Supplemental Methods

Materials and Regents. Recombinant human BMP-2, α-tubulin antibody, collagenase IA, protease inhibitor cocktail, GW9662, and phosphatase inhibitor cocktail I and II were purchased from Sigma-Aldrich. Rosiglitazone was obtained from Cayman Chemicals. Recombinant human VEGFA and PDGFB were bought from R&D Systems and Apelin-13 was from American Peptide Inc. PPARγ (E8) and apelin antibodies were obtained from Santa Cruz Biotechnology, Inc. Apelin and αSMA antibodies for immunohistochemistry were obtained from Abcam. β-catenin antibodies were purchased from Millipore. Anti-BMPR2 was from BD Biosciences, Protein G-sepharose beads, HRP-conjugated rabbit and mouse secondary antibodies, and ECL and ECL Plus kits were ordered from GE Healthcare. Caspase 3/7 assay kit was obtained from Promega. siRNA duplexes were purchased from Dharmacon. Magnetic beads (Dynabeads) for PMVEC isolation were purchased from Invitrogen and the CD31 antibody for mouse PMVEC isolation from BD Pharmingen. Nitro-fatty acids (NFA) synthesized according to (1) were obtained under an MTA from Dr. Bruce Freeman, University of Pittsburgh, Pittsburgh PA.

Cell Culture. Primary PAEC from large vessels (ScienCell) and human PMVEC (Lonza) were grown in commercial EC media supplied by ScienCell (Cat: 1001). We also isolated human PMVEC from control and IPAH patients (see below) and these cells were cultured under the same conditions as the commercially obtained primary ECs. Cells were subcultured at a 1:6 ratio in gelatin-coated dishes and flasks (BD Falcon and Corning) and used at passages 4–8. Cells were starved in basal media (ScienCell) with 0.5% FBS and 1% gentamycin/amphotericin overnight before adding the ligands or the vehicle. SF (0% FBS) conditions used the same culture media with only antibiotics added.

Isolation of Human and Mouse PMVEC. Mouse PMVEC were isolated by digesting whole lung tissue with collagenase IA (0.5mg/ml) for 45 minutes at 37∞C. The cell suspension was filtered through 70μm cell strainers, and then centrifuged at 250g for five minutes. The cell pellet was then washed three times with PBS and the cell suspension was incubation with sheep anti-rat IgG magnetic beads (Invitrogen; Cat: 110.35) coated with rat anti-mouse CD-31 antibody (BD Pharmingen; cat: 553370) to select out PMVEC for culture. Characterization of the cell culture after isolation was performed by labeling with Dil-conjugated Ac-LDL (Dil-Ac-LDL) and CD31 staining. Human PMVEC were isolated from fresh lungs from control and IPAH patients obtained through the PHBI Network (see below). Lung tissue was digested with collagenase IA (1.0mg/ml) for 1h and followed the mouse PMVEC protocol. Anti-human CD31-coated beads were used for EC purification (Invitrogen; Cat: 111.55D). To ensure the purity of the culture we re-purified these cultures with CD31 beads after first passage. Staining using Dil-conjugated Ac-LDL (Dil-Ac-LDL) and CD31 show over 95% purity for ECs. The expression analyses were done at passage 2.

Western Immunoblotting. For protein expression analysis, PAEC or PMVEC were washed with ice-cold 1xPBS, and lysates were prepared by adding boiling lysis buffer (10mM Tris HCl, 1% SDS, and 0.2mM PMSF) containing protease and phosphatase inhibitors. Lysates were scraped into a 1.5-ml microcentrifuge tube, and boiled for 10 min before centrifugation. Supernatants were transferred to fresh microcentrifuge tubes and stored at -80∞C. The protein concentration was determined by the Lowry assay (Bio-Rad Laboratories). Equal amounts of protein were loaded onto each lane of a 4–12% Bis-Tris gel and subjected to electrophoresis under reducing conditions. After blotting, polyvinylidene difluoride membranes were blocked for one hour (5% milk powder in 0.1% PBS/Tween) and incubated with primary antibodies overnight at 4^{o}C. Binding of secondary horseradish peroxidase (HRP)-conjugated-antibodies was visualized by ECL or ECL Plus. Normalization for total protein was performed by re-probing the membrane

with a mouse monoclonal ab against α-tubulin. The antibody concentrations used were: apelin (1:200), β-catenin (1:1000), BMPR2 (1:250), PPARγ (1:200) and α-tubulin (1:5000). Secondary antibodies were used with concentrations 1:2000-1:10000.

