# TIPS TO MANIPULATE MYOGENESIS: RETENTION OF MYOBLAST DIFFERENTIATION CAPACITY USING MICROSPHERE CULTURE

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## Abstract

Cell therapy is an emerging option for regenerating skeletal muscle. Improved delivery methods for anchorage-dependent myoblasts are likely to improve integration and function of transplanted muscle cells. Highly porous microspheres, produced using thermally induced phase separation (TIPS), have features ideally suited for minimally invasive cell delivery. The purpose of this study was to investigate, for the first time, the use of TIPS microspheres as highly porous microcarriers for manipulation of human skeletal muscle myoblasts (HSMM) under defined culture conditions. HSMM cells readily attached to the surface of poly (DL-lactide-co-glycolide) (PLGA) TIPS microcarriers, where they were induced to continue proliferating or to be driven towards differentiation whilst under static-dynamic culture conditions for 7 days. Switching from proliferation medium to differentiation medium for 7 days, resulted in increased protein expression of skeletal muscle cell contractile apparatus components, MyoD and skeletal muscle myosin heavy chain, compared with cells cultured on conventional culture plasticware for the same duration ($p < 0.001$). Growth of myoblasts on the surface of the microcarriers and their migration following simulated delivery, caused no change to the proliferative capacity of cells over 7 days. Results from this study demonstrate that TIPS microspheres provide an ideal vehicle for the expansion and delivery of myoblasts for therapeutic applications. Transplantation of myoblasts anchored to a substrate, rather than in suspension, will reduce the amount of *ex vivo* manipulation required during preparation of the product and allows cells to be delivered in a more natural state. This will improve the ability to control cell dosage and increase the likelihood of efficacy.

**Keywords**: Cell therapy, microcarrier, TIPS microspheres, skeletal muscle, biomaterials, proliferation, differentiation.

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## Introduction

Cell therapy is an emerging treatment for regenerating tissue affected by skeletal muscle disease and volumetric muscle loss. Culture of cells from a biopsy, for autologous transplantation, is an established process for regenerative medicine based therapies, with autologous cell therapy circumventing complications associated with host immune rejection. For skeletal muscle regeneration, autologous myoblasts are isolated, cultured and expanded to provide a quantity of cells estimated to be sufficient to achieve restoration of tissue function. Minimal manipulation of cells *ex vivo*, to produce the final product for therapeutic application, is preferable from an industrial and regulatory viewpoint. For example, centrifugal force, cryopreservation and antibiotic treatment are routinely used in conventional cell culture but may have implications on the use of cells intended as a therapeutic product (Sekiya *et al*., 2012).

Myoblast engraftment and survival are fundamentally governed by the cell niche. In particular, the extracellular matrix (ECM) provides a complex structure that supports cell adhesion, anchorage and facilitates signalling to direct cell fate *via* dynamic cell-matrix interactions (Chiron *et al*., 2012). Detachment and transplantation of cultured anchorage-dependent cell types, in a suspended non-anchored state, may be detrimental to fulfilling their myogenic potential. The interaction between cell adhesion proteins, including integrins, M-cadherins and vascular cellular adhesion molecule 1 (VCAM1), and myogenic differentiation is closely regulated (Costa, 2014; Przewozniak *et al.*, 2013). The role of integrins in myoblast maturation includes cell fusion and participation in myofibre cytoskeletal assembly during embryogenesis (Schwander *et al*., 2003). It is therefore likely that myoblast behaviour following transplantation will also be influenced by their anchorage state.

The physical properties of scaffold structures also influence the behaviour of myoblasts (Kilian *et al*., 2010). Surface topography, microstructure and mechanical properties may affect cell adhesion, proliferation and differentiation (Knight *et al.*, 2013). The use of spherical microcarriers is a widely used technique to expand anchorage-dependent cells in culture (Yao *et al*., 2013; Kang *et al.*, 2008). Microcarriers provide a large surface area for cell adhesion and proliferation *in vitro*. Additionally, they offer a temporary niche following *in vivo* delivery whilst cells are still attached to the microcarrier. This may be advantageous over cell expansion on conventional tissue culture substrates, which often necessitates chemical and/or physical detachment from the culture vessel prior to *in vivo* delivery. Cell detachment disturbs adhesion to extracellular matrix proteins and may damage the cell

membrane, resulting in reduced viability and suboptimal cellular products (Gilbert *et al.*, 2006; Ahmadi *et al.*, 2011).

We have developed a novel polymeric microsphere technology for use as a minimally invasive scaffold and delivery system for cells and drugs (Blaker *et al.*, 2008a; Blaker *et al.*, 2008b; Ahmadi *et al.*, 2011). The microspheres are composed of poly (D, L-lactide-co-glycolide) (PLGA), a biocompatible and biodegradable material, and are prepared using thermally induced phase separation (TIPS).

The aim of the current study was to investigate the attachment of primary cultures of human skeletal muscle myoblasts to TIPS microcarriers under specific culture conditions that induce either cell proliferation or differentiation, with a view to using cellularised TIPS microspheres as a therapeutic system.

## Materials and Methods

**Manufacture of TIPS microspheres**
PLGA TIPS microcarriers were prepared as previously described (Ahmadi *et al.*, 2011). Poly (D, L-lactide-co-glycolide) 75:25 (Purasorb PDLG7507, Purac Biomaterials, Gorinchem, Netherlands) was dissolved in dimethylcarbonate (1:24 (w/v)) (Sigma-Aldrich) using magnetic stirring overnight. The polymer solution was fed into a Nisco Encapsulator Unit (Nisco Engineering, Zurich, Switzerland) by a syringe pump (Harvard Apparatus, Kent, UK), connected by a silicone tube, at a constant rate of 3 mL min$^{-1}$. The vibration frequency of the nozzle was kept at 1.80 kHz and the amplitude of frequency at 100 %. The polymer droplets were delivered through a 150 μm sapphire nozzle.

