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Appendix 1
Supplemental Materials and Methods

Mesenchymal Progenitor Cells (MPCs) harvest and culture. MPCs were harvested from traumatized muscle tissue of patients undergoing surgical debridements from penetrating wartime injuries as previously described [1,2]. Briefly, the healthy margin of the debrided tissue was minced and incubated with Collagenase type 2 [0.5 mg/mL] (Worthington Biochemical, Lakewood, NJ) for two hours at 37°C with agitation. Following incubation, the tissue was filtered through cell strainers (100μM and 40μM, Falcon/Corning, Corning, NY) followed by RNA extraction and MPCs isolation. For RNA extraction, cells were resuspended in TRIzol (Qiagen, Germantown, MD) for RNA isolation followed by real-time miRNA PCR array and qRT-PCR. For MPCs isolation, the cells were plated and incubated at 37°C for 2 hours, followed by culture of the adherent cells until confluence (~2-weeks). Isolated MPCs were used for downstream *in vitro* studies.

RNA isolation and real-time miRNA PCR array. RNA extraction was performed as previously described [3]. Briefly, RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA) following the manufacturer's instructions and purified using RNeasy Mini-columns (Qiagen). The miRNA expression was analyzed using Cell Differentiation and Development RT^2 PCR miRNA Arrays (Qiagen) containing 88 different miRNA sequences and using an ABI7900HT real-time RT-PCR system (Applied Biosystems, Foster City, CA). Data analysis was performed using the RT^2 Profiler PCR Array Data Analysis software (Qiagen). Downstream analyses were performed with Gene Ontology Consortium (http://www.geneontology.org) and Ingenuity Pathway Analysis (IPA, release 2012, Qiagen).

RNA-Seq analysis. RNA-Seq was performed using standard sequencing pipelines for sample preparation and analyses. Briefly, samples were prepared following TruSeq™ RNA Sample Preparation Guide (Illumina, San Diego, CA). Sequencing was performed at the Genome Analysis Core Facility, National Institute of Arthritis and Musculoskeletal and Skin Diseases, National Institutes of Health using an Illumina HiSeq 2500 sequencer, TruSeq SBS Kit v3-HS sequencing kit and TruSeq SR Cluster Kit v3-cBot-HS cluster kit. Sequences were demultiplexed and converted to FastQ based on lane number and index. Reads were mapped to the human genome (hg10) using Tophat, and results were used to create BAM files. Downstream analyses were performed with Partek software following standard RNA-Seq pipelines, including QA/QC analysis, mRNA quantification and definition of groups for comparison. The transcript RPKM data was transformed to Log2 with an offset of 0.001, ANOVA was performed for comparison of the groups on log transformed RPKM values, and q-value was added as False Discovery Rate. Ingenuity Pathway Analysis software (IPA, release 2012) was used for annotation of each transcript. Downstream analyses were performed with Gene Ontology Consortium (http://www.geneontology.org), Ingenuity Pathway Analysis (IPA, release 2012,

Qiagen), Jaspar (http://jaspar.genereg.net), Transfac (http://www.gene-regulation.com) and MicroRNA.org database (http://www.microrna.org).

Quantitative PCRs (qRT-PCRs). For mRNA gene expression analysis, RNA was extracted using TRIzol (Thermo Fisher Scientific/Invitrogen, Carlsbad, CA) following manufacturer's instructions and purified using RNeasy Mini-columns (Qiagen). RNA concentration was measured with a Nanodrop spectrophotometer (ThermoFisher Scientific) followed by cDNA synthesis performed with RT^2 PCR assay system (Qiagen). Relative gene expression analyses were performed for *RUNX2*, *ALP*, *Osteocalcin*, *Aggrecan*, *COL2A1* and *SOX9* by qRT-PCR with a QuantStudio 7 Flex real-time PCR detection system or an ABI7900HT real-time RT-PCR system (Applied Biosystems) as previously described [3,4]. Gene expression was normalized using *Beta-Actin* or *GAPDH* as housekeeping control genes [3]. For miRNA expression analysis, complementary DNA specific to each miRNA was generated from total RNA purified using RNeasy Mini-columns (Qiagen) using TaqMan™ MicroRNA Reverse Transcription Kit (Applied Biosystems) according to manufacturer's protocol and subjected to qRT-PCR reaction using TaqMan™ Universal Master Mix II, no UNG (Applied Biosystems) and specifc primers and probes (Applied Biosystems). Expression was normalized using *miR-103* as previously reported [5]. All reactions were performed in triplicate.