Co-IP. At harvest PAEC were washed with ice-cold PBS, and lysates were prepared by scraping the cells in RIPA buffer (50mM HEPES, 300mM NaCl, 5mM $MgCl_2$, 1% NP-40, 1.2 mM EDTA) containing protease and phosphatase inhibitors. After centrifugation supernatants were transferred to fresh microcentrifuge tubes and stored at -80^{o}C. The protein concentration was determined by the Lowry assay (Bio-Rad Laboratories). We used 300μg of protein for Co-IP and 40μg of same lysate for the loading control. Protein lysates for IP were first cleared with protein G-sepharose beads (GE Healthcare) and after removal of the beads, lysates were incubated with antibodies overnight at 4^{o}C. G-sepharose beads were added and incubated for two hours before washing. After the last wash, the beads were resuspended in lysis buffer and used in western immunoblotting assays.

ChIP Assay. In all the ChIP experiments we used 10x10^{6} PAEC per sample. Antibodies against PPARγ (Santa Cruz; sc-7273X) or β-catenin (UPSTATE; Cat 06-734) were used for ChIP. The Farnham protocol, found on the website *http://www.genomecenter.ucdavis.edu/farnham/*, was used for all ChIP sample preparations. Standard PCR reactions using 4μl of immunoprecipitated DNA were performed to validate PPARγ-βC complex formation on the *apelin* promoter. PCR primers used: fwd: AATAGGGCGGAGGGAAAG and rev: TGCTCTGGCTCTCCTTGAC. PCR products were separated by 2% agarose gel electrophoresis and visualized by ethidium bromide. For ChIP-chip the immunoprecipitated DNA was amplified using the Whole Genome Amplification Kit (Sigma).

ChIP-chip Assay. Promoter tiling arrays were produced by Roche NimbleGen. In this study we used the 385K 2-array promoter set (Cat:05224136001; build HG18). This array system contains all annotated splice variants and alternative transcription start sites and covers approximately 4250bp promoter sequences, i.e., 3500bp upstream and 750bp downstream of the transcription start site. These arrays were hybridized and the data extracted according to standard operating procedures of NimbleGen Systems Inc. Signal map software, Nimblescan 2.5 was used to visualize and analyze the detected peaks.

RNAi. To achieve gene knockdown, siRNA duplexes specific for βC (L-003482-00; Dharmacon); BMPR2 (L-005309-00; Dharmacon), apelin (L-017023-01; Dharmacon) or non-targeting siRNA as control (D-001810-01, Dharmacon) were transfected into PAEC using lipofectamine 2000 (Invitrogen) as described (2). Knockdown efficiency for β–catenin and BMPR2 was determined in our previous reports (3). Apelin knock down was determined by qT-PCR and western immunoblotting (Supplemental Figure 2).

Gene-Expression Microarray Analysis. We isolated 250ng of mRNA from control, BMPR2 or β-catenin siRNA treated PAEC, using the Illumina Human Ref-12 BeadChip (Illumina, Inc.) whole- genome gene expression array analysis. Two samples were used per condition and each sample was hybridized on two arrays producing four hybridizations per condition. Each RNA sample was amplified using the Ambion Illumina RNA amplification kit with biotin UTP labeling. The Ambion Illumina RNA amplification kit uses the T7 oligo(dT) primer to generate single stranded cDNA followed by a second strand synthesis to generate double-stranded cDNA, which is purified. In vitro transcription is then carried out to synthesize biotin-labeled cRNA using T7 RNA polymerase. The cRNA is then purified, weighed and 750ng used to hybridize each array following standard Illumina protocols in which streptavidin-Cy3 is used for detection. Slides are scanned on an Illumina Beadstation and analyzed using BeadStudio (Illumina, Inc). Microarray data analysis is performed using Significance Analysis of Microarray (SAM) (4) with

