

SUPPLEMENTAL MATERIALS

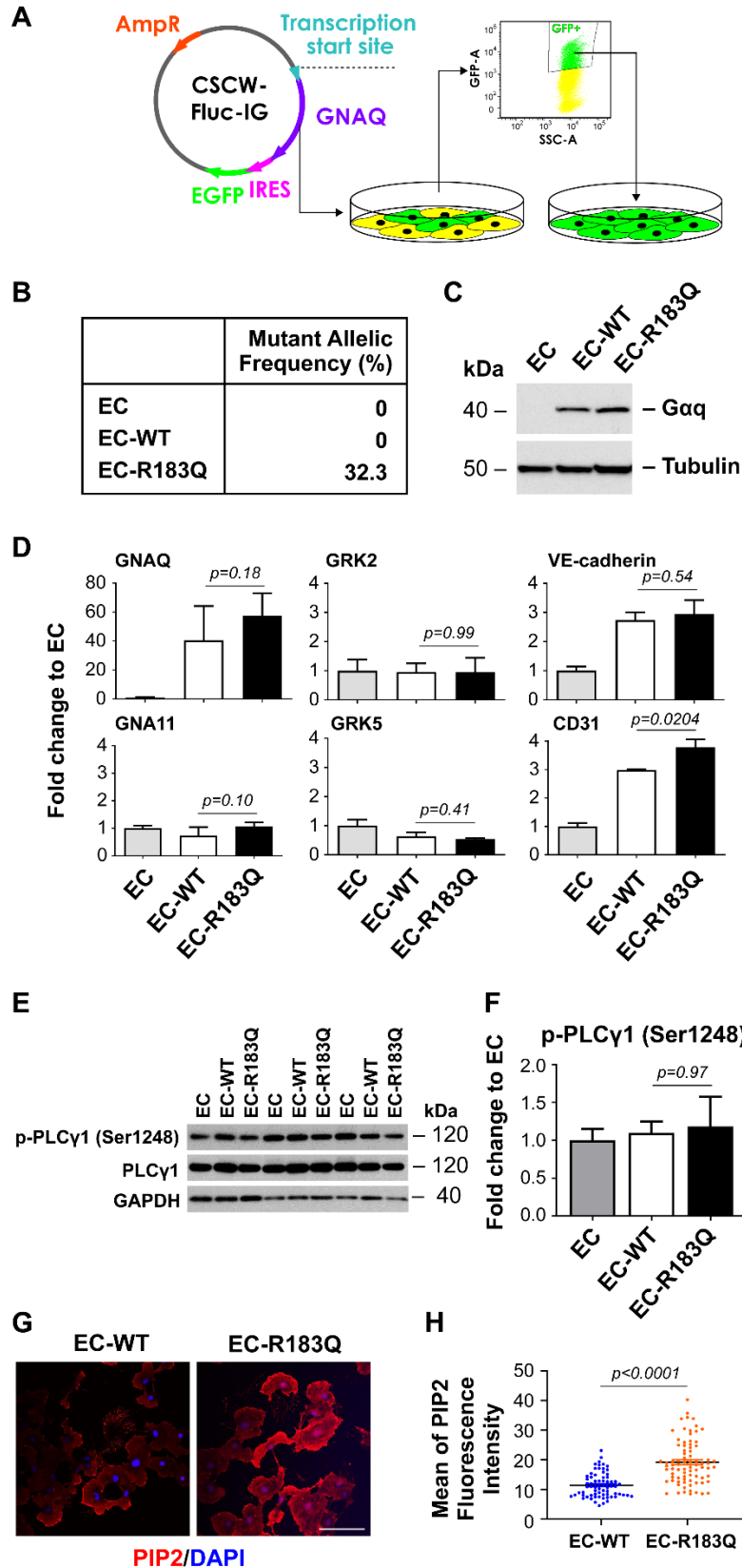
Title: Endothelial GNAQ p.R183Q increases angiopoietin-2 and drives formation of enlarged blood vessels

Running Title: Increased PLC β 3 activity and angiopoietin-2 in GNAQ p.R183Q endothelial cells

