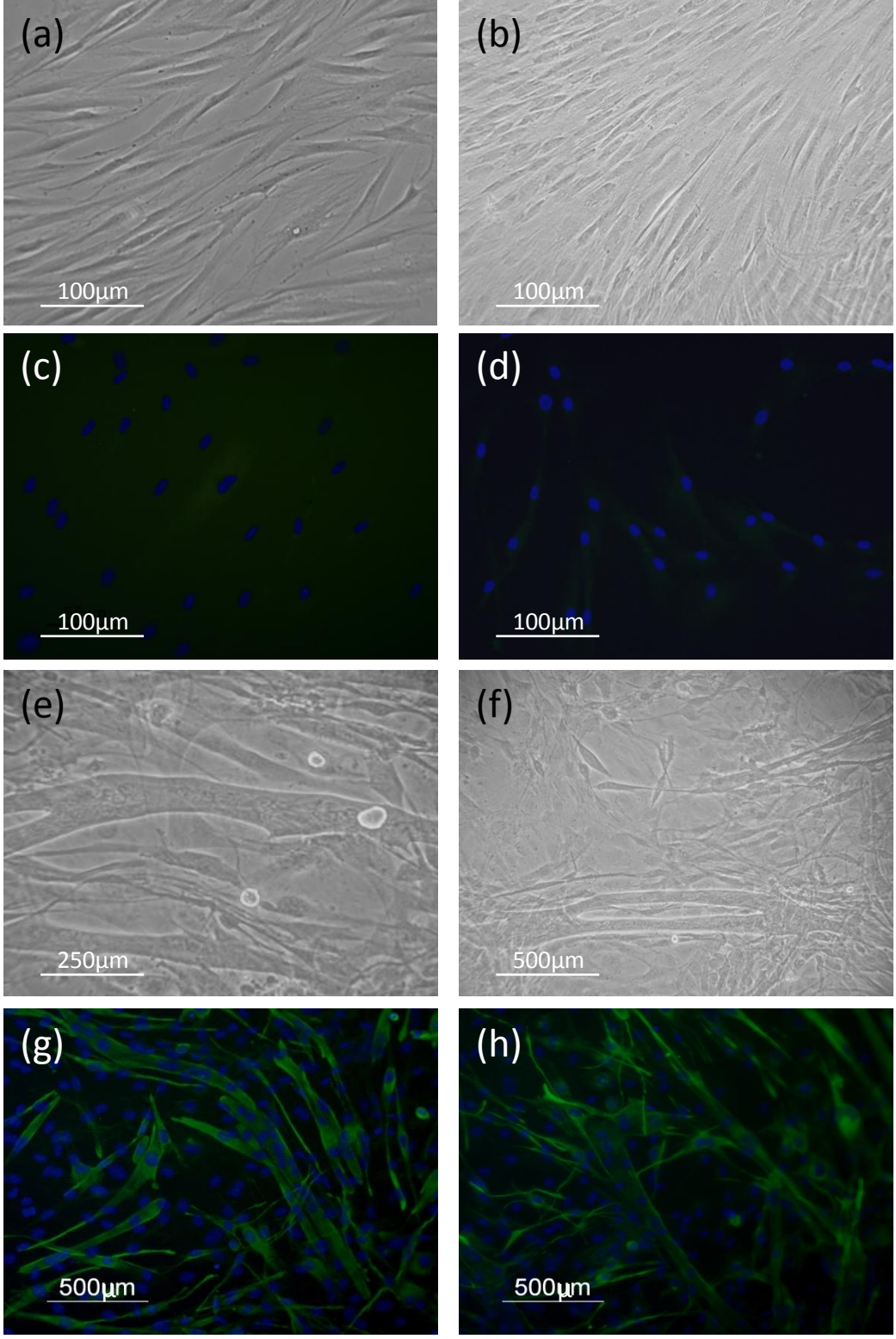
Figure 2



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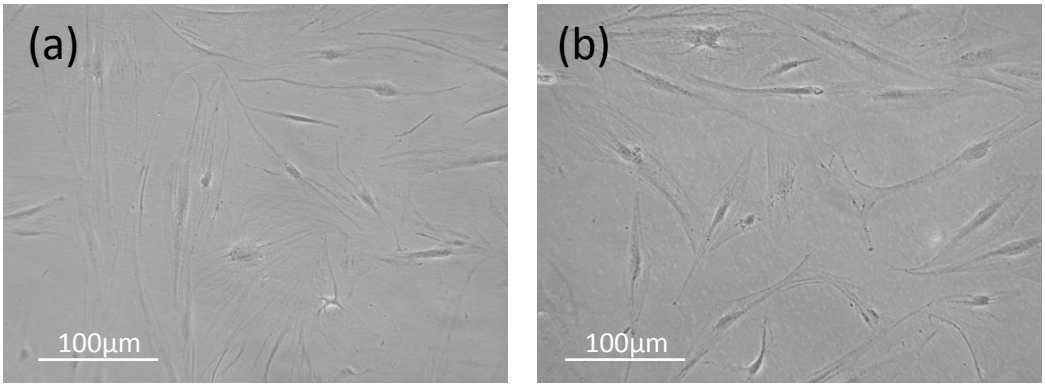
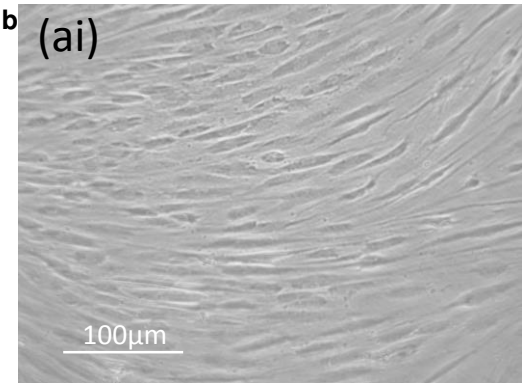


Fugures 4a & b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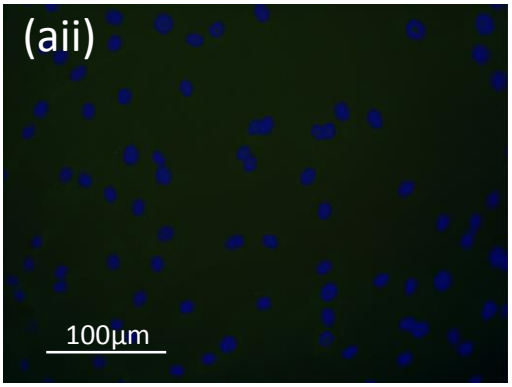
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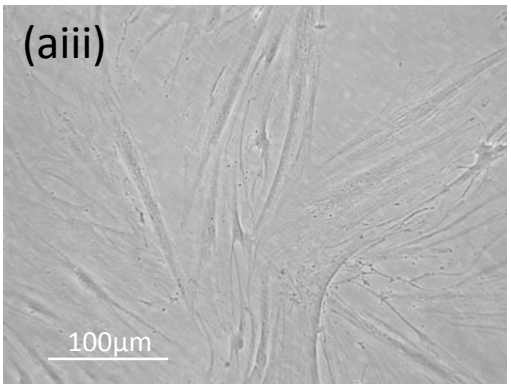
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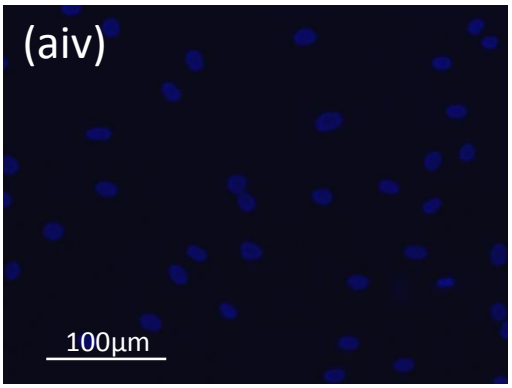
