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# Supplementary Materials for

## An Acellular Biologic Scaffold Promotes Skeletal Muscle Formation in Mice and Humans with Volumetric Muscle Loss

Brian M. Sicari, J. Peter Rubin, Christopher L. Dearth, Matthew T. Wolf, Fabrisia Ambrosio, Michael Boninger, Neill J. Turner, Douglas J. Weber, Tyler W. Simpson, Aaron Wyse, Elke H. P. Brown, Jenna L. Dziki, Lee E. Fisher, Spencer Brown, Stephen F. Badylak*

*Corresponding author. E-mail: badylaks@upmc.edu

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**The PDF file includes:**

## SUPPLEMENTARY MATERIALS

### Supplementary Methods

### Mouse model of VML

Scaffold preparation, surgical procedure, and tissue harvest were performed as previously described (*20*). Briefly, approval was obtained from the University of Pittsburgh Institutional Animal Care and Use Committee. Twenty-four female C57BL/6 mice were randomly assigned into either treated or untreated experimental groups. Both groups were subjected to a muscle defect consisting of unilateral resection of the tensor fascia latae quadriceps muscle. The VML defect accounts for a loss of approximately 90% of the fascia latae and 60% of the rectus femoris. In total, the defect represents a volumetric loss of approximately 75% of the quadriceps skeletal muscle compartment. This 75% loss is analogous to the VML injuries treated in the translational portion of the present manuscript. The defects within the treated group were filled with a biologic scaffold composed of ECM. Non-resorbable marker sutures were placed at the corners of the defect in both groups and were used to identify the defect margins. The time points for evaluation were 7, 14, and 180 days (n=4 per time point / group). Microscopic analysis included histochemistry and immunolabeling to examine PVSCs and skeletal muscle tissue.

### Physical therapy

*Screening.* The screening evaluation included a clinical examination performed by a licensed physical therapist (F.A., M.B.). A medical history was obtained and the subject

goals for participation in the study were documented. The greatest perceived functional limitations were identified. Active and passive ranges of motion were assessed using a goniometer, and strength of the affected region was assessed using a hand-held dynamometer. Functional assessments were established on a patient-by-patient basis and were aligned with the subject's self-reported greatest deficits and the clinical examination.

*Pre-surgical***.** Functional outcome variables were established *a priori* immediately following completion of the screening visit. Because each subject was unique in his clinical presentation, outcome variables that isolated tissue and functional deficits across specific joints were identified through a study team consensus. Outcome variables previously shown to be valid and reliable, and when possible aligned with the participants self-reported deficits were selected. This custom-designed pre-surgical rehabilitation protocol lasted 6-8 weeks, and only subjects who reached maximum strength and functional capacity upon completion of the pre-surgical physical therapy program, as determined by the treating physical therapist, proceeded to surgery.

*Post-surgical***.** Physical therapy that mimicked the pre-operative program was initiated within 24-48 hours post-surgery. Pain level, range of motion, strength and functional capacity were evaluated at each visit. Strength measures were assessed with a hand held dynamometer. Targeted exercises were performed within the subject's pain tolerance as early as the first postoperative day with the goal of stimulating muscle contraction and weight bearing across the scaffold implantation site. Subjects were instructed in a home exercise program, which they were encouraged to perform multiple times each day. Physical therapy visits were completed 3 times per week for the first 6

weeks post-surgery. The customized physical therapy program was then adjusted in frequency based upon the patients' functional improvement.