a two class unpaired approach to compare expression data between control and siRNA groups. The false discovery rate (FDR) was calculated using 1000 permutations. Gene fold changes were calculated by taking the average of the four hybridizations for each condition. Fold changes of 1.5 or greater were selected for further analysis. FDR cutoffs 10% and 20% were used to generate gene lists for the gene ontology analysis and combined ChIP-chip analysis, respectively. Functional annotations were performed using the program DAVID 2008 (http://david.abcc.ncifcrf.gov/) with the Gene Ontology Biological Process terms database.

mRNA Expression by qRT-PCR. The following pre-verified Assays-on-Demand TaqMan primer/probe sets (Applied Biosystems, Foster City, CA) were used: apelin (Hs00936329_m1;Mm00443562_m1), BMPR2 (Hs00176148_m1), ADM2 (Hs00363866_m1), CD31 (Hs00169777_m1), CD34 (Hs00990734_g1), PGF (Hs00182176_m1), SOX-18 (Hs00746079_s1), SMURF2 (Hs00224203_m1), VASH (Hs00208609_m1) and 18s (Hs03003631_g1).

Immunohistochemistry. Sections from folmaldehyde-fixed and paraffin embedded lung tissues were deparaffinized and rehydrated. Epitope retrieval was performed by boiling the sections in citrate buffer, pH 6.0. Sections were reacted with hydrogen peroxide to block endogenous peroxidase, washed and blocked with 1% goat serum. 100μl of anti-apelin ab (1:100;Abcam) were preincubated with 100μl of apelin peptide (10μg/100μl; American Peptide Inc.) or 100μl of PBS overnight at 4^{o}C. The sections were then incubated with these primary ab solutions (1:200) overnight at 4^{o}C. After streptoavidin-biotin amplification (LSAB2+ kit DAKO), the slides were incubated with 3, 3'-diaminobenzidine and counterstained with hematoxylin. The localization of altered immunoreactivity was noted by two independent examiners blinded to the diagnosis of PAH. Anti-αSMA ab (Abcam) was used in mouse lung sections and IHC was performed as above.

EC Survival, Proliferation and Migration Assays. PAEC or PMVEC survival was assessed by cell counts or MTT assays. For cell count experiments, ECs were seeded at $25x10^3$ ECs per well in a gelatin coated 24-well plate in 500μl volume of growth media and allowed to adhere and recover for 24h. Cell attachment was over 90% and identical in all tested wells. Cells were washed three times and then starved under SF conditions for 24h in the presence or absence of different ligands mentioned in the Figure Legends. Cells were trypsinized in 100μl volume and trypsin was blocked by adding 100μl of serum. Then the cell density (cells/ml) in the 200μl solution was counted in a hemocytometer (Bright-Line; Hausser Scientific). For the MTT assay, we plated 5000 ECs per well in a 96-well plate, 6 wells per condition. The MTT assay was done following the protocol of the manufacturer (ATCC). Cell apoptosis was assessed by the Caspase 3/7 assay (Promega) using cells plated as in our MTT assays. Caspase 3/7 activity was assessed after 12 or 24h of SF condition in the presence or absence of different ligands (3). At the time of harvest cells were incubated for 1h in 100μl of Caspase 3/7 Luciferase Reagent Mix (Promega), and total luminescence was measured in a 20/20 luminometer (Turner Biosystems, Inc). PAEC proliferation was assessed by cell counts and MTT assays. Cells were seeded as above and allowed to adhere for 24h. Cells were washed three times and then incubated in low serum (0.5%FBS) overnight. In these low serum conditions quiescent ECs were then stimulated with various ligands for 24h and cell numbers were analyzed as above. To assess cell migration, we used the modified Boyden chamber assay. Cells were added to gelatin-coated microporous inserts in 24-well plates, and the migratory stimulus was added to the well in the bottom of the chamber. The cells that had migrated through the bottom of the insert six hours later were fixed and stained with the Diff Quick Kit. The cells in three different fields (200x) at the center of each well were counted under the microscope and an average obtained.