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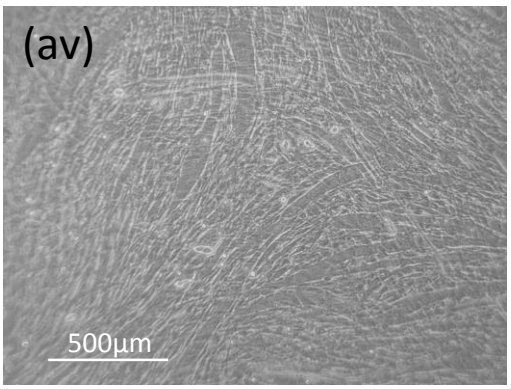
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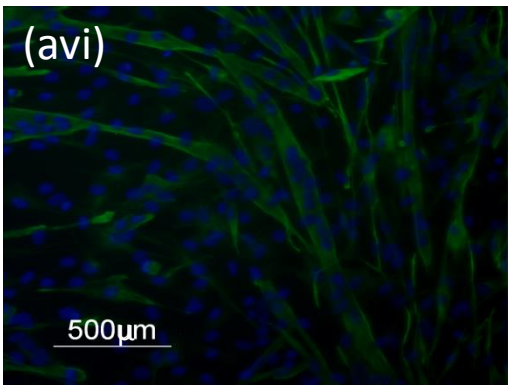
