Supplementary Online Content

Aksamitiene E, Baker AL, Roy S, et al. Biochemical effects of exercise on a fasciocutaneous flap in a rat model. *JAMA Facial Plast Surg.* Published online March 9, 2017. doi:10.1001/jamafacial.2016.2162

eMethods. Details of the Study Methods

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eReferences

This supplementary material has been provided by the authors to give readers additional information about their work.

eMethods

Sample preparation

The homogenates were collected into 2 mL Eppendorf tubes and spun down at 13,000×g for 10 minutes at 4°C to clear from debris and insoluble-material. The supernatant of lysate was stored in aliquots at •20°C until analyzed. Protein concentration was determined using the Pierce 660 nM protein assay kit (Pierce Biotechnology/Thermo Fisher Scientific, Rockford, IL) and adjusted to contain 3 μg/μl protein by adding extraction lysis buffer. Samples for electrophoresis were prepared by mixing 130 μL of total cell lysate with 20 μL 0.5 M DTT and 50 μL 4× LDS sample buffer (Thermo Fisher Scientific). Thus the final protein concentration in each sample was ~2 μg/μl. Samples for LDS•PAGE were prepared separately and then pooled if needed.

Electrophoresis

50 μg of protein was loaded and electrophoretically resolved by molecular weight in NuPAGE Bis•Tris 4%•12% gradient gels (Thermo Fisher Scientific) at 145V.