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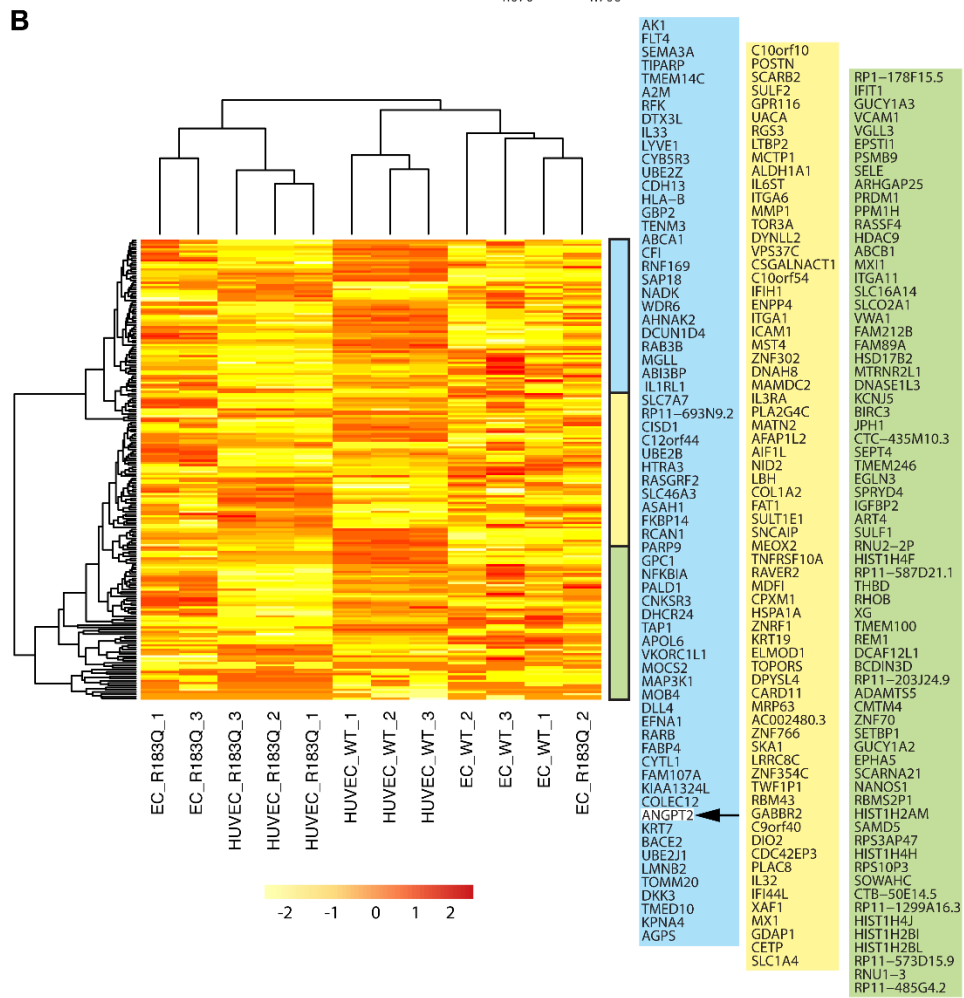
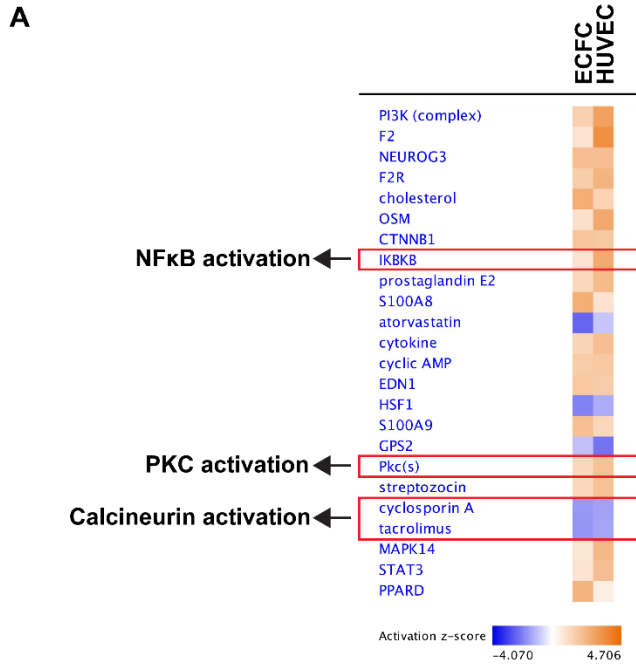