Polymer droplets were collected in a polyethylene beaker containing 250 mL of liquid nitrogen. The frozen droplets were equilibrated in the liquid nitrogen, transferred to 50 mL Falcon™ tubes (BD Biosciences, Oxford, UK) and stored frozen at -80 °C for 10 min. Samples were freeze-dried (MicroModulyo, Fisher Scientific) for 24 h to allow sublimation of residual dimethylcarbonate. For the experiments conducted in the current study PLGA TIPS microcarriers were sieved to produce microspheres with a mean diameter of 337.5 ± 87.5 μm.

*Attachment of human skeletal muscle myoblasts to TIPS microspheres*
Microspheres were added to 1.5 mL microfuge tubes (Eppendorf, Hamburg, Germany) at 80 ± 5 *per* tube. Industrial methylated spirit contains 99 % ethanol and 1 % methanol, which was used as an alternative to pure ethanol for this set of experiments. A solution of 70 % (v/v) industrial methylated spirit (IMS) (99 % ethanol, 1 %

methanol) in deionised water and proliferation medium (1:5 v/v) was added to the microspheres sequentially and centrifuged at 14,000 rpm, followed by incubation at 37 °C for 18 h.

Human primary human skeletal muscle myoblast cells (Lonza, Basel, Switzerland) were cultured in proliferation medium consisting of Ham's F10 medium (Gibco, Carlsbad, USA) containing 20 % foetal bovine serum (FBS) (Gibco, Carlsbad, USA), 100 U/mL penicillin and 100 μg/mL streptomycin, 0.25 μg/mL amphotericin B (Sigma-Aldrich, Dorset, UK), 10 ng/mL basic fibroblast growth factor (bFGF) (Peprotech, New Jersey, USA) and incubated at 37 °C, 5 % $CO_2$.

For HSMM cell attachment to TIPS microspheres, cells were trypsinised, counted and adjusted to $1 \times 10^6$ cells *per* 1 mL. The culture medium-alcohol solution was removed and the microspheres were washed twice with 500 μL PBS before adding 500 μL proliferation medium. Microspheres in medium were transferred into individual wells of a 96-well ultra-low attachment plate (Corning, New York, USA). A cell suspension of 40,000 cells in a total volume of 100 μL was added to each well containing microspheres and incubated at 37 °C, 5 % $CO_2$ on a microplate shaker (SciQuip, Shropshire, UK). The plate was shaken for 1 min at 250 rpm. The plate was subsequently shaken at the same speed for 1 min *per* h throughout the incubation period.

*Culture of HSMM on TIPS microspheres in proliferation and differentiation medium*
PLGA TIPS microspheres and cells were prepared for attachment and incubation in proliferation medium as described above. Cells were maintained for 1 or 7 d in 100 μL of proliferation medium under static-dynamic incubation conditions with the culture medium being replaced every 2 d (Pavlath, 1996) (Table 1).

Cellularised microspheres were incubated in Differentiation Medium 1, consisting of Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich, Dorset, UK), 2 % horse serum (Gibco, Carlsbad, USA), 2 mM L-glutamine (Sigma-Aldrich, Dorset, UK), 100 U/mL penicillin (Sigma-Aldrich, Dorset, UK), 100 μg/mL streptomycin (Sigma-Aldrich, Dorset, UK) for 24 h. After, Differentiation Medium 1 was replaced with 100 μL Differentiation Medium 2 containing 15 % horse serum in DMEM and changed every 2 d for 7 d (Pavlath, 1996) (Table 1).

**Measurement of HSMM cell proliferation**
HSMM cells were attached to TIPS microspheres in 24-well ultra-low attachment plates under static-dynamic culture conditions. To each well 3 mL of proliferation medium was added and samples were incubated for 1, 3, 5 and 7 d with the culture medium changed every

**Table 1**. Composition of primary human skeletal muscle myoblast culture medium for proliferation and

| Proliferation medium | Differentiation medium 1 | Differentiation 2 |
|---|---|---|
| Ham's F-10 Medium | Dulbecco's Modified Eagle Medium | Dulbecco's Modified Eagle Medium |
| 20 % Foetal calf serum | 2 % Horse serum | 15 % Horse serum |
| 1 % pen./strep. | 2 mM L-glutamine | 2 mM L-glutamine |
| 10 ng/mL bFGF | 100 U/mL penicillin | 100 U/mL penicillin |
| 0.1 μmol/L dexamethasone | 100 μg/mL streptomycin. | 100 μg/mL streptomycin. |

2 d. A BrdU-ELISA assay was performed following the manufacturer's instructions (Roche, Basel, Switzerland). The microspheres were transferred from the ultra-low attachment plates into sterile microfuge tubes. To limit the interference of the microspheres, the amount of BrdU-incorporated into proliferating cells in the HSMM attached to the TIPS microspheres was analysed using colourimetric detection of the supernatant product at 490 nm using a colourimetric plate reader (Multiskan, Thermo Scientific, Massachusetts, USA).