**Immunolabeling**

Frozen or formalin fixed tissue sections from the rodent study were fixed in ice cold 50:50 methanol acetone for 5 minutes, or 10% neutral buffered formalin for 18 hours respectively: at room temperature and washed in phosphate buffered saline (PBS). To reduce non-specific antibody binding, tissue sections were blocked in blocking buffer (1% (w/v) bovine serum albumin, 2% (v/v) normal horse serum, 0.05% (v/v) Tween-20, 0.05% (v/v) Triton X-100 in PBS) for 1 hour. Tissue sections were then incubated in primary antibodies diluted in blocking buffer with mouse on mouse blocking reagent (Vector Labs) according to manufacturer's protocol at 4°C for 16 hours. The primary antibodies used for the immunolabeling studies were: (i) mouse monoclonal CD146 (Abcam, Cambridge, MA) at a 1:350 dilution for identification of perivascular cells; (ii) rabbit polyclonal Neurogenin-2 (NG2, Millipore) at a 1:200 dilution for identification of perivascular cells; (iii) monoclonal anti-βIII tubulin (Thermo-Fisher/Pierce) at a dilution of 1:200 for identification of neurons; and (iv) monoclonal anti-desmin (Abcam) and (iv) monoclonal anti-sarcomeric myosin heavy chain (MHC) [Developmental Studies Hybridoma Bank (DSHB) at the University of Iowa] at 1:200 dilution for identification of muscle cells. After washing in PBS, tissue sections were incubated in fluorophore conjugated secondary antibodies (Alexa Fluor donkey anti-mouse 488 or 594 or donkey anti-rabbit 488, Invitrogen). After washing again with PBS, nuclei were counterstained with DAPI and slides were coated with anti-fade mounting media (Dako).

The human tissue biopsy samples were embedded in optimal cutting temperature compound (OCT, Sakura Finetek) and snap frozen in liquid nitrogen. Frozen tissue sections (5um) were fixed in ice cold 50:50 methanol:acetone for 5 minutes at room temperature and washed in PBS.

Tissue sections were blocked in blocking buffer [1% (w/v) bovine serum albumin, 2% (v/v) normal horse serum, 0.05% (v/v) Tween-20, and 0.05% (v/v) Triton X-100 in PBS] for 1 hour. The primary antibodies were applied, diluted in blocking buffer, at $4^{o}$C for 16 hours. The primary antibodies used for the immunolabeling studies were: (i) 1:350 mouse monoclonal anti-CD146 (Abcam); (ii) 1:200 rabbit polyclonal anti-Neurogenin-2 (NG2; Millipore); (iii) 1:200 488 conjugated goat polyclonal anti-von Willebrand Factor (vWF) (USBiological) and; (iv) 1:200 mouse monoclonal anti-desmin (Abcam) and (v) monoclonal anti-sarcomeric MHC (DSHB at the University of Iowa). After washing in PBS, the secondary antibodies were applied (Alexa Fluor donkey anti-mouse 488 or 594 or donkey anti-rabbit 488, Invitrogen). After washing again with PBS, nuclei were counterstained with DAPI and slides were coated with anti-fade mounting media (Dako).

Tissue sections were evaluated using a Zeiss Axio-observer Z1 microscope using a 20X, 0.4-NA objective with a 1.6X Optovar magnification changer (Carl Zeiss). A minimum of three fields of view from each of the surgical sites or biopsy samples were examined from each preclinical model ($n = 4$) and human patient ($n = 5$), respectively.

**EMG in the mouse model**

Mice were anesthetized with 2% isofluorane, and both the treatment area and the femoral nerve were exposed. A pair of fine wire electrodes (38 G, Teflon-coated with 1.5-mm

deinsulated tips) spaced 3-4 mm apart were placed around the femoral nerve approximately 1 cm rostral to the defect area, and used to evoke muscle twitch. A custom bipolar silicone epimysial electrode was placed within the defect via blunt dissection along the fascial plane dividing the tensor fascia latae and rectus femoris muscles. The silicone cuff electrically insulated the defect area from native muscle dorsal to the defect while the exposed electrodes remained in contact exclusively with remodeled tissue. The recording leads were spaced 1.5 mm apart along the rostrocaudal axis positioned in the center of the defect, and were grounded to the tail to reduce electrical noise and artifact.

CMAPs were evoked via stimulation of the femoral nerve. Symmetric biphasic voltage-controlled pulses with a 200 μs cathodic leading phase were applied at a rate of 3 pulses per second and a 20-second train duration (Model S88X Stimulator, Grass Technologies, Natus Neurology Inc.) for each stimulation event. Electrical responses within the defect were amplified (50X gain) and filtered (30 – 3000 Hz bandpass) prior to measurement (BMA 400 bioamplifier, CWE Inc.). A range of stimulation intensities between 0.3 and 8 V were applied in succession to characterize muscle fiber recruitment, and the range of stimulation intensities was repeated 2-3 times for each animal. The EMG response from each pulse train was averaged and recorded (Tektronix TDS 3012C Digital Phosphor Oscilloscope, Tektronix Inc.) for analysis of the peak-to-peak voltage ($V_{PP}$) and root mean square voltage ($V_{RMS}$) responses during a time window 1-8 ms following stimulation.