PASMC Proliferation and Apoptosis Assays. Cell count and MTT assays were used to evaluate cell proliferation. For both assays, the same number of cells was used per well as described

above in experiments using PAEC. After seeding, the cells were allowed to adhere for 24h. The PASMC were then washed three times and starved in low serum (0.1%FBS) for 48h. These quiescent cells were then stimulated with different ligands or conditions, mentioned in figure legends. Proliferation was measured by cell counts and MTT assays and apoptosis by the Caspase 3/7 assay following the protocol used for PAEC with the exception that the assay was conducted after a longer, 48h treatment period in the absence or presence of apelin in SF medium.

Experimental Design to Assess the Effect of Apelin Administration on PAH in TIE2CrePPARγ$^{flox/flox}$ Mice. 12-15 week old WT and *TIE2CrePPARγ$^{flox/flox}$* mice were treated with daily i.p. injections of either vehicle (PBS) or apelin (200μg/kg) for 14 days. Cardiac function and output were measured after 12 days of apelin or vehicle treatment by echocardiography under isoflurane anesthesia (1%, in 1L O_2/min) using a Vivid 7 ultrasound machine (GE Medical Systems) and a 13-MHz linear array transducer. The RVSP was measured under isoflurane anesthesia (1.5% in 2L O_2) by inserting a 1.4F catheter (Millar Instruments) via the right jugular vein as described previously (5). The RV mass was measured by the weight of the RV relative to LV plus septum as described previously (6). Lungs were perfused with normal saline, fixed in 10% formalin overnight, and then embedded in paraffin for routine histology (H&E, Movat pentachrome), as previously described (5, 6). A subset of left lungs was injected with barium-gelatin via the pulmonary artery–to identify peripheral pulmonary arteries for morphometric analysis. Barium-injected, transverse left lung sections were stained using the Movat pentachrome method. From all mice, we took the same full thickness section in the mid-portion of the barium-injected left lung parallel to the hilum and embedded it in the same manner. Pulmonary arterial muscularization was assessed at x400 magnification by calculating the proportion of fully and partially muscularized peripheral (alveolar wall and duct) PAs to total peripheral PAs in 5 random fields. All measurements were carried out by investigators blinded to the genotype and experimental condition.

References:

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Supplemental Table 1:

Gene ontology analysis of gene-expression changes after loss-of BMPR2

Category	P value
Anatomical structure development	5.0E-6
Wound healing	2.0E-5
Angiogenesis	4.4E-4
Vascular development	9.1E-4
Cell differentiation	4.9E-3

Supplemental Table 2:

Gene ontology analysis of gene-expression changes after loss of β-catenin

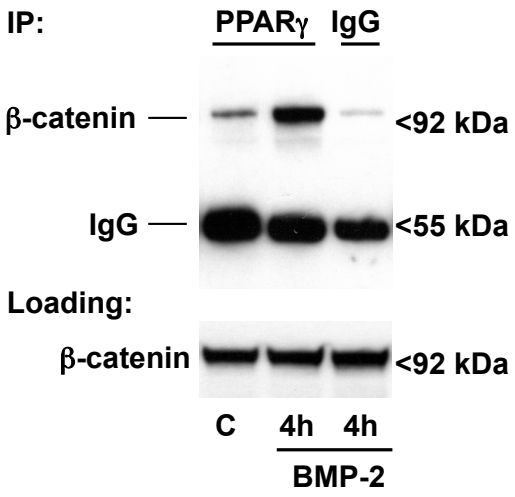
Category	P value
Anatomical structure development	1.9E-5
Cell proliferation	1.4E-4
Angiogenesis	8.9E-4
Vascular development	1.4E-3
Cell-matrix adhesion	1.7E-3
Cell differentiation	2.3E-3
Cell motility	3.4E-3