### In-cell Western analysis for skeletal muscle cell phenotype markers in HSMM attached to TIPS microspheres

HSMM attached to the surfaces of TIPS microspheres incubated for either 1 d in proliferation medium, 7 d in proliferation medium, or 7 d in differentiation medium, or HSMM attached to tissue culture plastic for either 1 d in proliferation medium, 7 d in proliferation medium, or 7 d in differentiation medium, were washed in PBS followed by fixation in 4 % formaldehyde in PBS for 30 min. Cells were permeabilised by washing five times in 0.1 % Triton X-100 in PBS for 5 min *per* wash. Non-specific binding was blocked using Odyssey blocking buffer (Li-Cor Biosciences, USA) for 1 h at room temperature. The samples were incubated with antibodies to Ki67 (0.2 mg/mL), Pax7 ( 1 mg/mL), MyoD (1 mg/mL), myogenin (0.1 ng/mL) (Sigma-Aldrich, Dorset, UK), or skeletal muscle myosin heavy chain (30 μg/mL; Developmental Studies Hybridoma Bank, University of Iowa, Iowa, USA) at 4 °C overnight. Samples were washed extensively in PBS containing 0.1 % Tween 20 five times for 5 min *per* wash. Samples were incubated with IR Dye 800 CW secondary antibody (1:800) with CellTag™ 700 stain (1:500) (Li-Cor Biosciences, Cambridge, UK) in Odyssey blocking buffer for 1 h at room temperature. The TIPS microspheres did not cause interference with the infrared detection of the protein detection. The number of cells attached to the TIPS microspheres and in a monolayer was normalised using the CellTag™ 700 stain, which was also used for well-to-well variation between the samples. Samples were extensively washed in PBS containing Tween 20 for 5 min *per* wash.

After a final wash all liquid was removed and the plate was scanned on the Odyssey Classic Infrared Imaging System (Li-Cor Biosciences, Cambridge, UK) using both 700 nm and 800 nm detection channels at 169 nm resolution, medium quality with a focus offset of 4.0 mm. Quantitative in-cell Western analysis was performed using Image Studio Lite version 3.1 (Li-Cor Biosciences, Cambridge, UK).

### qPCR analysis for skeletal muscle cell phenotype markers in HSMM attached to TIPS microspheres

TRIzol (Invtrogen, Paisley, UK) was used to extract RNA from HSMM attached to TIPS microspheres and incubated for either 1 d in proliferation medium, 7 d in proliferation medium, or 7 d in differentiation medium, or HSMM attached to tissue culture plastic either for 1 d in proliferation medium, 7 d in proliferation medium, or 7 d in differentiation medium. RNA content was quantified using a Nandrop 2000 system (Thermo Scientific, Surrey, UK). Total RNA was reverse transcribed using the Verso™ cDNA kit (Thermo Scientific, Surrey, UK) to generate complementary DNA from total RNA. Samples were measured for 1 ng/μL total RNA. Real time PCR measurements were analysed relative to the internal control, GAPDH on a Roche LightCycler® II 480 system (Roche, Basel, Switzerland). Each reaction was performed in duplicate and contained 10 pmol of specific primers (Table 2), SYBR Green Mastermix (Roche, Basel, Switzerland) and 1 μL cDNA in a 20 μL total reaction volume. A control consisting of water without cDNA was also included. Cycle threshold (Ct) values were obtained for each gene of interest and calculated relative to GAPDH as the internal standard to be represented as ΔCt values. The calculated relative gene expression was normalised to controls of GAPDH expression level of 1.0.

### Aspect ratio analysis of HSMM on TIPS microspheres

HSMM cells were attached to TIPS microspheres using static-dynamic incubation in 96-well plates. An equivalent number of cells *per* unit surface area were attached to the surface of 22 mm diameter gelatin-coated glass coverslips (VWR, Leicestershire, UK) (Table 3). Cells were incubated

**Table 2**. Human skeletal muscle myoblast forward and reverse primers for qPCR.

| Gene | Forward Primer | Reverse Primer |
|------|----------------|----------------|
| **Ki67** | AATACGTGAACAGGAGCCAGC | GTTGTTCCCTGAGCAACACTG |
| **Pax7** | ACTGTGCCCTCAGGTTTAGTG | CGTCGTCCTCCTTCTTGTCC |
| **MyoD** | CTTGAGGGGCTAGGTTCACG | TGGGTTACGGTTACACCTGC |
| **Myogenin** | GATCATCTGCTCACGGCTGA | CAGATGATCCCCTGGGTTG |
| **SkMHC** | AGCGTGAGCTGGAAGATGTC | GCCCTCCTCTAGTCGGAAGA |

**Table 3**. Calculation of cell seeding for accurate representation of cell number on the surface area of TIPS microsphere and tissue culture plastic control. The average number of cells attached *per* microsphere, within the 250-425 μm size range, was used to calculate the relative surface area coverage for a 12-well cover slip.

| Attachment object | Smallest microsphere | Largest microsphere | Average microsphere | 12-well coverslip |
|-------------------|---------------------|---------------------|---------------------|-------------------|
| **Diameter (μm)** | 250 | 425 | 337.5 | 22,100 |
| **Radius (μm)** | 125 | 212.5 | 168.75 | 11050 |
| **Surface area (μm)** | 196,000 | 567,000 | 358,000 | 1,530,000,000 |
| **Cell No. at 1** d | | | 647 | 2,765,111 |

on TIPS microspheres for 1, 3, 5 and 7 d under static-dynamic incubation conditions in proliferation medium. Culture medium was replaced every 2 d. Cells were washed and fixed in 4 % formaldehyde prior to phalloidin staining. Briefly, cells were washed, permeabilised in 0.1 % for 5 min in Triton X-100, blocked in 1 % bovine serum albumin for 30 min and incubated in Alexafluor-488 Phalloidin methanolic solution (Molecular Probes, Life Technologies, Carlsbad, USA) diluted in PBS for 20 min at room temperature. Samples were mounted onto a microscope slides in DAPI mountant (Vectashield, Vector Laboratories, Peterborough, UK). Images were acquired using a Leica SP8 confocal microscope (Leica, Solms, Germany) and the aspect ratio was calculated from cell length divided by cell width using the measurement tool in ImageJ.