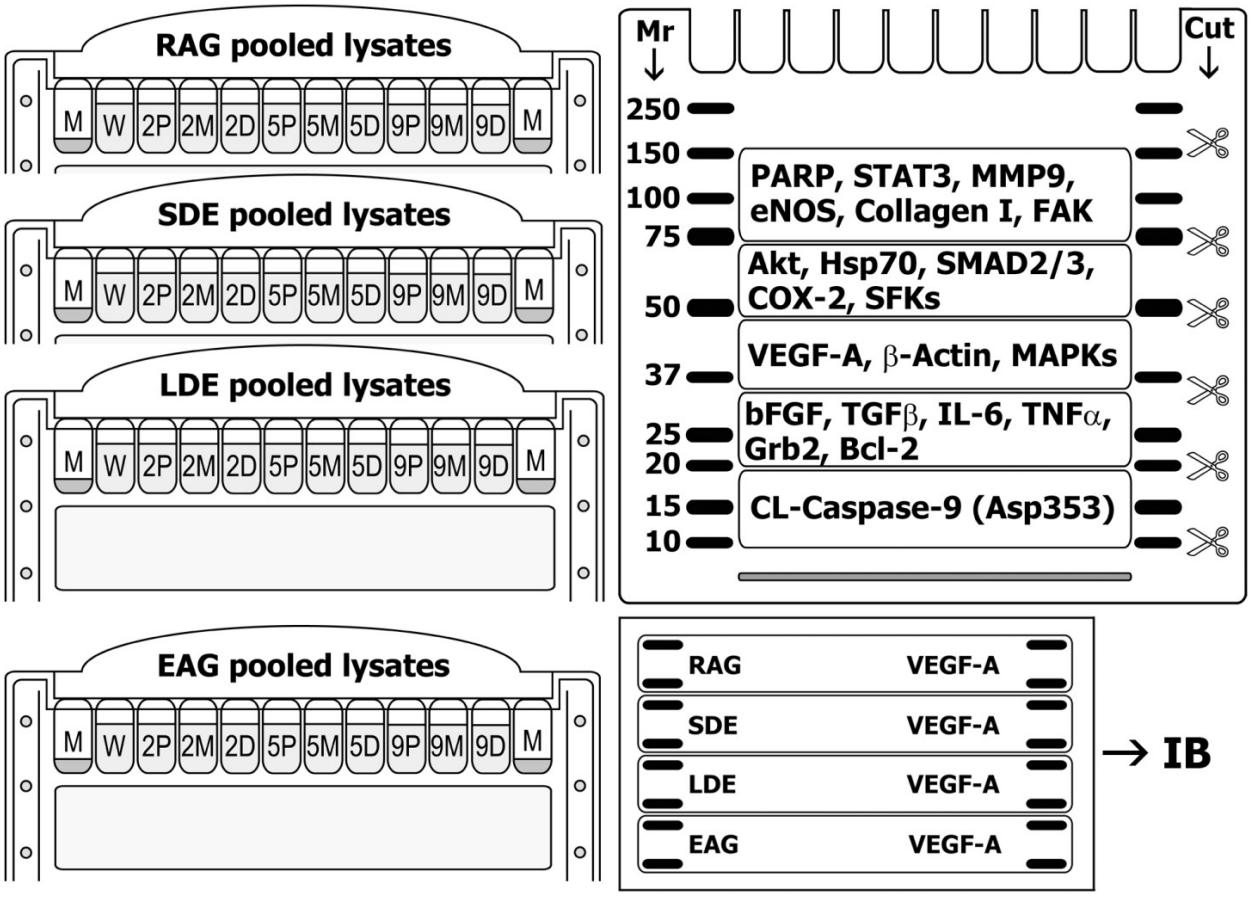
Immunoblotting

Electrophoretically separated proteins were transferred onto 0.22 μm nitrocellulose (NC) membranes (Bio-Rad Laboratories, Hercules, CA) at 30V for 90 min in XCell II Blot modules (Thermo Fisher Scientific) using either conventional immunoblotting (IB) or Multi•strip Western Blotting (MSWB), a technique designed to compare large numbers of samples on the same blot as described previously [1] (**eFigure 1)**. The following monoclonal (mAb) or polyclonal (pAb) primary antibodies were used in the study: rabbit anti•Cleaved Caspase•9 (Asp353) mAb, anti•Bcl•2 (clone 50E3) mAb, anti-β- Actin (13E5) mAb, anti-PARP antibody pAb, anti-phospho-Akt (Ser473) (D9E) XP® mAb, anti• phospho•Akt (Thr308) (D25E6) XP® mAb, anti•pan•Akt (C67E7) mAb (all purchased from Cell Signaling Technology, Danvers, MA and used at 1:1000 dilution), rabbit anti•VEGF•A (ab46154) pAb (Abcam, Cambridge, MA; used at 1:2000 dilution), mouse anti•Flk•1 Antibody (A•3) mAb, rabbit anti•phospho•Flk•1 (Tyr 951) pAb (which recognizes Tyr 947 in rat species), anti•Grb2 (C• 23) pAb (all obtained from Santa Cruz Biotechnology, Dallas, TX and used at or 1:500 dilution). Horseradish peroxidase•conjugated horse anti•mouse (Cell Signaling Technology) or goat anti•rabbit (Pierce Biotechnology/Thermo Fisher Scientific) secondary antibodies were used at dilutions 1:10,000 and 1:50,000, respectively.

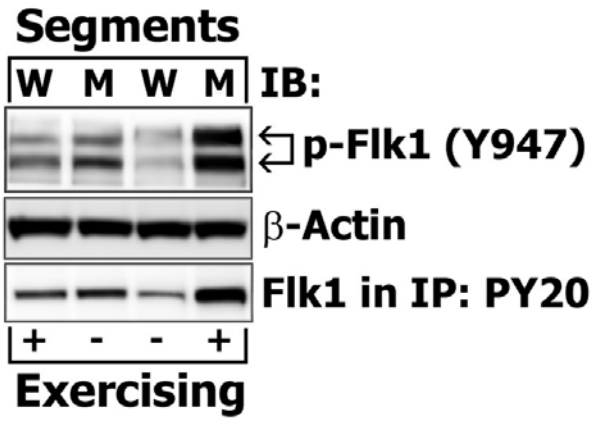
Immunoprecipitation

Tyrosine•phosphorylated proteins were precipitated from supernatants of pooled lysates using anti•Phosphotyrosine (PY20) agarose beads (Sigma•Aldrich, St. Louis, MO) as described previously [2]. Chemiluminescent signals of protein bands were detected using SuperSignalTM West Dura Extended Duration Substrate (Thermo Fisher Scientific) quantified densitometrically using KODAK Model 440CF Digital Science Image Station and dedicated software to obtain signal intensity values in arbitrary units (AU).

eFigure 1. Principles of Simultaneous Protein Analysis by Multistrip Western Blotting (MSWB)