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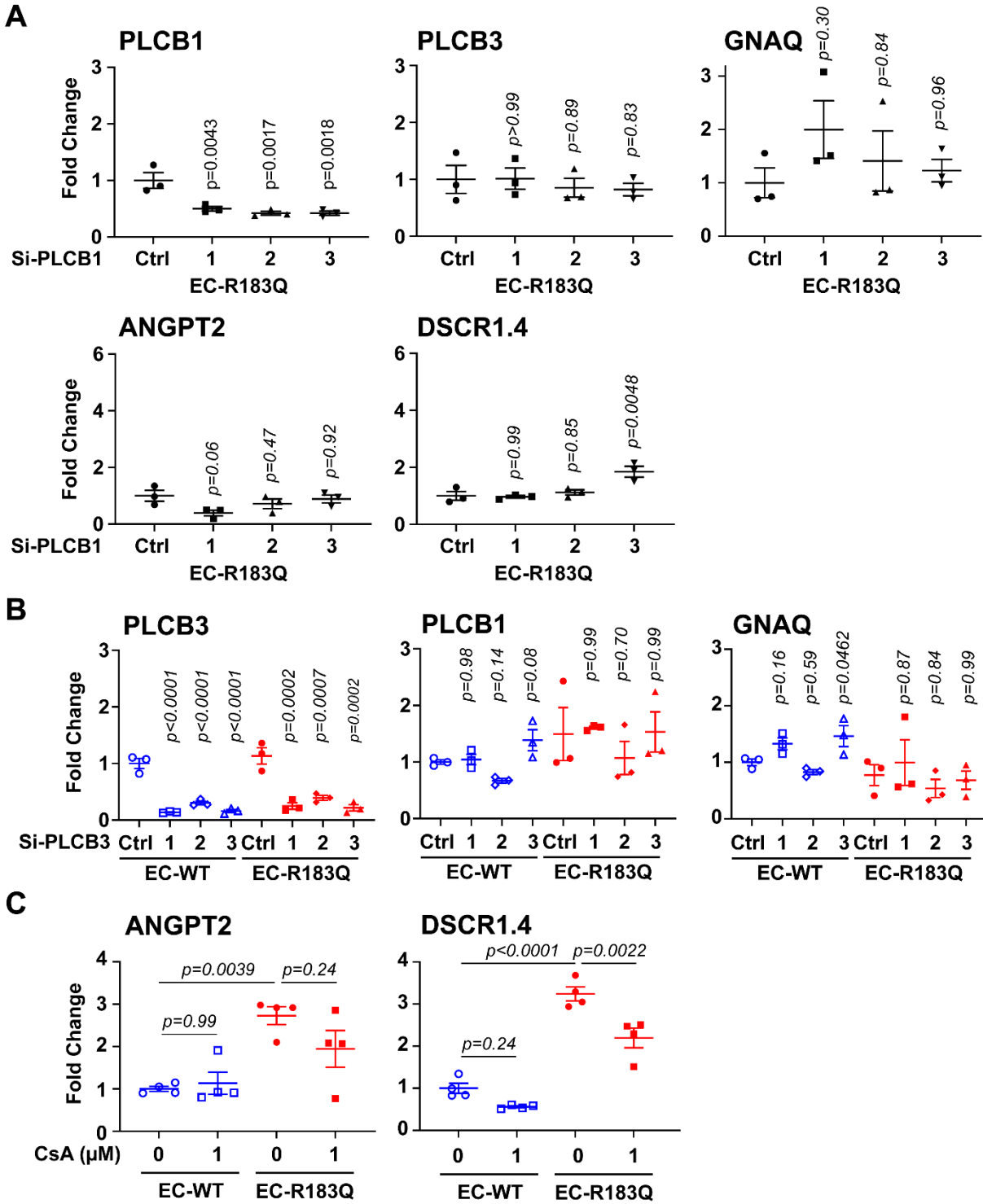
Online Figure I

