Therapeutic Regeneration of Muscle Using a Skeletal Muscle-Mimicking Matrix is Maximized in Necrotic Contexts

[1,2]Kuraitis, D; [1]Musarò, A, [2]Suuronen, EJ
[1]University of Rome La Sapienza, Rome, Italy; [2]University of Ottawa Heart Institute, Ottawa, Canada

Introduction:

Diseases of skeletal muscle are highly prevalent in today's society. Afflicted individuals typically experience muscle loss and dysfunction via active degeneration or atrophic/wasting pathways. Degenerating muscle is hallmarked by necrosis and the breakdown of muscle cells. Atrophic muscle is instead characterized by a slow loss of muscle cells through attrition and reduced functional capacity. Muscle turnover is coordinated by muscle progenitor cells called satellite cells (SCs). After activation (by stimuli such as necrosis, injury or exercise), SCs may mature and fuse into myotubes in order to contribute to regenerating muscle. Previous studies have shown that an injectable matrix composed of natural extracellular matrix (ECM) components, which physically mimics skeletal muscle, is able to direct pluripotent embryonic stem cells towards a muscle lineage *in vitro* and also augment rapid muscle regeneration (myogenesis) when applied to degenerating ischemic skeletal muscle *in vivo* (1). This study sought to: i) investigate whether or not the same biomaterial therapy can augment myogenesis in other disease states; ii) characterize interactions between the hydrogel and SCs; and, iii) explore the role of necrosis in matrix-mediated myogenesis.

Materials and Methods:

All experiments were approved by the University of Rome and were carried out in accordance with national law. Collagen I and chondroitin sulfate-C were cross-linked with EDC/NHS, and then maintained on ice until applied *in vivo* or spread onto culture dishes for *in vitro* studies. Murine models of necrotic (*mdx*; 2 week) or atrophic myopathy (*MLC/SOD*^{G93A}; 4 month) were anesthetized and the extensor digitorum longus (EDL) muscle – heavily involved in motility – was exposed and immediately received a 5ul intramuscular injection of PBS or matrix, or no injection (sham). After 2 weeks (*mdx*) or 2 months (*MLC/SOD*^{G93A}), muscle function was assessed using a treadmill test and RNA from the EDL was analyzed for myogenesis-related gene transcripts using qPCR.

Hindlimbs of 4-week old C57BL/6 mice were digested with dispase and collagenase and maintained on collagen I-coated tissue culture dishes under high serum growth conditions to generate SC populations (2). SCs were then seeded onto collagen I or matrix coatings under differentiation conditions of low serum, ± the addition of necrotic myocyte debris (NMD) prepared from dead myoblasts to act as a necrotic stimulus. After 24h, cells were imaged and harvested for qPCR analysis. Supernatant was collected from SC cultures and applied to differentiating C2C12 myoblast cultures and also screened using a cytokine array.

All *in vivo* studies were performed with n=4 and *in vitro* studies with n=6. Data between two experimental conditions was analyzed using a student's t-test and data between multiple groups was analyzed using an analysis of variance with Tukey's post-hoc using Prism 4.0.

Results:

Mdx mice receiving matrix ran at least 40% further than sham or PBS-treated mice (p<0.02) and resisted fatigue, exhausting themselves at speeds of 22 m/s, whereas sham and PBS-treated mice could not continue beyond 18 m/s (p<0.02). Myogenesis-associated transcripts pax3, myf5, desmin and myogenin were up-regulated up to 6-fold in matrix-treated *mdx* mice (p<0.05), but not muscle

creatine kinase (p=0.5). No functional improvements in matrix-treated atrophic mice were observed, nor were there any differences in transcriptional profiles between treatments (p>0.3).

When exposed to the matrix, the frequency of SC-derived myotubes increased (13.9±1.3/field-of-view vs. 5.0±0.9; p<0.0001), regardless of the presence of the NMD stimulus. However, exposure to the matrix while in the presence of the necrotic stimulus accelerated the growth of the myotubes, producing myotubes that were at least 50% longer and 20% thicker (p<0.02). Myotubes generated under matrix-NMD stimuli were also observed to spontaneously beat after 24 h and the expression of myf5, myoD, myogenin and mef2c mRNA was increased up to 13-fold in these cells (p<0.04). When the supernatant from these cultures was applied to C2C12 myoblasts, only the supernatant from matrix-NMD exposure accelerated the maturation of myoblasts, as evidenced by a greater fusion index (16.1% vs. 6.5-10.0%; p<0.05) after 48h and also increased transcription of myf5, myogenin and mef2c (up to 192-fold; p<0.03). Supernatant was screened to identify which factors are being produced by SCs under matrix and NMD co-stimuli: levels of fibroblast growth factor-2, hepatocyte growth factor, and stromal cell-derived factor-1 were increased by at least 50% (p<0.05).

Discussion:

Application of the matrix had a profound effect on the ability of the dystrophic *mdx* mice to run. The improvement in muscle function was echoed in the molecular analysis, where increases in myogenesis-associated transcripts were observed. Unfortunately, neither functional nor molecular indications of myogenesis were apparent in the atrophic model. These results suggest that there are particular contexts in which the ECM-mimicking matrix may be able to augment myogenesis, and these contexts were further explored with *in vitro* studies.

Exposure of SCs to the matrix had a mild effect on their numbers and robustness; however, co-exposure to a necrotic stimulus greatly amplified myotube formation, evidenced by accelerated growth, increased myogenesis-associated transcripts, and the observation of early beating. Furthermore, under matrix and NMD co-stimuli, these SCs secreted paracrine factors, which on their own accelerated myogenesis when transferred to independent myoblast cultures. Together, these results suggest that the matrix is a powerful catalyst for SC maturation and myogenesis, but that may be most efficacious when it is deployed for disease states characterized by necrotic contexts.

This study offers broad implications: i) matrix treatment is able to augment myogenesis, but may mechanistically require a necrotic microenvironment; ii) under a necrotic context, the matrix is able to augment myogenesis via both SC differentiation and also paracrine effects; iii) rather than attempting to work around or control necrosis, necrotic stimuli may be exploited using biomaterial therapy in order to achieve functional results.

References:

(1) Kuraitis *et al*., *Eur Cell Mater* (2012) 24:175-95.

(2) Musaro & Barberi, *Method Mol Biol* (2010) 633:101-11.