Supplemental Table 3:

Genes selected for qRT-PCR confirmation of gene-expression microarray data

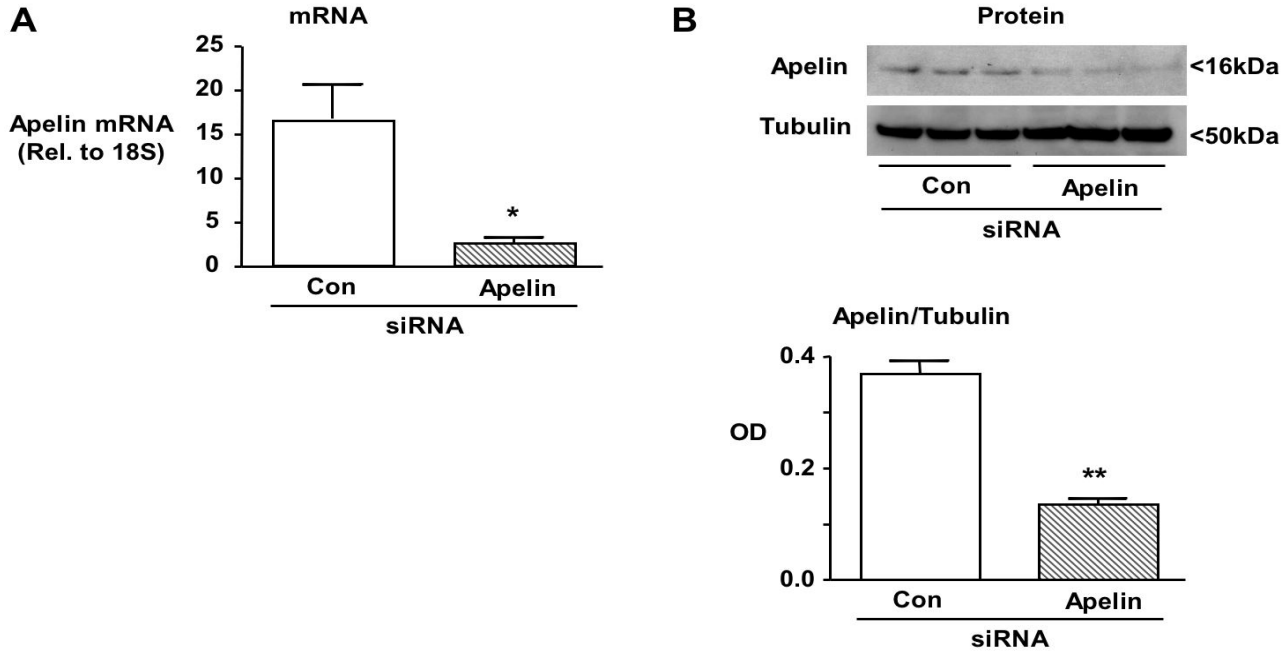
Gene	Definition	BMPR2 / Con siRNA, log	BMPR2 / Con siRNA, q%	β-catenin / Con siRNA, log	β-catenin / Con siRNA, q%
ADM2	Adrenomedullin 2	0.81	1.52	-1.02	2.48
CD31	PECAM1	-0.66	5.23	-0.36	4.60
CD34	CD34 antigen	-1.72	5.23	-1.91	1.15
PGF	Placental growth factor	-1.50	2.36	-1.43	0.94
SOX18	SRY-box 18	-1.41	6.72	-1.53	1.89
SMURF2	SMAD specific ubiquitin ligase 2	1.18	31.81	1.12	9.71
VASH1	Vasohibin 1	-0.81	12.61	-1.14	1.89

Supplemental Table 3: List of genes that were selected for qRT-PCR confirmation of gene-expression microarray data (see also Figure 4). These genes showed, at least in one siRNA condition, a significant >1.5-fold change in gene expression (log2 space on table) and showed, at least in one condition, q<10% based on SAM analysis.