Online Figure I. Characterization of EC-WT and EC-R183Q. (A) Schematic of lentiviral transduction and GFP+ cell sorting. (B) Mutant allelic frequency was determined by ddPCR and (C) Gαq protein level was detected by western blot. Non-transduced ECFC (EC) shown for comparison. (D) qPCR shows gene expression in EC-R183Q compared to EC-WT and non-transduced EC. *P*-values calculated by one-way ANOVA followed by Sidak's multiple comparisons in EC-WT vs. EC-R183Q. (E-F) Western blots detected no difference in the expression of p-PLCγ1(Ser1248) or PLCγ1 in EC-R183Q, EC-WT and non-transduced EC. N=3. *P*-values calculated by one-way ANOVA followed by Sidak's multiple comparisons in EC-WT vs. EC-R183Q. (G) PIP2 staining (red) in quiescent EC-R183Q versus EC-WT. Nuclei counterstained with DAPI (blue). Scale bar, 100 μm. (H) The mean of fluorescence intensity (MFI) measured in 70 cells from each (G) showed increased PIP2 in EC-R183Q. *P*-values calculated by unpaired, two-tailed Student-*t* test.



Online Figure II

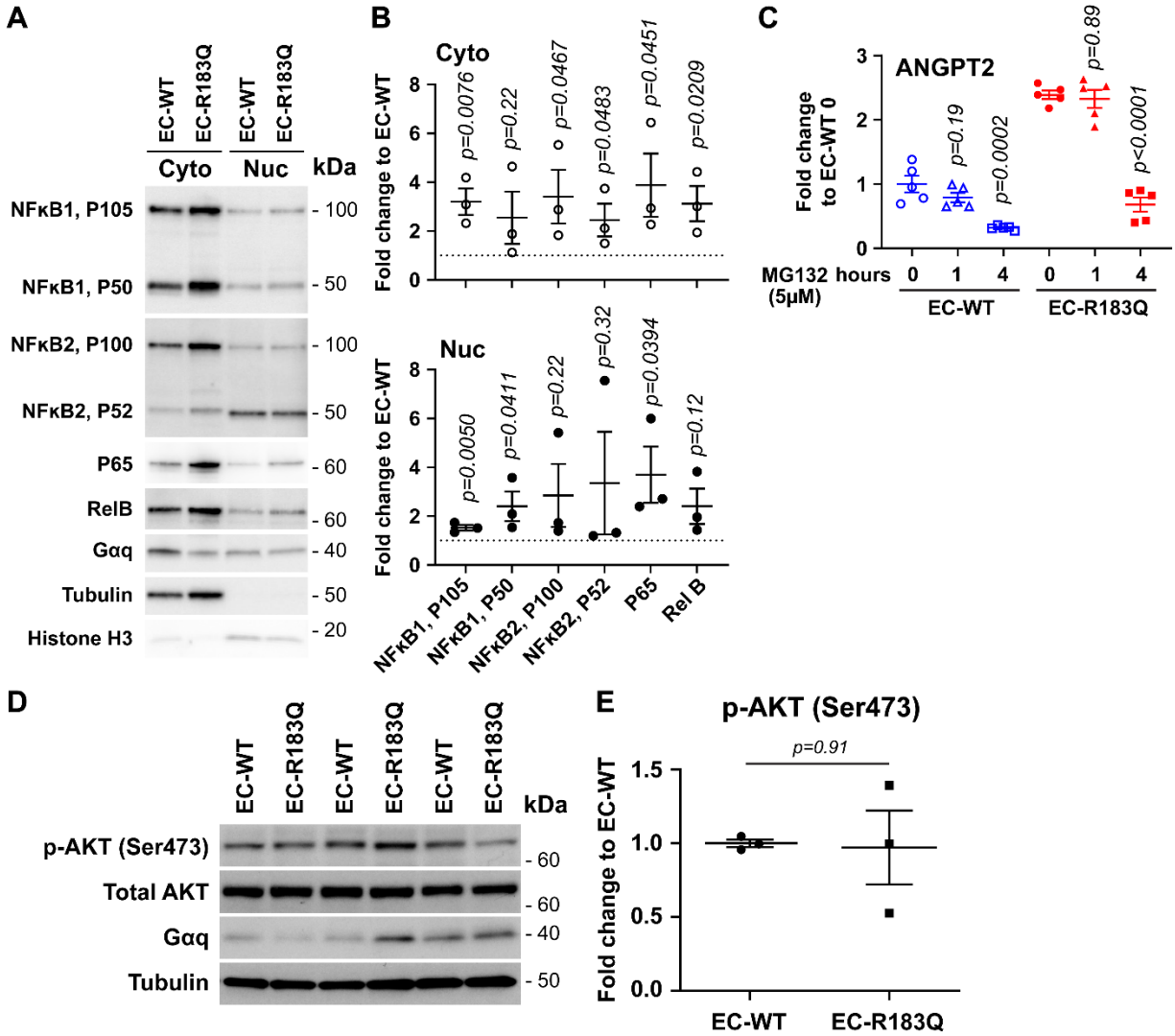
Online Figure II. RNA-seq of EC-R183Q. (A) Differentially expressed genes between R183Q and WT *GNAQ* expressed in ECFC or in HUVEC (n=3 biological replicates for each) were analyzed for upstream regulators with concordant differential expression between R183Q and WT ECs using Ingenuity Pathway Analysis. NF- κ B, PKC, and calcineurin activation are highlighted. (B) A heatmap of fold change-concordant genes (FDR <0.1, scaled log₂ CPM (Counts Per Million) expression levels) was generated in R. Euclidean distance was used with an average clustering method in the pheatmap R package. The heatmap shows clustering of three HUVEC mutant R183Q lines from three HUVEC wildtype controls (x-axis) with differences based on differential gene expression. For R183Q ECs, samples from two cell lines clustered together while a third was placed in a clade with three EC wildtype controls. ANGPT2 is indicated (arrow). Scale bar indicates expression levels.



