METHODS AND MATERIALS

Animals Seventy one TG Sprague-Dawley male rats with 40-fold cardiomyocyte-specific overexpression of the α_{1A}-AR[1], thirty TG FVB mice with 66-fold cardiomyocyte specific α_{1A}-AR overexpression[2, 3] and their respective NTLs were housed on a 12-hour light-dark cycle with standard chow and water. Development of the transgenic rats and mice was performed with approval of the St. Vincent's Hospital Animal Ethics Committee, whereas the breeding and the experiments performed at both Rutgers-New Jersey Medical School and Victor Chang Cardiac Research Institute were approved by their IACUC and all animals were maintained in accordance with the guidelines for the Guide for the Care and Use of Laboratory Animals (National Research Council, 8th Edition 2011). The *in vivo* and histology studies were performed in TG rats, whereas the *in vitro* cell culture experiments were studied in TG mice.

Myocardial infarction Coronary artery ligation was performed as previously described[1]. Briefly, male α_{1A}-TG rats and their NTLs, weighing between 300-450g, were treated with buprenorphine (0.05mg/kg s.c.) and cefazolin (50mg/kg i.m.), and were then anesthetized with sodium pentobarbital (60mg/kg i.p.). Rectal temperature was monitored and body temperature maintained at 37°C with an automatic heating lamp. After intubation of the trachea, rats were ventilated with a tidal volume of 0.5ml/kg at a rate of 90 breaths per minute, a left thoracotomy was performed at the fourth intercostal space and myocardial ischemia was induced by permanent ligation of the left anterior descending coronary artery (LAD). After completion of the ligation, the chest was closed and buprenorphine (0.05mg/kg s.c.) was given as post-operative medication. For the MEK inhibitor, U0126 (catalog# U-6770, LC Labs, MA), which was previously used at a different dosage of 1-400μg/kg/day via osmotic pumps[4-6], To ensure the full blockade of

MEK, a dose of 400μg/kg/day was chosen to be delivered via an osmotic pump subcutaneously from the day of LAD occlusion continuously for 4 wk. SU5416 (catalog# S8442, SigmaAldrich, MO), which has been tested at 25mg/kg per day before to fully block VEGF [6-9], was administered in both NTL and TG rats with same dose, i.p. for 4 wk.

Echocardiography At 4-6 wk after myocardial infarction, rats were anesthetized by injection of sodium pentobarbital, (60mg/kg i.p.). Transthoracic echocardiography was performed as previously described[10]. The animal body temperature was carefully monitored and maintained as close to 37°C as possible during the entire procedure. Using a VisualSonics Vevo 770 ultrasound system, LV internal dimensions were measured in systole and diastole using leading-edge methods and guidelines of the American Society of Echocardiography[11]. LV systolic function was estimated using the Vevo 770 software for calculation.

Myocardial blood flow assessment At 1 day, 1 wk, 2 wk and 4 wk after MI, myocardial blood flow was assessed using the microsphere technique. Briefly, TG rats and their NTLs were catheterized using sterile surgical technique as described previously[12]. An 8-inch in length and 0.023 in diameter (internal) fluid filled catheter (Braintree Scientific RPT 037) was inserted via the right carotid artery into the left ventricle, and a second catheter of the same size was inserted into the left femoral artery. After recovery from anesthesia, the rats received approximately 1,000,000 stable-isotope labelled color microspheres (Gold; BioPAL, Worcester, MA; catalog# C-10H10,15μm diameter) that were injected into the LV via an intraventricular catheter. A reference blood sample was collected simultaneously at a rate of 0.5mg/ml for 2 minutes. On completion of the protocol, animals were euthanized with a saturated KCl solution and the hearts collected. The LV was isolated and sliced in the short axis into 2 mm thick rings. The center of the ischemic zone was dissected and demarcated as the

infarct zone, whereas the tissue within 1.5mm distance from the edge of the infarct zone was collected and considered as the adjacent zone, and tissue within non-ischemic zone and directly opposite to the ischemic zone was considered as the remote zone. All individual heart samples, reference blood samples and both kidney samples were sent to the Biophysics Assay Laboratory (BioPAL, Worcester, MA) for microsphere analysis. The analysis with similar blood flow in both kidneys demonstrated adequate mixing of the microspheres. In addition, the statistical differences also confirm the accuracy.

Tissue preparation for capillary density To measure vessel density, heparinised animals were euthanized with an overdose of pentobarbital (120mg/kg i.p.) and then the ascending aorta was dissected and cannulated for perfusion of a vasodilation solution and a fixation solution[29]. Briefly, 10ml of 1 x Dulbecco's Phosphate-Buffered Saline (DPBS) with heparin (100U/ml), 10ml of vasodilation solution [1 x DPBS with adenosine (100mM) and sodium nitroprusside (10mM) and bovine serum albumin (0.05% (wt/vol)], and 10ml of fixation solution [(4% (wt/vol) paraformaldehyde solution)] were infused consecutively at a constant pressure of 80mmHg.

Histology staining Hearts were collected from TG and NTL rats and fixed in10% formalin. After embedding in paraffin, LV rings were sectioned at 5μm thickness and processed for the following:

Interstitial fibrosis - Tissue sections were stained with Picro-Sirius Red (PSR) to identify interstitial fibrosis deposition. The percentage of total fibrosis was quantified using ImagePro-Plus software, as previously described[30].

Scar size/viable tissue - Using PSR staining, scar size was assessed by quantification of the fibrotic area and calculated as a percentage of the whole myocardial area. Viable tissue was identified as the non-fibrotic area, which was quantified and expressed as the percentage of the ischemic zone.

