Molecular Medicine

Supplemental Data

Transforming Growth Factor-β1 Downregulates Vascular Endothelial Growth Factor-D Expression in Human Lung Fibroblasts via the Jun NH_2-Terminal Kinase Signaling Pathway

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SUPPLEMENTARY MATERIALS AND METHODS

Cell Culture

Human umbilical vein endothelial cells (HUVECs) (Lonza, Walkersville, MD) were maintained in endothelial basal medium (EBM)-2 supplemented with fetal bovine serum (FBS) and growth factors in Endothelial Growth Media (EGM)-2 SingleQuot kit (Lonza). Cells were incubated at 37°C in a humidified 5% CO_2 atmosphere and used as positive controls for VEGFR-2 and VEGFR-3 expression.

ELISA

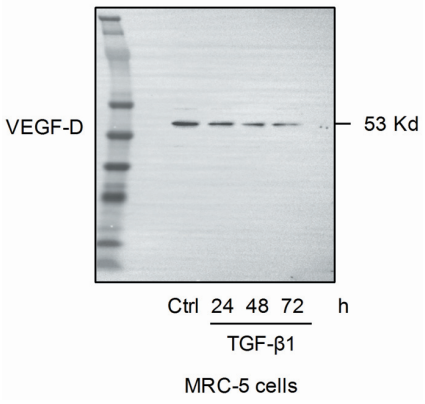
Culture supernatants from control siRNA- and VEGF-D siRNA- transfected cells were concentrated using standard acetone, trichloroacetic acid (TCA) precipitation protocols (1, 2) or Amicon Ultra centrifugal filter units (Millipore, Billerica, MA). The levels of VEGF-D in the concentrated samples were measured using a human VEGF-D DuoSet ELISA Development kit (R&D Systems) according to the manufacturer's recommendations.

Sircol Assay

Collagen concentration in culture supernatants from control siRNA- and VEGF-D siRNA- transfected MRC-5 cells was determined by Sircol assay (Biocolor, County Antrim, UK) according to the manufacturer's recommendations.

Cell Contraction Assay

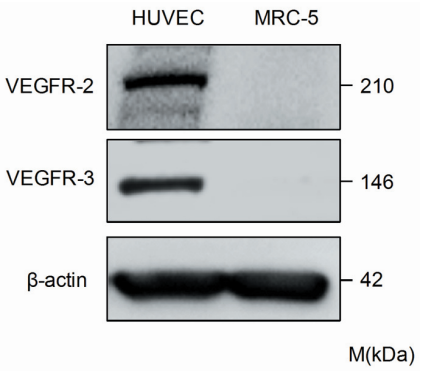
Control siRNA- and VEGF-D siRNA-transfected MRC-5 cells were resuspended and mixed with collagen gel solution (Cell Biolabs, San Diego, CA) at a concentration of 2×10^5 cells/ml. 0.5ml of the cell-collagen mixture was immediately added in 24-well tissue culture plates, incubated at 37 °C for 1 h, and then overlaid with culture media. The gels were subsequently released from the plates to initiate contraction. Images of gels were captured at 48h using ChemiDoc XRS+ imaging system (Bio-Rad) and analyzed using ImageJ software (NIH).