The muscle fiber recruitment curve (stimulation intensity vs. $V_{PP}/V_{RMS}$) was characterized by parameters that define a sigmoid function (*42*, *44*). The maximum peak-to-peak voltage ($V_{PP\text{-}max}$) and maximum RMS voltage ($V_{RMS\text{-}max}$) were defined by the

amplitude of the CMAP response during maximal, asymptotic stimulation. The stimulation threshold ($S_{thresh}$) was defined as the minimum stimulation voltage required to evoke a CMAP response, and the half-max stimulation ($S_{1/2}$) and maximum stimulation ($S_{max}$) were defined as the minimum stimulation voltages required to evoke one half of the maximal and the maximal CMAP response, respectively. The slope of the muscle recruitment curve was defined as the maximum linear slope in the peak-to-peak voltage/stimulation intensity relationship.

## Supplementary Figures

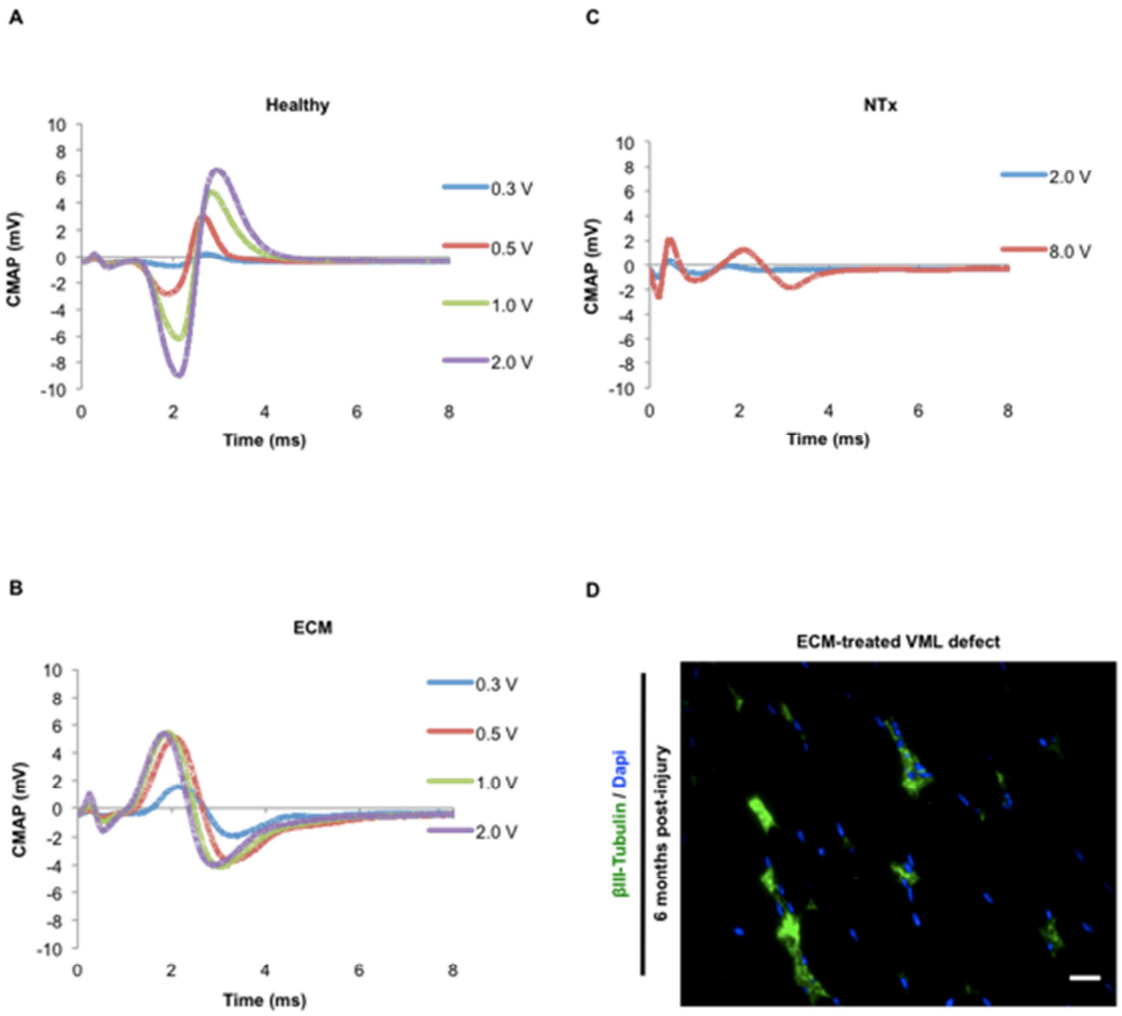


**Figure S1: CMAP at different stimulation intensities in mice**. (**A** to **C**) Representative waveforms for healthy, ECM-treated, and untreated (NTx) VML mouse model defects. The CMAP response begins after approximately 1 ms. (**D**) Representative image of β-III tubulin immunolabeling for neurons present within the ECM scaffold implantation site after 6 months. Scale bar, 50 µm.