Ki67 positive myocytes - Using Ki67 (Life Technologies, catalog#18-0192Z) and WGA double immunostaining, Ki67 positive myocytes were quantified and expressed as the percentage of total cardiomyocytes within the ischemic zone[31].

Cell size - Cardiomyocyte size was determined using rhodamine-conjugated wheat germ agglutinin (WGA), which stains all cell membranes, allowed for discrimination of myocytes and non-myocytes based on their size and phenotype. The circumference of each cardiomyocyte was traced and quantified using ImagePro-Plus software.

Capillary density - Using isolectin staining (isolectin IB4-Alexa448, catalog# I21414 Invitrogen), capillaries were identified as single endothelial cell layer with a diameter less than 25μm. Capillary density was quantified at 40x magnification as absolute number per unit myocardial area.

Angiogenic gene screening Microarray analysis was performed on cardiomyocytes isolated from TG and NTL rats to determine changes in gene expression of angiogenic markers[32, 33]. Upregulation of angiogenic genes was further confirmed by quantifying their mRNA levels using quantitative real-time PCR (qPCR).

Cardiomyocyte isolation Cardiomyocytes from both TG rats and 8-12 week old male mice and their corresponding NTLs were isolated using a modified Langendorff perfusion system. Briefly, the heart was first perfused with perfusion buffer (NaCl 120.4mmol/L; KCl 14.7mmol/L; KH2PO4 0.6mmol/L; Na2HPO4 0.6mmol/L; MgSO4▪ 7H2O 1.2mmol/L; NaHCO3 4.6mmol/L; Na-HEPES 10mmol/L; taurine 30mmol/L; BDM10mmol/L; glucose 5.5mmol/L) for 4min at 37°C at 3-4ml/min and then with enzyme buffer (perfusion buffer 50ml, containing collagenase II (catalog# LS004176, Worthington, NJ) 2.0mg/ml) for 12-15min. At the end of the digestion procedure, live myocytes were collected.

RNA extraction Cardiomyocytes were homogenized in Trizol (1ml/5 x 10^6 cells; Roche, Switzerland) and chloroform (0.2ml) was added. After vortexing and incubating at room temperature for 10min, samples were centrifuged at 12,000rpm at 4°C for 15min. Isopropanol (1.5ml) was added and the resulting mixture incubated at room temperature for 10min before centrifugation at 12,000rpm for 15min. The supernatant was removed and the pellet was washed with 1ml 75% ethanol. An additional centrifugation was performed at 10,000rpm at room temperature for 10 min and the resulting RNA pellet was dried and resuspended in nuclease free water. RNA quality was tested using NanoDrop (Thermo Scientific, DE) for the following criteria: concentration >1.0µg/µl; $A_{260/280}$ >2.0. RNA integrity was confirmed by electrophoresis, with a 28S:18S ratio of 2:1 being considered satisfactory for microarray gene analysis.

Tube formation assay Cardiomyocytes isolated from 8-12 week old male NTL or TG mice were cultured in the presence or absence of the α_{1A}-AR agonist, A61603 (25 nM). After 48 hours, the conditioned medium from cardiomyocytes was collected and centrifuged at 1000rpm for 10 minutes followed by filter sterilization using 100µm syringe filters. The conditioned medium was then aliquoted and stored at -80º.C until further use. For the tube formation assay, human umbilical vein endothelial cells (HUVECs) (catalog# CRL-1730™,ATCC, Manassas, VA) were used. Matrigel (reduced growth factor, BD Bioscience) was placed in 96-well tissue culture plates and allowed to polymerize at 37^0C for 30 min. HUVECs at a density of 5000 cells/well were then plated on Matrigel in the presence of either unconditioned medium or the cardiomyocyte conditioned medium. For VEGF-A inhibition, the cardiomyocyte conditioned medium was pre-treated for 1 hour with either a control IgG or anti-VEGF-A neutralizing antibody (catalog# sc-152, Santa Cruz) before being added to HUVECs. After 6 hours, the

tubules were imaged at 10x magnification followed by quantification. Data were expressed as the number of tubules in the conditioned medium normalized to that of unconditioned medium.

Quantification of VEGF-A expression levels Cardiomyocytes isolated from 8-12 wk old male TG mice of their NTLs were treated with α_{1A}-AR agonist, A61603 (25nM), and/or the α_1-AR inhibitor, prazosin (1μM), and/or the MEK inhibitor, U0126 (0.5μM). After 48 hours, RNA was extracted and VEGF-A levels were quantified relative to an internal control, HPRT (Hypoxanthine-guanine phosphoribosyltransferase) by qPCR. Cardiomyocytes were harvested in lysis buffer treated with DNase I and resulting RNA was reverse transcribed according to manufacturer's instructions using TaqMan gene expression Cells-to-C_t kit. (catalog# 4399002, Life Technologies). The mRNA was quantified using real-time qPCR (40 cycles at 15s step at 95ºC and a 1 min step at 60ºC) on a 7500 ABI-Prizm sequence detector (Applied Biosystems) using standard TaqMan probes (Applied Biosystems) for VEGF-A and HPRT. The conditioned medium from TG or NTL cardiomyocytes was collected and VEGF-A protein levels determined using ELISA (mouse VEGF Quantikine ELISA kit, catalog#DVE00, R&D systems).

Statistical Analysis: Data are expressed as mean ± SE. Statistical significance was determined using Student's *t*-test or ANOVA plus Bonferroni *post hoc* test evaluations, where appropriate. Comparison of hemodynamics of post-MI rats treated with MEK inhibitor, U0126, or the VEGF (Flk-1/KDR) receptor antagonist, SU5416, were determined using two-way ANOVA. $p<0.05$ was taken as a minimal level of significance.

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