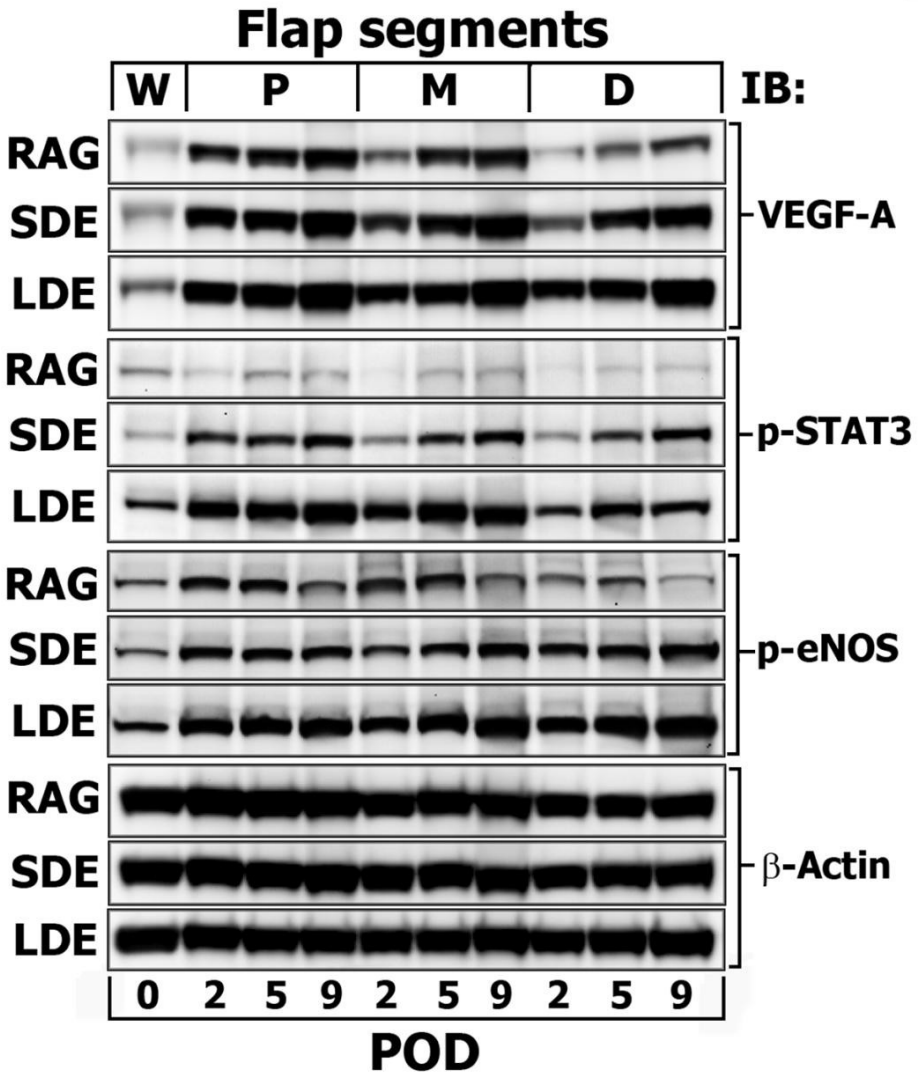


The lysates of the proximal (P), middle (M) and distal (D) flap segments or a whole tissue retrieved from the defect site (W) that were obtained from individuals within sedentary/resting animal group (RAG), exercising animal group (EAG), short distance traveled rat subgroup (SDE) and long distance traveled rat subgroup (SDE) at day 0 (W) or at 2nd, 5th and 9th post-operative days are pooled and electrophoretically separated in different 12-well gels along with protein standards marker (M). The areas containing an appropriate protein of interest (POI) of specific molecular weight (Mr) in the middle were excised from each gel. The gel strips were then aligned horizontally onto a filter paper, which was incorporated into a blotting sandwich. Thus each POI (e.g. 42 kDa VEGF-A) was transferred onto a single nitrocellulose membrane sheet, which was further subjected to regular immunoblotting steps (blocking, washing, incubation with primary and then secondary antibodies, immunodetection) under the identical conditions, enabling side-by-side comparison of signal intensities of protein bands.



eFigure 2. VEGFR Activation by Exercise

Pooled lysates of middle flap segments harvested from RAG (- exercising) and EAG (+ exercising) rats at POD 9 or POD 0 (W) were either subjected to electrophoresis and then immunoblotting (IB) with indicated antibodies or to immunoprecipitation (IP) using 50 µL mouse anti-phosphotyrosine (clone pY20) agarose-beads to isolate phosphotyrosine- harboring proteins and then subsequently detect phosphorylated Flk1 by IB.



eFigure 3. Impact of Cardiovascular Exercise Intensity on Time Course of VEGF, p-STAT3, and p-eNOS Expression in Different Flap Segments

Side-by side comparison of relative endogenous VEGF- A, p-STAT3 (Tyr705), p-eNOS (S1177) and β-Actin (control) protein levels among resting (RAG), short distance exercised (SDE) and long distance exercised (LDE) animal groups in proximal (P), middle (M), distal (D) segments of the flaps harvested on 2, 5 or 9 post-operative days (POD) and in a whole (W) skin tissue excised from each donor site on POD 0 (baseline). Tissue homogenates for each time point were pooled either from RAG or from SDE or LDE rats and subjected to LDS-PAGE followed by MSWB as described under "Supplementary Material" methods section.

eReferences

1. Aksamitiene E, Hoek JB, Kiyatkin A. Multistrip Western blotting: a tool for comparative quantitative analysis of multiple proteins. *Methods Mol Biol.* 2015;1312:197-226.

2. Aksamitiene E, Achanta S, Kolch W, Kholodenko BN, Hoek JB, Kiyatkin A. Prolactin-stimulated activation of ERK1/2 mitogen-activated protein kinases is controlled by PI3-kinase/Rac/PAK signaling pathway in breast cancer cells. *Cellular signalling.* Nov 2011;23(11):1794-1805.