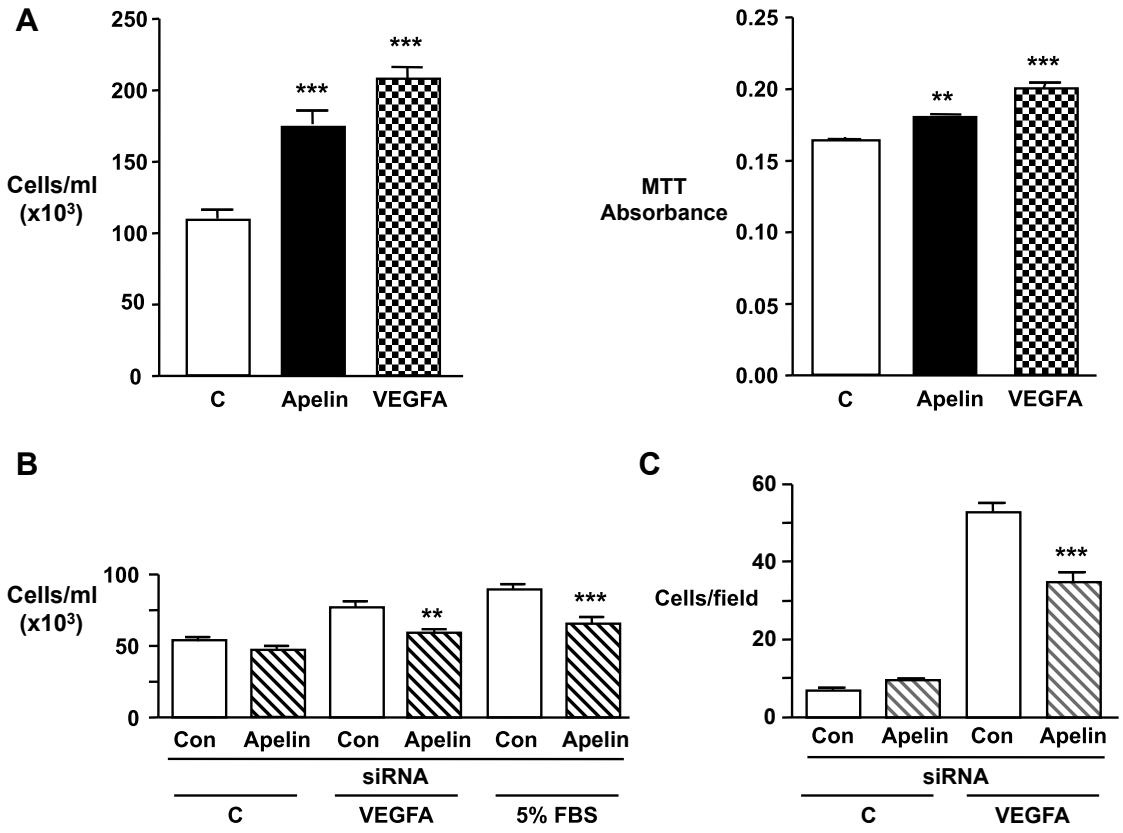
Supplemental Figure 1: Western immunoblot showing β-catenin levels associated with PPARγ in vehicle and BMP-2 treated PAEC.

IgG control is included to show specificity of co-IP with PPARγ antibody.