### Statistical analysis

All experiments were analysed in replicates of five of the same cells, unless stated otherwise. The data were analysed using the unpaired two-tail Student's *t*-test and one-way ANOVA using GraphPad Prism v.6.00 statistical software (GraphPad Software, San Diego, USA). The data were presented as mean ± standard deviation. A *p* value of < 0.05 indicated statistical significance.

### Results

**Proliferation of HSMM Attached to TIPS microspheres**
The proliferation of HSMM cultured on TIPS microspheres in proliferation medium at days 1, 3, 5 and 7 was measured using a BrdU-ELISA. The assay was performed on aliquots of TIPS microspheres incubated under static-dynamic conditions (Fig. 1). The proportion of proliferating HSMM cells remained consistent when cultured on TIPS microspheres over 7 d. No significant difference was observed between groups.

**Characterisation of phenotypic status of HSMM on TIPS microspheres**
*In-cell Western analysis of skeletal muscle cell phenotype markers*
HSMM cells were cultured on TIPS microspheres for 1 and 7 d in proliferation medium and 7 d in differentiation medium. Cells incubated on conventional flat tissue culture substrates for each group were analysed in parallel. Protein expression of myogenic regulatory factors in HSMM under proliferation and differentiation conditions was measured using in-cell Western analysis and quantified relative to cell number using the housekeeping CellTag 700™ protein. The signal intensity for each specific protein probed for was calculated *per* cell by normalising signal to cell number (Fig. 2a-e). Myoregulatory factor protein expression in HSMM attached to TIPS microspheres incubated in differentiation medium for 7 d exhibited higher values for Ki67 ($p < 0.01$), Pax7 ($p < 0.005$), MyoD ($p < 0.001$), myogenin ($p < 0.001$) and skeletal muscle myosin heavy chain ($p < 0.001$) compared with HSMM attached to TIPS microspheres at day 1. HSMM attached to TIPS
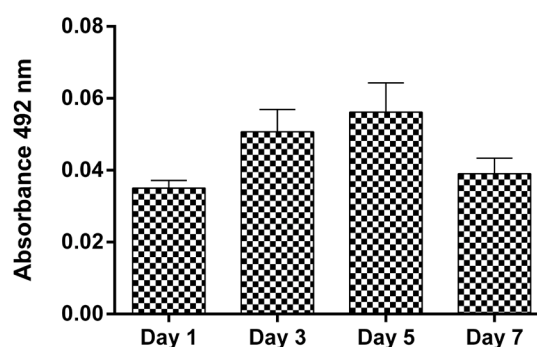
## HSMM Proliferation on TIPS Microspheres



**Fig. 1**. Proliferation of human skeletal muscle myoblasts on TIPS microspheres over 1-7 d in proliferation medium under static-dynamic incubation conditions. TIPS-attached myoblasts showed consistent proliferation over 7 d in proliferation medium.

microspheres and incubated in proliferation medium for 1 or 7 d showed no difference in myoregulatory factor expression between the different time points.

*qPCR analysis of skeletal muscle cell phenotype markers*
The phenotypic status of HSMM attached to TIPS microspheres was quantitatively analysed for myoregulatory factor expression using qPCR. HSMM attached to TIPS microspheres were incubated in proliferation medium for 1 or 7 d, and in differentiation medium for 7 d before qPCR was performed using relative quantification at day 1. Control groups consisting of cells attached to conventional tissue culture plastic surfaces were also analysed. All data were compared to GAPDH gene expression and compared with the Ct cycle of the calibrator obtained with the cells incubated on TIPS microspheres for 1 d (Fig. 3a-e). The expression of Ki67 did not change in HSMM cells attached to TIPS microspheres between days 1-7. HSMM attached to TIPS microspheres expressed Pax7, MyoD and myogenin at significantly higher levels in samples incubated for 7 d in proliferation and differentiation medium compared with the expression at day 1. The relative expression of skeletal muscle myosin heavy chain in HSMM attached to TIPS microspheres cultured for 7 d in differentiation medium resulted in a 14-fold increase compared with expression in HSMM on TIPS microspheres at day 1.