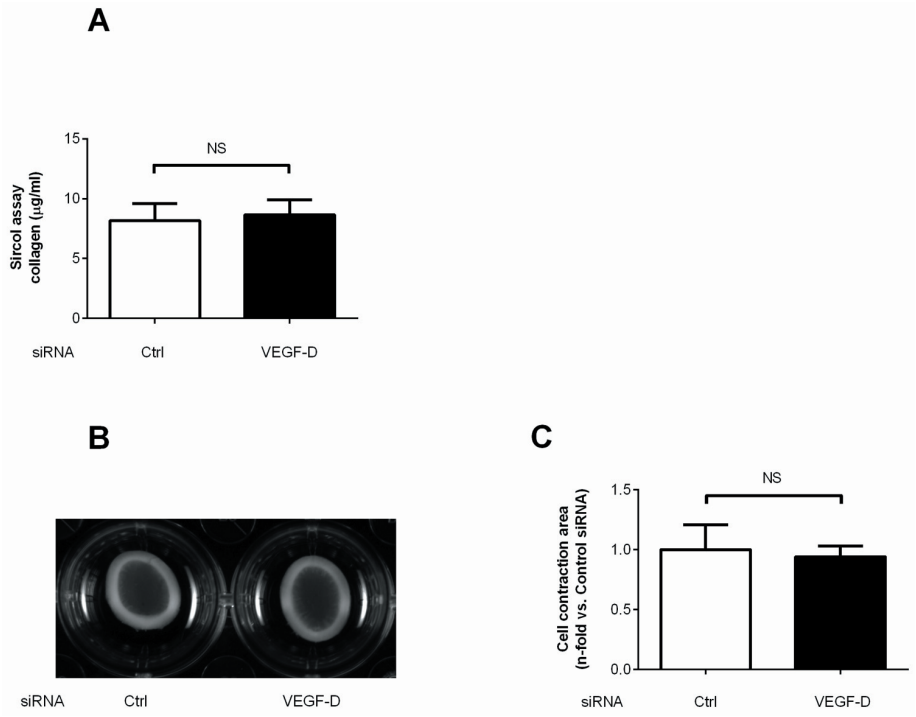
Supplementary Figure S1. Full-length unprocessed VEGF-D is the only VEGF-D isoform detected in MRC-5 cell lysates. MRC-5 fibroblasts were stimulated with TGF-β1 (5 ng/ml) for the indicated time. Equal amounts of protein from whole cell lysates were analyzed by western blotting with antibodies against VEGF-D. Results show a single immunoreactive band of ~53Kd consistent with the full-length unprocessed VEGF-D.

SUPPLEMENTARY REFERENCES

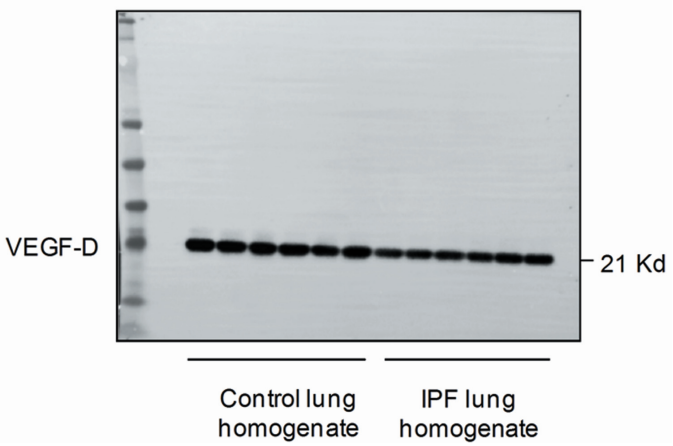
1. Hwang BJ, Chu G. (1996) Trichloroacetic acid precipitation by ultracentrifugation to concentrate dilute protein in viscous solution. *BioTechniques* **20:** 982-984.
2. Fic E, Kedracka-Krok S, Jankowska U, Pirog A, Dziedzicka-Wasylewska M. (2010) Comparison of protein precipitation methods for various rat brain structures prior to proteomic analysis. *Electrophoresis* **31:** 3573-3579.



Supplementary Figure S2. MRC-5 cells do not express detectable levels VEGFR-2 and VEGFR-3. Equal amounts of protein from MRC-5 and HUVEC whole cell lysates were analyzed by western blotting with antibodies against VEGFR-2 and VEGFR-3. β-actin was used as a loading control.



Supplementary Figure S3. VEGF-D knockdown in MRC-5 cells does not affect collagen synthesis and cell contractility. (A) MRC-5 cells were transfected with control siRNA or VEGF-D siRNA for 48 h. Collagen content in cell culture medium was measured using Sircol soluble collagen assay. (B) Effect of siRNA-mediated VEGF-D silencing on MRC-5 cells contractile efficiency in collagen gel. (C) Cell contraction was expressed as the fold of gel area relative to control siRNA transfection. Data represent mean ± SEM of three independent experiments. NS, not significant, by Student *t* test.



Supplementary Figure S4. Mature VEGF-D is the only VEGF-D isoform detected in human lung homogenates. Equal amounts of protein from human lung homogenates were analyzed by western blotting with antibodies against VEGF-D (n=6 samples per group), showing a single immunoreactive band at ~21Kd, consistent with the molecular weight of the C-and N-terminally cleaved VEGF-D.