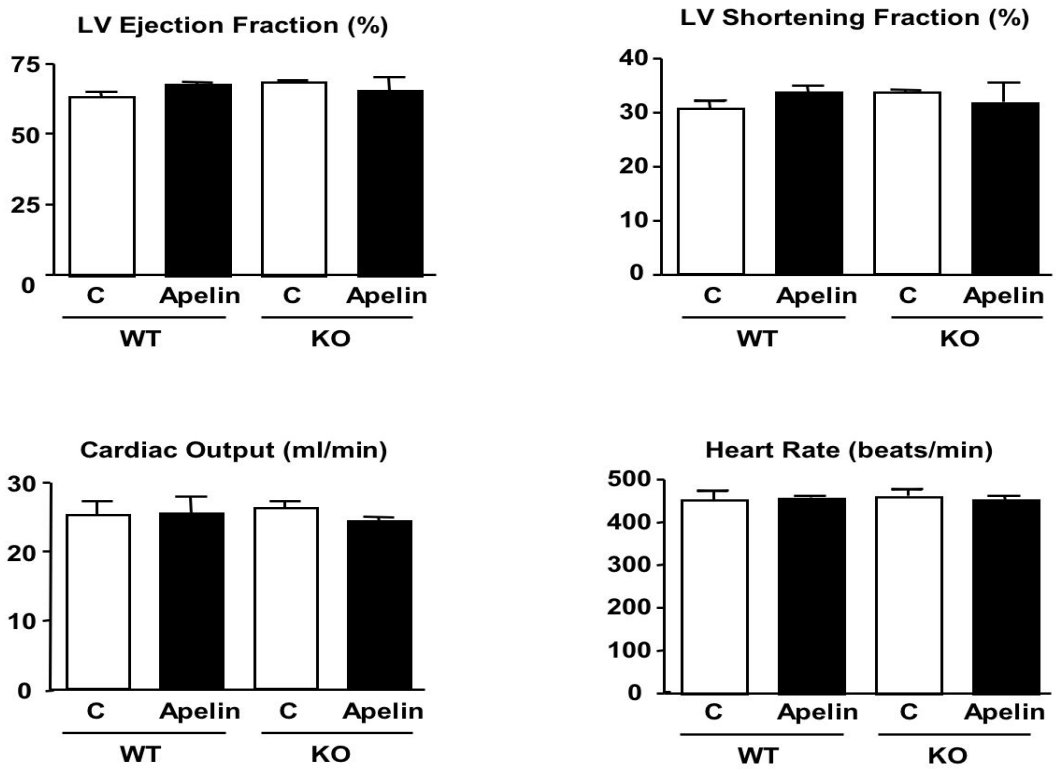
Supplemental Figure 2: Assessment of apelin knock-down in PAEC.

Non targeting (Con) or apelin siRNA were transfected in PAEC and knock-down efficiency assessed. Cells were harvested 36 h after transfection for RNA **(A)** and protein analysis **(B)**. Taqman qRT-PCR shows ~75% knock down in apelin mRNA expression with apelin siRNA and western immunoblot analysis confirms ~60% knock down in apelin protein. *P<0.05, **P<0.01, vs. Control siRNA (Con siRNA) by Student's T-test.



Supplemental Figure 3: Apelin promotes PAEC proliferation and migration.

(A) PAEC were treated in 0.5% FBS (low serum) with vehicle (C), apelin (100nM) or VEGFA (50ng/ml) for 24 h. Cell proliferation was analyzed by cell count or MTT assay. **(B)** Non-targeting (Con, white bar) or apelin siRNA (striped bar) transfected cells were stimulated with 5% FBS or VEGFA (50ng/ml) and cell count was used to analyze the proliferation rate of PAEC after 24h. **(C)** For migration assays, cells were transfected as in **B**. Thirty-six hours after transfection 40,000 cells were placed in a Boyden chamber to measure migration induced by vehicle, (C) or VEGFA (50ng/ml). Bars represent mean ±SEM from 3 separate experiments with 3 replicates per condition for cell count and migration assays, and 6 replicates for MTT assay. **P<0.01, ***P<0.001 vs. Control (C) in **A**, or vs. the respective control siRNA (Con siRNA) in **B**; One-way ANOVA with Bonferroni's multiple comparison test.



Supplemental Figure 4: Cardiac Function of TIE2CrePPARγ$^{flox/flox}$ mice.

Cardiac echocardiography was used to evaluate LV function (ejection fraction and shortening fraction), cardiac output and heart rate of wild type (WT) and *TIE2CrePPARγ$^{flox/flox}$* mice (KO). At the time of cardiac echocardiogram, mice were treated with vehicle (C; PBS) or apelin (200μg/kg) for 12 days. Bars represent mean ±SEM from 3 separate mice/group. No statistically significant differences were observed.