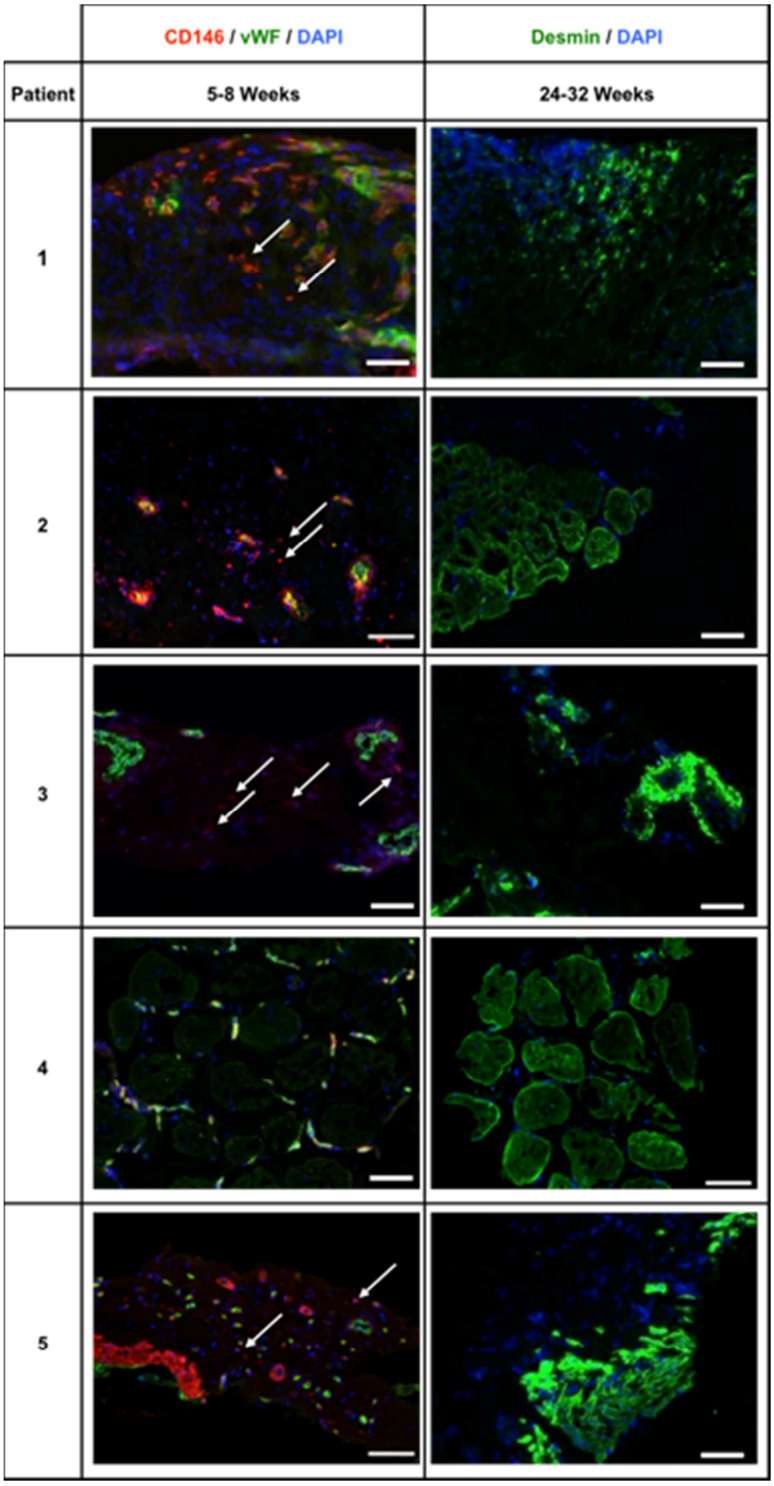


**Figure S2: Histomorphology in human subjects with VML.** Ultrasound-guided biopsies from each patient showing CD146+ PVSCs within and outside of (arrows) their typical perivascular niche 5-8 weeks after ECM scaffold implantation. Desmin+ muscle cells were present in biopsies from each patient 24-32 weeks after ECM scaffold implantation. Scale bars, 50 µm.

## SUPPLEMENTARY TABLES

**Table S1. Semiquantification of histological findings from the mouse model of VML.** The anatomic location of PVSCs and the presence of angiogenesis and muscle cells were identified at 7 days and 14 days post injury in untreated ($n$ = 4) vs. ECM treated ($n$ = 4) VML defects. The presence of muscle cells was also identified after 6 months in both treatment groups ($n$ = 4). Angiogenesis was evaluated by the presence of CD31+ vasculature structures. CD146+NG2+ PVSCs were identified independent of CD31+ vessels. Desmin+ and myosin heavy chain (MHC)+ cells identified the presence of skeletal muscle cells.

| | | 7 Days after injury | | | 14 Days after injury | | | 6 Months after injury |
|---|---|---|---|---|---|---|---|---|
| | Animal number | Angio-genesis | CD146+NG2+ cells independent of vessels | Desmin+/ MHC+ cells | Angio-genesis | CD146+NG2+ cells independent of vessels | Desmin+/ MHC+ cells | Desmin+/ MHC+ cells |
| Un-treated VML defects | 1 | +++ | - | - | ++ | - | - | - |
| | 2 | +++ | + | - | + | - | - | - |
| | 3 | ++ | - | - | +++ | + | - | - |
| | 4 | +++ | - | - | ++ | - | - | - |
| | | | | | | | | |
| ECM-treated VML defects | 1 | ++ | + | - | ++ | +++ | - | ++ |
