The Effects of Substrate on Muscle-Derived Stem Cell Differentiation

Rebecca A Long[1], Johnny Huard[1,2], Michael B Chancellor[3], and Michael S Sacks[1,2]
[1] Department of Bioengineering, University of Pittsburgh, Pittsburgh, PA, USA
[2] McGowan Institute for Regenerative Medicine, University of Pittsburgh, Pittsburgh, PA, USA
[3] Department of Urology, University of Pittsburgh, Pittsburgh, PA, USA

Introduction:
Current limitations in muscle tissue engineering arise from the inability to form innervated, vascularized, and well-organized tissues *in vitro*. There has been success engineering a 3D muscle tissue *in vitro* through cell self-assembly on collagen, laminin, or fibrin gels; however, these "myooids" are limited in size due to lack of innervation and vascularization (1). Muscle-derived stem cells (MDSC) have been injected into many tissues *in vivo,* ranging from bone to myocardium (2), and have been shown to restore dystrophin when injected into *mdx* mice (3). However, MDSC have not been well characterized on scaffold materials. In order effectively utilize MDSC for *in vitro* tissue engineering applications, more fundamental studies are necessary. Since cell organization is necessary for tissue engineered muscle constructs, substrates made from natural gels provide an optimal surface for self-organization. The current study examines collagen gel and coating, fibrin gel, and gelatin as substrates for MDSC growth in static culture.

Methods:
Cell isolation and expansion
PP6 cells were isolated from a rat hind limb and expanded in Dr. Huard's laboratory using the pre-plate technique. PP6's were expanded in DMEM with 10% HS, 10% FBS, 1% PS, and 0.5% CEE. *Substrate preparation*
SYLGARD (PDMS, Fisher Scientific) was used (10:1) to coat 35 mm tissue culture dishes and allowed to cure for 48 hours. Dishes were rinsed 3x in DI H2O. Then, groups were divided in the following: collagen coating (2mg/mL, Vitrogen), collagen gel (anchored and free floating, 2 mg/mL at 37^{o}C 1 h, Vitrogen), gelatin gel (anchored and free floating, 5%), fibrin gel (anchored and free floating, 20 mg/mL fibrinogen with 10 U/mL thrombin, Sigma). After substrates were prepared, PP6 cells were seeded (passage 18-22) 1x10^{5} cells per dish. Half of the 42 samples were in static culture under standard tissue culture conditions for up to 10 days. The remaining 21 samples were switched to differentiation media (DMEM supplemented with 1% HS, 1% FBS, 1% PS) after 4 days.
Biological assays
In order to examine cell proliferation, a PicoGreen dsDNA quantitation kit (Molecular Probes, Eugene, OR) was used on cell lysates. In order to determine the extent of differentiation, a creatine phoshophokinase (CPK) assay (Sigma) was used on all samples. The data were normalized by total protein concentration, which was determined using Coomassie Blue total protein kit (Fisher Scientific) as per the manufacturer's instructions and using a spectrophotometer set at 595 nm.
Immunofluorescence
At 10 days total culture time, samples were fixed in 10% formalin for paraffin embedding and sectioning. Sectioned samples were blocked with 10% horse serum for 1 hour, myosin heavy chain (Sigma) 1:250 for 1 hr, biotinylated anti mouse (Vector) 1:200 for 1 hr, S/A cy3 (Sigma) 1:500 for 30 minutes, and DAPI (Sigma) for 10 minutes. Samples were viewed on a Nikon Eclipse Microscope.
Statistical analysis
Data from biological assays were analyzed using a two way ANOVA with $p<0.05$ for significance (SigmaStat 3.0).

Results / Discussion: Myotube formation was observed on all constructs in static culture. DNA quantification showed that MDSC proliferated a significantly greater amount ($p<0.05$) on collagen gel compared to collagen coating or gelatin gel. When exposed to differentiation media, MDSC decreased in proliferation on fibrin gel only.

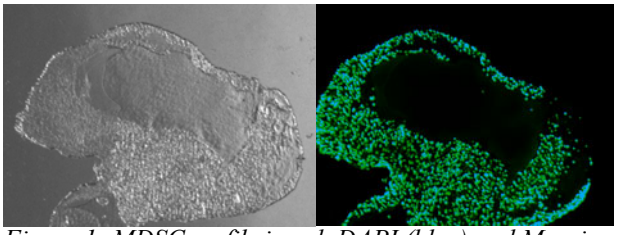

Figure 1: MDSC on fibrin gel. DAPI (blue) and Myosin Heavy Chain (green).

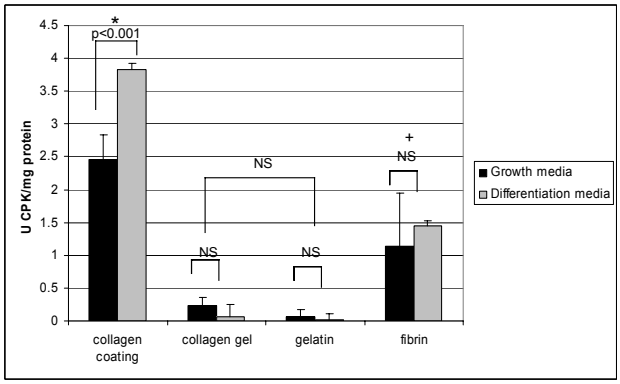

*Figure 2: Creatine phosphokinase activity of MDSC in static culture. N= 3 mean +/- sem. *, + p<0.01*

Conclusions: Preliminary results indicate that fibrin gel as well as collagen-I coating provide the best surfaces for MDSC differentiation into mature skeletal muscle *in vitro* (Fig 2). These findings suggest that substrate and culture conditions may be optimized in order to form an organized avascular contractile tissue, which may be used to study fundamental aspects of contractile tissue formation using MDSC.

References
1. Huang Y et al. *J Appl Physiol* 2005 98: 706-713.
2. Young BH et al Clin Orthop Relat Res. 2002 Oct;(403 Suppl):S243-51.
3. Cao B et al Cell Cycle. 2004 Feb;3(2):104-7.