ONLINE SUPPLEMENTAL METHODS

Title: Guidelines for the Isolation, Culturing and Characterization of Murine Vascular Smooth Muscle Cells: A report for the International Society of Cardiovascular Translational Research

Authors: Neeta Adhikari, Ph.D[1], Kadambari Chandra Shekar, M.S.[1], Rodney Staggs, M.S.[1], Zaw Win, B.S.[2], Kerianne Steucke, B.S.[2], Yi-Wei Lin, M.S.[3], Li-Na Wei, Ph.D[3], Patrick Alford, Ph.D[2], and Jennifer L. Hall, Ph.D[1]*

Address correspondence to:

Jennifer L. Hall, Ph.D.
Division of Cardiology, Department of Medicine
Lillehei Heart Institute, University of Minnesota
4-138 CCRB
2231 6th Street SE
Minneapolis, MN 55455
jlhall@umn.edu
Phone 612.625.4144
Fax 612.301.8298

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METHODS

Immunofluorescence and live cell imaging

VSMCs were stained for SM22α and Acta2 using anti-rabbit SM22α antibody (1:1000, Abcam) and monoclonal anti-Acta2 (1:1000, Clone 1A4, Sigma) overnight at 4°C followed by anti-rabbit Alexa Fluor -594 and 488 secondary antibodies, respectively, for one hour in the dark at room temperature. Nuclei were counter stained with DAPI. Images were taken with a 63x objective on a Zeiss Upright Microscope. Live images of VSMCs were taken with 20x and 40x objectives.

Western Blot

Western blotting was performed as previously described [1]. Membranes were probed with antibodies for sm22α (ab14106. Abcam, 1:2500, Secondary Anti Rabbit HRP 1:1000, Santa Cruz Biotechnology) and β-actin (monoclonal, Sigma, 1:5000, Secondary Anti mouse HRP, 1:1000, Santa Cruz Biotechnology). Densitometry was used to quantitate protein expression. Data shown as means ± SE and compared using Student's *t* test. A p value of <0.05 was considered significant.

Proliferation

Proliferation of VSMCs at P1 and P2 were assessed by the uptake of EdU according to the manufacturer's protocol (Click-iT kit, #10339, Molecular Probes). Briefly, VSMCs grown in 8-chamber slides were incubated with 10μM of EdU for the final 24 hours of 5 days of culture. Following fixation and permeabilization, the cells were stained with Alexa Fluor 594 azide and counter stained with DAPI. EdU positive cells were visualized under a Zeiss Upright microscope. Percent proliferation is represented as a ratio of EdU positive cells to the total number of DAPI positive cells. Cells were co-stained for sm22α to confirm VSMC morphology. Data shown as means ± SE and compared using Student's *t* test. A p value of <0.05 was considered significant.

Single cell RNA Sequencing

Cell suspension of VSMCs at P1 (5 days after isolation) and P2 (5 days after sub-culturing of P1) was submitted to the University of Minnesota Genomic Core facility for capturing on the 10-17 μm C1 chip. The selection of the C1 chip utilized in this study

was based on the average size of murine VSMCs (16.9 ± 0.6 μm) The cells were stained with green-fluorescent calcein-AM to indicate viability by intracellular esterase activity and red-fluorescent ethidium homodimer-1 to indicate loss of plasma membrane integrity (Cytotoxicity Kit, Invitrogen) prior to capture and visualized on a Zeiss Confocal microscope to assess the viability and number of cells in each well. 22 cells from the P1 cohort and 45 cells from the P2 cohort were processed for cDNA synthesis.

The concentration of cDNA in P1 cells ranged from 0.3-2.0 ng/μL and from 0.17-0.93 ng/μL in P2 cells. Integrity of cDNA in a subset of 10 randomly chosen samples from each cohort was assessed by electrophoresis on a Bioanalyzer (Agilent). The remaining single cell suspension after the captures was processed concurrently as bulk cell population control. The cDNA concentration of the P1 bulk population was 14.9 ng/μl while of P2 was 85.73 ng/μl. A total of 69 dual indexed RNA-seq libraries were prepared using Nextera XT reagents. The libraries were sequenced on a 50PE run on the HiSeq 2500. More than 120 million pass filter reads for the lanes were achieved and all barcodes were detected at reasonable levels. Average Q scores for all libraries were well above 30.

After passing an initial quality control with FastQC, sequences were aligned to the mouse GRCm38.75 genome using Tophat. A raw count of sequences aligning to known genes was taken with HTSeq, these were then globally normalized in R using the DESeq bioconductor library. Fluidigm's SINGuLAR analysis software was used to identify outliers prior to analysis.

To begin the analysis, outliers in both the cohorts were identified by utilizing SINGuLAR analysis and removed from any downstream analysis. Three single cell experiments were removed from the analysis.

Single Cell Contractility by traction force microscopy

Sample preparation: Polyacrylamide gels conjugated with FN were prepared as previously published[2]. Briefly, PDMS stamps were incubated with 50 µg/mL of FN for 1 hr. PDMS stamps were dried with air and placed in conformal contact with a plasma treated 15 mm glass coverslip for 30 min at 37 ˚C. A 5mL aliquot of 7.6 kPa PA gel solution (10/0.13% acrylamide/bis concentration) doped with 50 µL of 0.2 µm fluorescent microbeads were prepared. Prior to gel polymerization, 10 µL of TEMED and 50 mL of 1 mg/mL acrylic acid NHS-ester were added to the solution. 1 M HCl was added to the prepolymer solution to bring the pH to 7.0-7.4. Then, 25 µL of 10% w/v APS was added to initiate polymerization. A 10 µL droplet of the prepolymer solution was placed onto Bind-Silane treated 35mm glass-bottomed petri dishes. The FN coated 15 mm glass coverslips were then placed on the droplet. The gels were allowed to polymerize for 1 hr. After removing the glass coverslip, gels were passivated in 4% w/v BSA for 1 hr. Gels were stored at 4 ˚C overnight in PBS prior to cell seeding.

Traction force measurement: Murine VSMCs (P1 and P2) were seeded at a density of 2,000-5,000 cells per 35 mm dish and allowed to attach overnight in growth medium. Cells were then serum starved in serum-free medium for 24 hr prior to experimentation. Immediately prior to experimentation, the dishes were placed in 5 mL of Tyrode's buffer.

The dishes containing cells were allowed to equilibrate for 15 min in a temperature controlled microscope chamber at 37 ˚C. Bright field and fluorescent images were taken after equilibrium. Cells were allowed to relax by treatment with 100 μM of HA-1077, a Rho-kinase inhibitor, for 30 min. A set of brightfield and fluorescent images were taken after relaxation. Basal tone was determined by comparing images of fluorescent beads at equilibrium and after relaxation. Bead displacements were determined using an iterative particle image velocimetry (PIV) algorithm using 2.5 μm x 2.5 μm discretized windows [3].Traction stresses were determined using the same discretization from bead displacement data using an unconstrained FTTC algorithm [4]. Strain energy for each cell was determined as previously published [5], from measured bead displacement data and calculated traction stresses. A minimum of 23 cells were analyzed for each condition. P1 and P2 data were compared using a Mann-Whitney U test basal tone. A p value of <0.05 was considered significant.

References:

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