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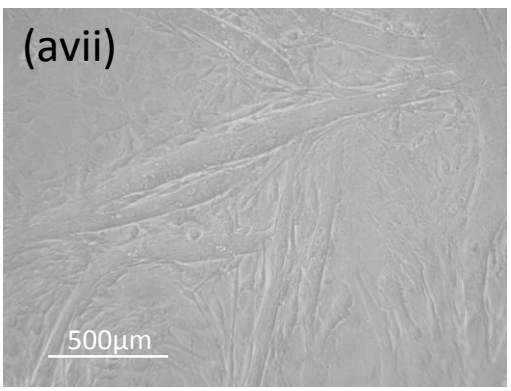
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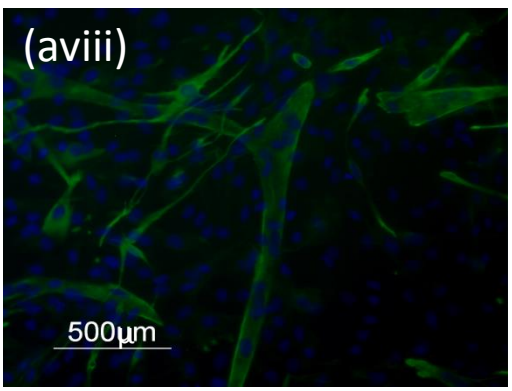
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