**Aspect ratio analysis of HSMM on TIPS microspheres**
The effect of culturing HSMM on TIPS microspheres on the cell morphology was explored using cell aspect ratio analysis of phalloidin-stained cells and compared with HSMM attached to tissue culture plastic surfaces (Fig. 4). Quantitative analysis of aspect ratio indicated that cells incubated in proliferation medium and differentiation medium for 7 d had a higher aspect ratio ($p < 0.01$), compared with cells cultured on tissue culture plastic under the same conditions. Confocal microscopy revealed the cell cytoskeleton to be morphologically elongated, when cultured on TIPS microspheres for 7 d, compared with
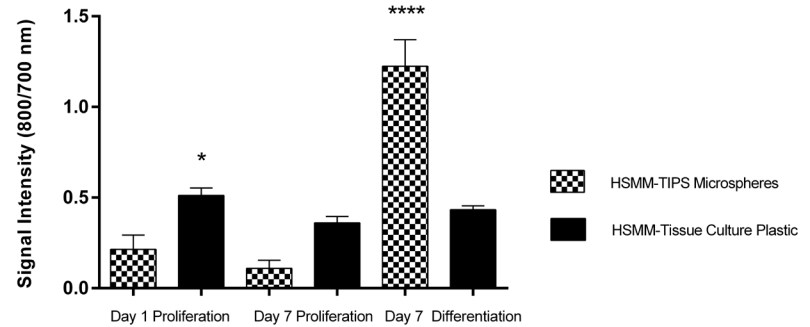
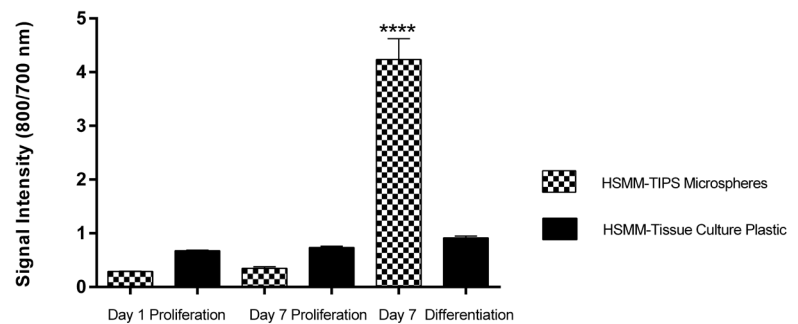**Ki67 Expression in HSMM on TIPS Microsphere vs. TC Plastic**  a
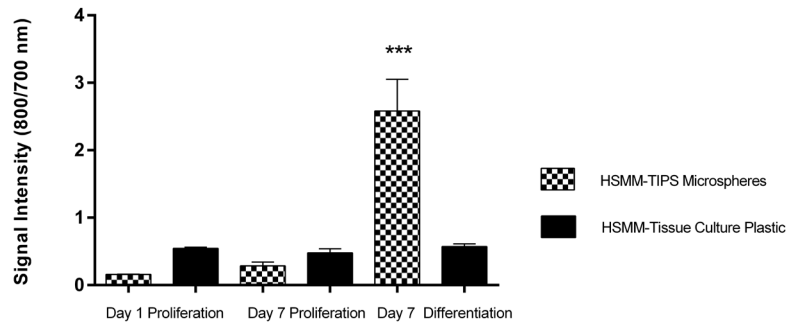


**Pax7 Expression in HSMM on TIPS Microsphere vs. TC Plastic**  b
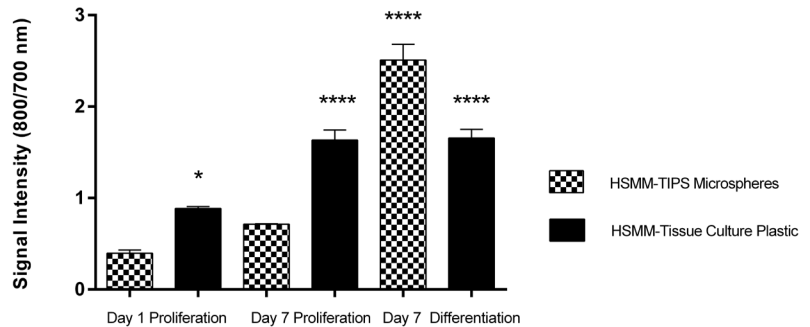


**MyoD Expression in HSMM on TIPS Microsphere vs. TC Plastic**  c



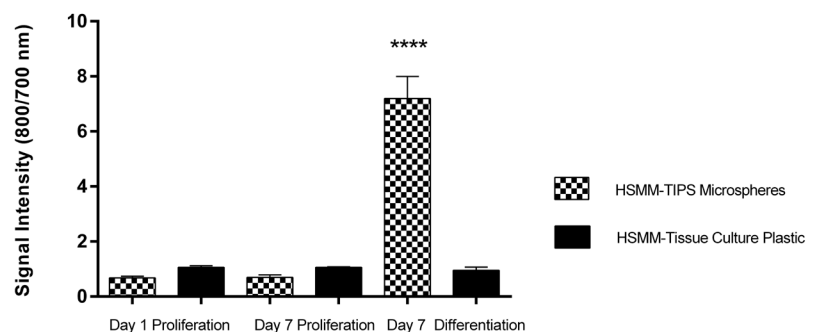**Myogenin Expression in HSMM on TIPS Microsphere vs. TC Plastic**  d