Online Figure III

Online Figure III. Gene expression in presence of siRNA targeted to PLC β 1 and PLC β 3

and cyclosporine A treatment. (A) EC-R183Q transfected with three PLC β 1-siRNA and a control siRNA were assayed for gene expression by qPCR. PLC β 1 was significantly inhibited by siRNA-PLC β 1 while PLC β 3, GNAQ, ANGPT2, and DSCR1.4 were not affected. *P*-values calculated by one-way ANOVA followed by Dunnett's multiple comparisons. (B) EC-WT and EC-R183Q treated with three different PLC β 3-siRNAs and a control siRNA were assayed by qPCR. PLC β 3 was significantly inhibited by siRNA-PLC β 3 while PLC β 1 and GNAQ were not affected. *P*-values calculated by one-way ANOVA followed by Dunnett's multiple comparisons. (C) EC-WT and EC-R183Q treated with 1 μ M calcineurin inhibitor Cyclosporine A (CsA) (N=4) were assayed for gene expression by qPCR. *P*-values calculated by one-way ANOVA followed by Tukey's multiple comparisons. One-way ANOVA showed *P*=0.0027 for ANGPT2 and *P*<0.0001 for DSCR1.4.



Online Figure IV

Online Figure IV. Expression of NF-κB proteins and p-AKT (Ser473) in EC-R183Q. (A)

Cells were lysed and separated into cytosolic (Cyto) and nuclear (Nuc) fractions for western

blot. (B) Western blot band intensities, normalized to Gaq band intensity, are presented as the

fold change to EC-WT (dashed lines). The NF-κB proteins were increased in both cytosolic