Osteogenic induction. Osteogenic differentiation was performed as previously described [1,2,6]. Briefly, cells were seeded at a density of 20,000 cells/cm^2 and cultured with osteogenic medium (DMEM with 10% FBS, supplemented with 10 nmol/L dexamethasone, 10 mmol/L ß-glycerolphosphate, 50 mg/ml ascorbic acid-2-phosphate and 10 nmol/L 1,25 dihydroxyvitamin D3 (Biomol International L.P., Plymouth Meeting, PA) for 1-, 2- or 3-weeks or using commercial osteogenic media (StemPro™ Osteogenesis Differentiation Kit, ThermoFisher Sicentific) for 1- or 2-weeks.

Alizarin red staining. Alizarin red staining was performed as previously described [2]. Briefly, after 2- or 3-weeks of osteogenic induction, cells were fixed with 60% Isopropyl alcohol and stained for 3 minutes with 2% (wt/vol) Alizarin Red S pH=4.2 (Rowley Biochemical, Danvers, MA) to detect mineralization.

Transfection with miRNA-mimics. Transfection of the miRNAs *miR-1*, *miR-206*, *miR-133a*, *miR-133b*, *miR-26a* was performed in MPCs using 25 nM mimic miRNAs (Sigma-Aldrich, St. Louis, MO) and Lipofectamine 2000 (Invitrogen) following manufacturer's instructions and compared to a negative control miRNA (Sigma-Aldrich). After transfection, cells were induced to osteogenic differentiation for 1-, 2- or 3-weeks followed by alizarin red staining or qRT-PCR analysis as described above.

Luciferase assay. Luciferase assay activity was performed 48 hours after co-transfection of MPCs with *miR-1* and *miR-206* mimic miRNAs (Sigma-Aldrich) and SOX9 3'UTR Lenti-reporter-Luciferase Vector [ratio 2:1 (miRNA mimics:3'UTR vectors)] (ABM, Richmond, BC, Canada). Co-transfection with a pLenti-UTR-Luciferase-Blank Vector (ABM) was used for comparison, and co-transfection of each luciferase vector with a negative control miRNA

(Sigma-Aldrich) was used as control. Luciferase activity was performed using the Luciferase Assay Kit (ABM) following manufacturer's instructions and luciferase signal was determined by luminometry using an Infinite 200 Pro Tecan plate reader (Tecan, Männedorf, Switzerland).

Western blot analysis. Total protein extracts were prepared using RIPA Lysis and Extraction Buffer supplemented with 1X Halt™ Protease Inhibitor Cocktail (both from ThermoFisher Scientific) and protein quantification was performed with a Micro BCA™ Protein Assay Kit (ThermoFisher Scientific) following manufacturer's instructions. Equal amounts of protein (20 µg) were separated by NuPAGE Novex 4% to 12% Bis-Tris gel electrophoresis and transferred using the iBlot Blotting System onto nitrocellulose membranes (Invitrogen). Blots were probed with antibody against human SOX9 (Cell Signaling, Danvers, MA) followed by anti-rabbit horseradish peroxidase-conjugated secondary antibody (EMD/Millipore/Sigma-Aldrich). Immunoreactive proteins were detected and visualized using Immobilon Western Chemiluminescent HRP Substrate (EMD/Millipore/ Sigma-Aldrich). All blots were probed with GAPDH (Cell Signaling) antibody as loading control.

Appendix References

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