**Fig. 2**. Protein expression levels of (**a**) Ki67, (**b**) pax7, (**c**) MyoD, (**d**) myogenin and (**e**) skeletal muscle myosin heavy chain in HSMM attached to TIPS microspheres *versus* expression in HSMM attached to tissue culture plastic surfaces Relative expression in all groups was compared with HSMM attached to TIPS microspheres after 1 d in proliferation medium (*$p < 0.05$, **$p < 0.01$, ***$p < 0.005$, ****$p < 0.001$).
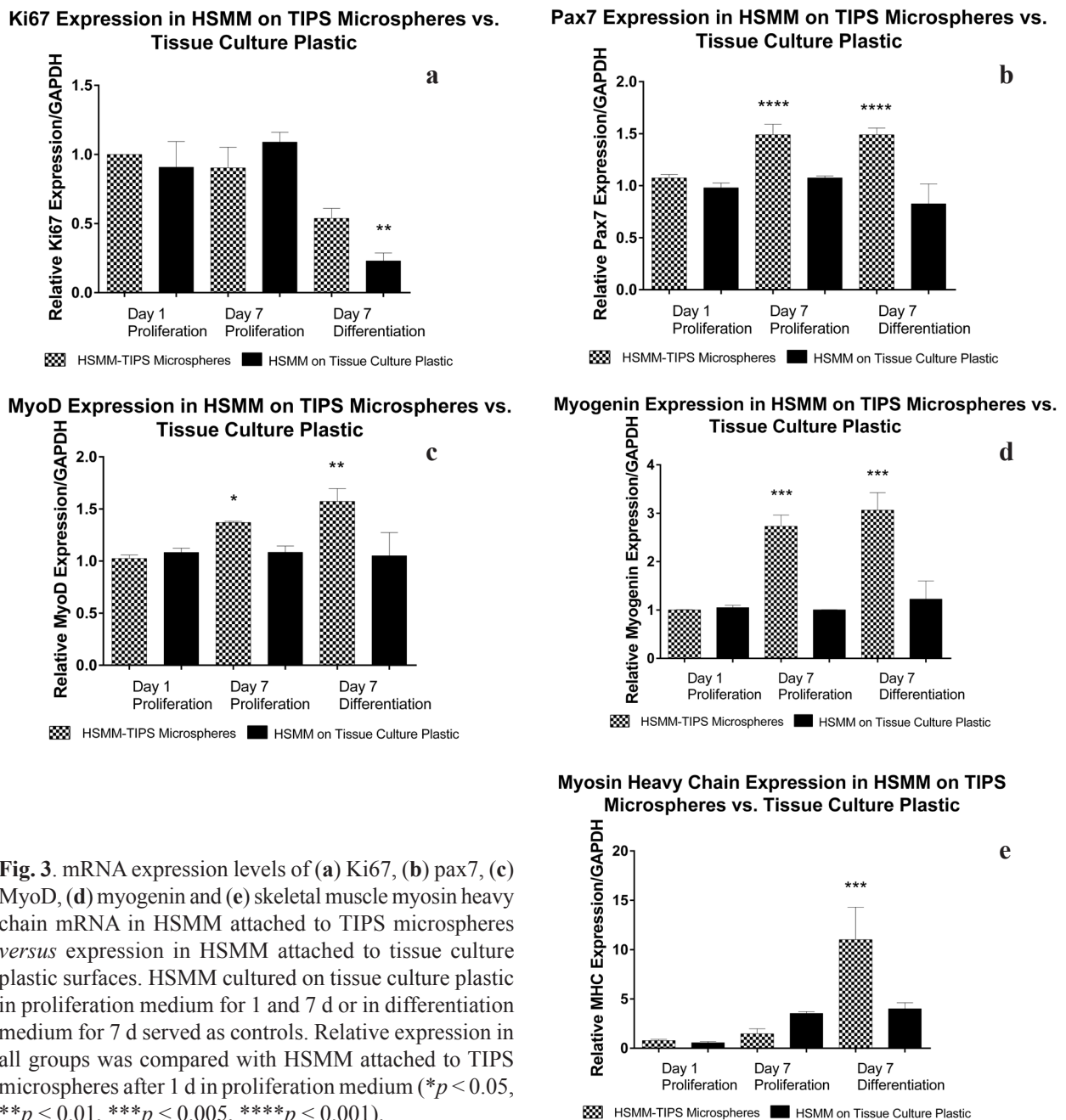
**MHC Expression in HSMM on TIPS Microsphere vs. TC Plastic**  e

**Fig. 3**. mRNA expression levels of (**a**) Ki67, (**b**) pax7, (**c**) MyoD, (**d**) myogenin and (**e**) skeletal muscle myosin heavy chain mRNA in HSMM attached to TIPS microspheres *versus* expression in HSMM attached to tissue culture plastic surfaces. HSMM cultured on tissue culture plastic in proliferation medium for 1 and 7 d or in differentiation medium for 7 d served as controls. Relative expression in all groups was compared with HSMM attached to TIPS microspheres after 1 d in proliferation medium (*$p < 0.05$, **$p < 0.01$, ***$p < 0.005$, ****$p < 0.001$).

the cytoskeleton arrangement of cells cultured on tissue culture plastic for the same period of time. Cells cultured on tissue culture plastic in differentiation medium for 7 d were also significantly elongated, compared with cells cultured on tissue culture plastic for 1 d in differentiation or proliferation medium ($p < 0.05$).

## Discussion

### Characterisation of HSMM attached to TIPS microspheres
Delivery of myoblasts to treat skeletal muscle injury often requires cell expansion *in vitro*. Cell delivery using 3D scaffolds has previously been used for proliferation *in vitro* (Knight *et al*., 2013; Chang *et al*., 2007). The role