| | 2 | +++ | - | - | +++ | ++ | - | ++ |
| | 3 | +++ | - | - | ++ | +++ | - | +++ |
| | 4 | +++ | - | - | +++ | +++ | - | ++ |

**Table S2. Patient exclusion and inclusion criteria.** Patients were screened against *a priori* established exclusion and inclusion criteria.

| Exclusion criteria | Inclusion criteria |
|---|---|
| Inability to provide informed consent | Age:<br>18 to 60 years and able to provide inform consent. |
| Poor nutrition | Subjects:<br>Military and civilian |
| Cancer diagnosis within last 12 months | Time since injury:<br>Within last 18 months. Target of 18 months or less, but subjects may be enrolled with injury outside this range if the PI determines there is viable muscle in the injured compartment by clinical exam and imaging studies. |
| Complete muscle/tendon gaps greater than 5 cm | Structural deficit:<br>25% (of muscle mass) |
| Infection | Functional deficit:<br>25% |
| Known coagulopathy | Injuries may encompass a single muscle belly or compartment. Whether an area is expected to be repaired by sutures will be determined from imaging studies and physical examination. |
| Diagnosis of schizophrenia or bipolar disorder | Eligible for study procedures 3 months post injury |
| Chronic disease such as congestive heart failure, liver disease, renal disease, or diabetes | Willing and able to comply with follow up exams, radiographic studies, physical therapy, muscle biopsy, and lab tests. |
| Active and unstable disease state or infection anywhere in the body per doctor's evaluation and determination | |
| Pregnancy | |
| Hypersensitivity to bovine serum or porcine products | |