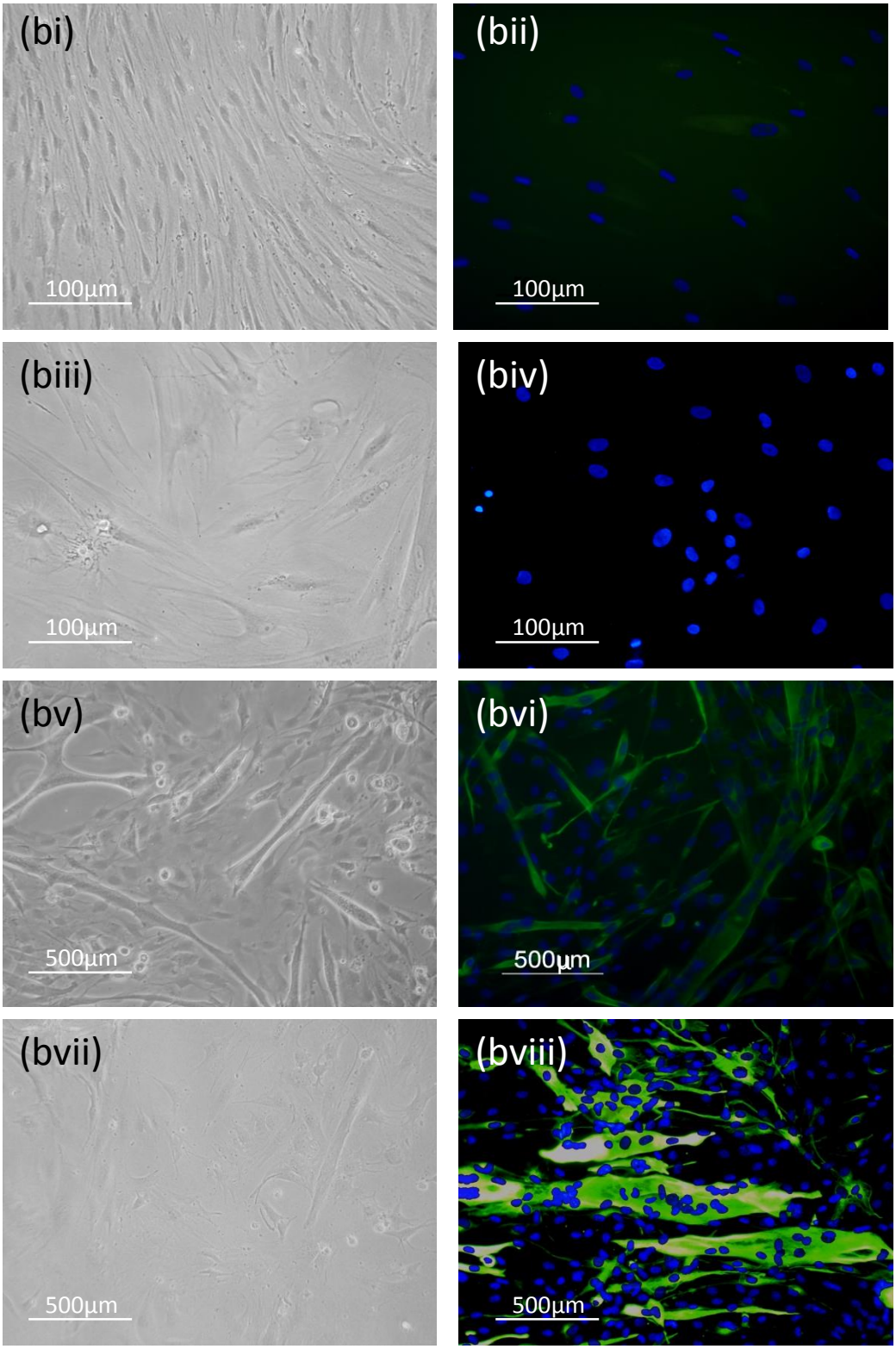
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(bvii)
500µm
(bviii)
500µm

Figure 5

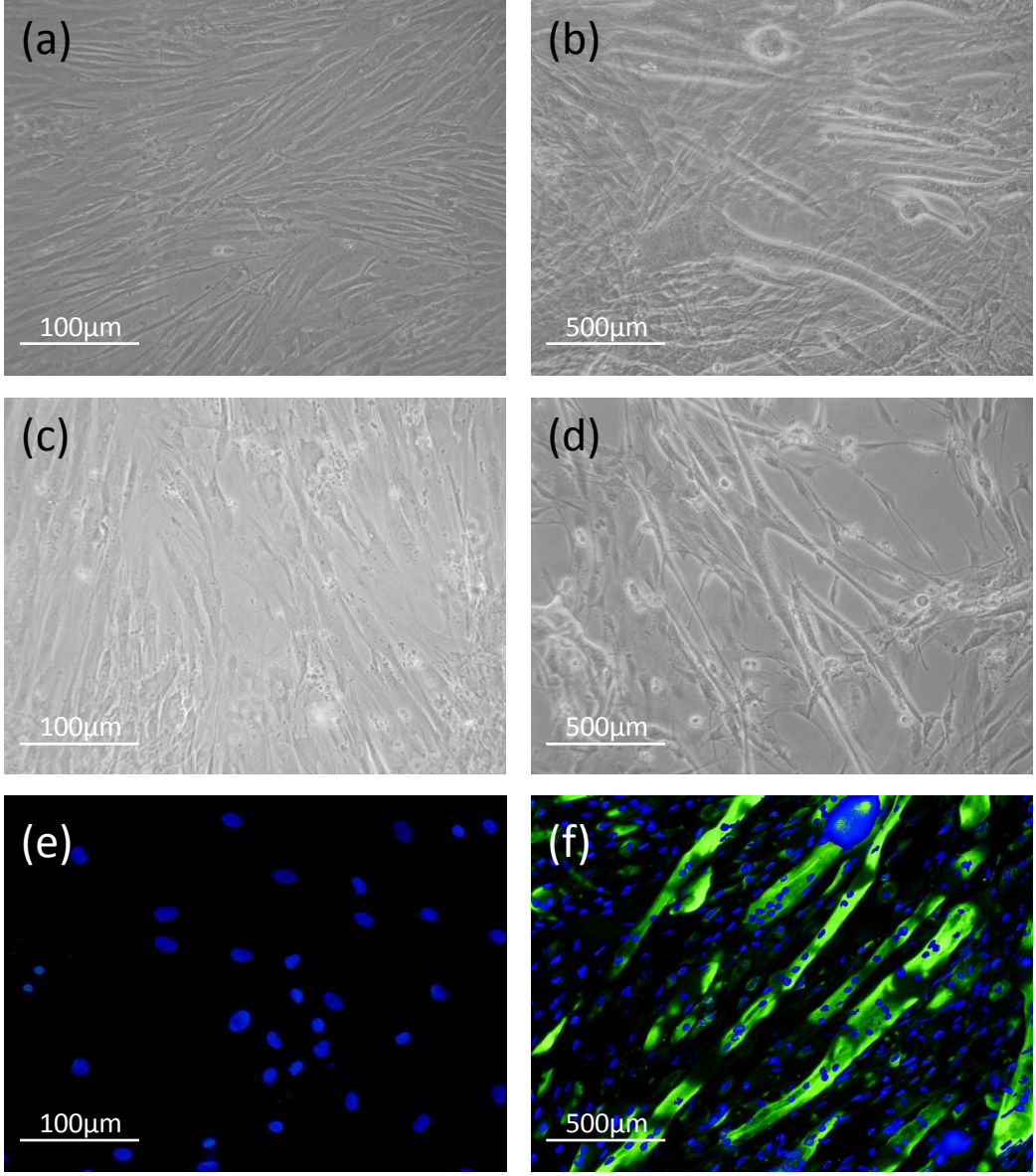
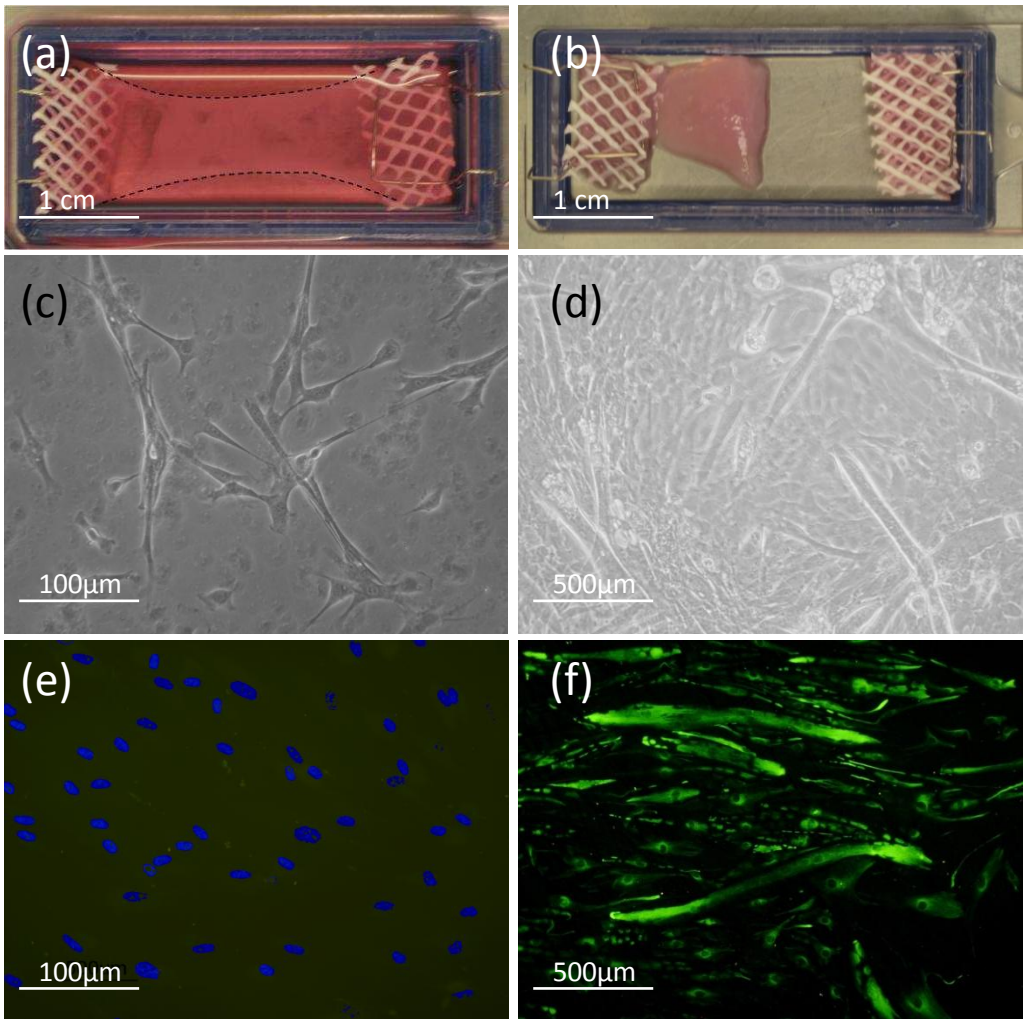
Figure 6