of a scaffold is to provide a template for regenerating tissue and may influence cell behaviour. This includes cell attachment, migration, proliferation, and differentiation to a specific cell phenotype, which could impact on the rate of tissue formation and efficacy of the treatment (Smith *et al*., 2009; Wei and Ma, 2008). In the current study the effect of attaching HSMM cells to TIPS microspheres was investigated in the context of their phenotypic status. A proliferation assay, measuring incorporation of BrdU into DNA of proliferating cells, revealed no significant change to cell proliferation during 1-7 d incubation in proliferation medium. This finding would suggest that HSMM retain their proliferative status when cultured on the surface of TIPS microspheres and do not spontaneously differentiate. This could be of value during a bioprocessing and therapeutic setting, which may facilitate tissue regeneration
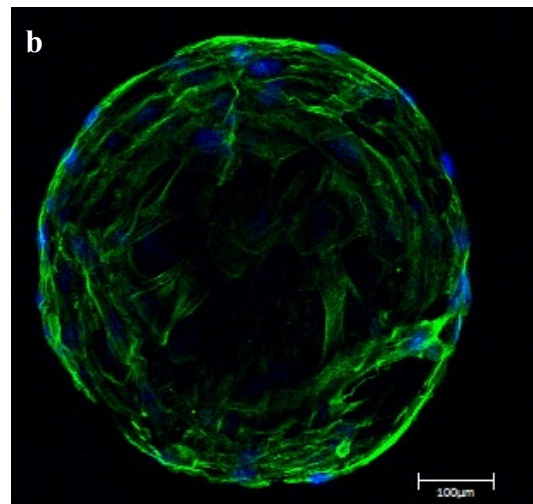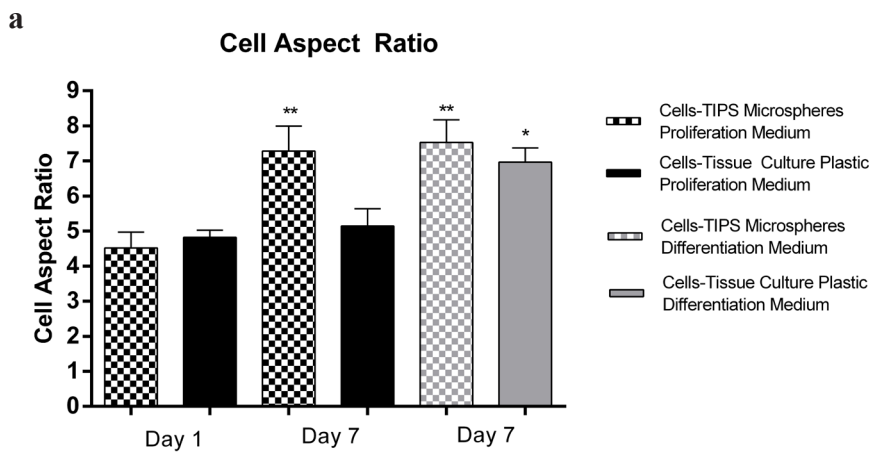
**a**





**Fig. 4**. (**a**) Cell aspect ratio of HSMM attached to TIPS microspheres *versus* HSMM attached to conventional tissue culture plastic surfaces. HSMM were cultured in proliferation medium for either 1 or 7 d or in differentiation medium for 7 d. Changes to the aspect ratio under different culture conditions were compared with cells attached to the surface of TIPS microspheres in proliferation medium, after incubation in proliferation medium for 1 d (*$p < 0.05$, **$p < 0.01$). (**b**) DAPI/Phalloidin-stained human skeletal muscle myoblasts attached to TIPS microspheres (100 μm scale bar).

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in a clinical setting. A void of defective tissue requires a sufficient mass of cells for replacement of the previously lost injured tissue.

**Manipulation of phenotypic lineage**

A current challenge for cell based therapy products is the control of cell fate upon transplantation (Ankrum *et al.*, 2014). For muscle regeneration, it is currently uncertain whether it is preferable for cells to be transplanted in a proliferative state as myoblasts, or differentiated as myotubes or myofibres. In culture, the presence of myotubes will eventually induce the cessation of cell proliferation. *In vivo*, this could also be influenced by the release of cytokines and other factors present at the site of injury (Chiron *et al.*, 2012). The current study demonstrates it is possible to manipulate the phenotype of myoblasts attached to TIPS microspheres *in vitro* under defined conditions.

Myoblast differentiation was initiated by the addition of 2 % horse serum, which leads to an increased level of MyoD that stimulates creatine phosphokinase activity and formation of contractile apparatus (Zalin, 1972; Lawson and Purslow, 2000; Yeh *et al.*, 2014). Cells attached to TIPS microspheres, cultured in differentiation medium, exhibited an increase in myoregulatory factors Ki67, pax7, myogenin, and myosin heavy chain. The co-expression of markers of proliferation and differentiation after

incubation in differentiation medium, suggests that these incubation conditions result in phenotypic heterogeneity in the cells attached to the microspheres. The mixed phenotype corresponds with data available from the cell product manufacturer that indicated the cells were > 60 % desmin positive (Lonza). Delivery of a phenotypically heterogeneous population of HSMM on TIPS microspheres may be beneficial, by offering engraftment of cells with contractile apparatus as well as more proliferative cells that could further increase the number of cells at the target site. Interestingly, MyoD expression was not increased in HSMM attached to TIPS microspheres incubated in differentiation medium. Previous studies have shown that in a heterogeneous population of myogenic cells, MyoD expression may be reduced as reserve cells are generated to delay further differentiation (Yoshida *et al.*, 1998).

Myoblast differentiation is a multifaceted process that implicates withdrawal from the cell cycle and morphological changes that include elongation, alignment and fusion of myoblasts to form multinucleated myofibres. HSMM mRNA analysis of myogenic regulatory factors demonstrated increased expression of myogenin and myosin heavy chain in cells cultured on tissue culture plastic in differentiation medium. This was translated to a significant increase in the protein expression of myosin heavy chain in HSMM attached to TIPS incubated in differentiation medium for 7 d. The increased expression

of myosin heavy chain in HSMM cells attached to the microspheres may be due to differences in the surface topography or substrate stiffness offered by microspheres compared with tissue culture plastic. Substrate stiffness has important implications for muscle regeneration strategies. For example, sarcomeres will not form *in vitro* unless myoblasts grow on a surface with stiffness representative of physiological muscle stiffness (Engler *et al*., 2004). The experiments conducted were based on repeats from one cell donor. Moving forward with this research will require further characterisation of cells attached to the TIPS microspheres using different cell donors.

Delivery of myoblasts on a 3D structure may enhance the survival of transplanted cells. A current limitation for many cell based therapies is the lack of a sufficient vascular supply to sites of cell transplantation (De Coppi *et al*., 2005). It is estimated that cells need to be no more than 0.1 mm away from a capillary to receive oxygen and nutrients as well as to remove waste products (Goodsell, 2003). Packing of the TIPS microspheres results in an open scaffold structure with predictable interstices between adjacent particles. This will facilitate the passage of nutrients and waste, increasing the likelihood of cell survival.

### Cell elongation on TIPS microspheres

The current study investigated changes to the aspect ratio of myoblasts attached to TIPS microspheres under proliferation and differentiation conditions. Cell aspect ratio is calculated by dividing cell height by cell width and used to quantify cytoskeletal arrangement. This has previously been used by (Crouch *et al*., 2009), to study the anisotropic and geometric cell behaviour in relation to biomaterial surface topography (Crouch *et al.*, 2009). HSMM were observed to have elongated on TIPS microspheres after 7 d culture in proliferation and differentiation medium. There was no difference in the elongation of cells in proliferation medium at day 1 on TIPS microspheres compared with cells on tissue culture plastic at days 1 and 7 in proliferation medium. Therefore, the cytoskeletal changes observed may have resulted from the initiation of differentiation cues. Elongation of cells in proliferation medium at day 7 indicates that some cells undergo spontaneous differentiation on TIPS microspheres. This may account for apparent differences of myoblasts incubated on TIPS, compared with on monolayer surfaces. The cause of this is unknown, but the 3D topography of the microsphere surface may elicit similar cues to the ECM. Cellular morphology is directed by the ECM and is thought to be an inherent cue to control myogenic differentiation, regardless of the use of chemical induction factors (Chiron *et al*., 2012; Baker, 2012).

### Cell delivery *via* TIPS microspheres

Delivery of cell suspensions derived from conventional methods using 2D tissue culture substrates, requires their detachment from the culture vessel prior to delivery. This process interferes with cell adhesion to extracellular matrix proteins and may disrupt cell membranes, resulting in changes to cell viability and function (Gilbert *et al*., 2006; Yang *et al*., 2007; Ahmadi *et al*., 2011). Such detrimental

effects could diminish the efficacy of the transplanted cell product and contribute to poor clinical outcomes. In this study, TIPS microspheres were tested as a substrate for the attachment of anchorage-dependent cells. The migration of cells from the site of implantation into other tissues may be reduced by the targeted delivery of cells using TIPS microspheres, resulting in potentially improved efficiency of cell number. TIPS microspheres possess a large surface pore, providing an internal cavity and increased surface area. The internal cavity feature promotes host tissue infiltration into the microsphere, to avoid it being extruded from the implantation site (Blaker *et al.,* 2008a; Keshaw *et al*., 2010). Thus, the migration of transplanted cells from the site of injury is likely to be reduced. Improved host-tissue infiltration into the microsphere cavity, along with diffusion of oxygen and nutrients to the transplanted cells is likely to facilitate engraftment and cell survival.

### Future directions

Effective regeneration of volumetric muscle loss, using the type of approach outlined in the current study, will require further understanding of the optimal phenotypic distribution of cells being delivered so that they match the conditions of the target site. This is likely to differ, depending on the condition being treated. Further studies will also be necessary, to determine whether the transplanted cells differentiate and provide functional contractile properties similar to native skeletal muscle.

## Conclusion

Results from the current study demonstrate that the phenotypic status of myoblasts, attached to TIPS microspheres, can be controlled independently to the delivery of conventional chemical stimuli. The efficient attachment of myoblasts to the microspheres and their continued proliferation offers an assistive technology for minimally invasive delivery of cells for muscle therapy.

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## Discussion with Reviewers

**S. Richardson:** The authors highlight that the TIPS microspheres have an internal cavity, which they suggest would promote host tissue infiltration. Can the authors speculate as to whether the cavity will influence local adhered cell phenotype compared to cells on the outer surface, possibly due to differences in cell density or local cell-cell interactions?

**Authors:** To date, this specific area has not been studied in terms of the phenotypic status of the attached cells. This unknown may cause induced differentiation of cells which are likely to be in closer proximity to each other. However, as the cells are likely to be seeded as a monolayer in the cavity the cells are likely to be exposed to similar local cell-cell interactions as on the outer layer.

**U. Rüegg:** What will be done in order to investigate whether this technique will provide functional improvement of skeletal muscle?

**Authors:** The next stage of development for this approach is to demonstrate the functional restoration of injured skeletal muscle. The group is currently investigating strategies to deliver myoblasts attached to the surface of the microparticles to a discrete skeletal muscle injury.

**R. Oldershaw:** How do you envisage the application of the TIPS microparticles within the context of other clinical indications?

**Authors:** This composition of TIPS microparticles has been demonstrated to promote excellent cell attachment properties for a variety of anchorage-dependent cell types. For some conditions which require targeted cell delivery, the delivery of cells on the surface of TIPS microparticles may promote more localised repair and regeneration. Current cell therapy approaches to conditions such as type II diabetes, localised muscular dystrophies and myocardial infarction usually require the systemic delivery of a high number of cells in suspension to account for cell death and non-specific delivery. The unique advantages of the TIPS microparticles permit the targeted delivery of cells to a discrete injury, using a minimally invasive approach.

**Editor's Note:** Scientific Editor in charge of the paper: Jürg Gasser.