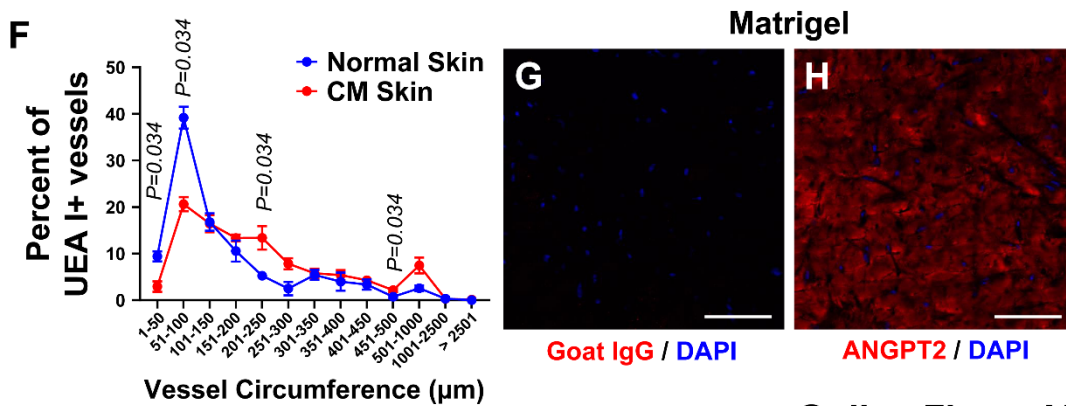
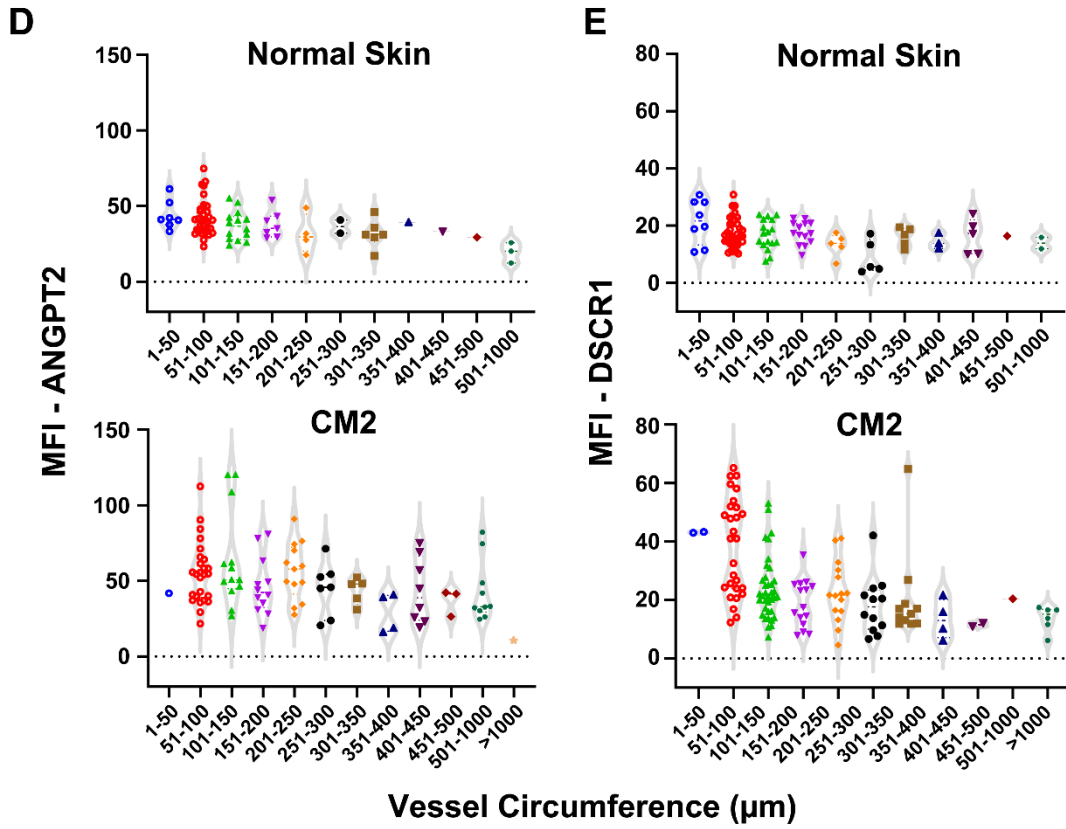
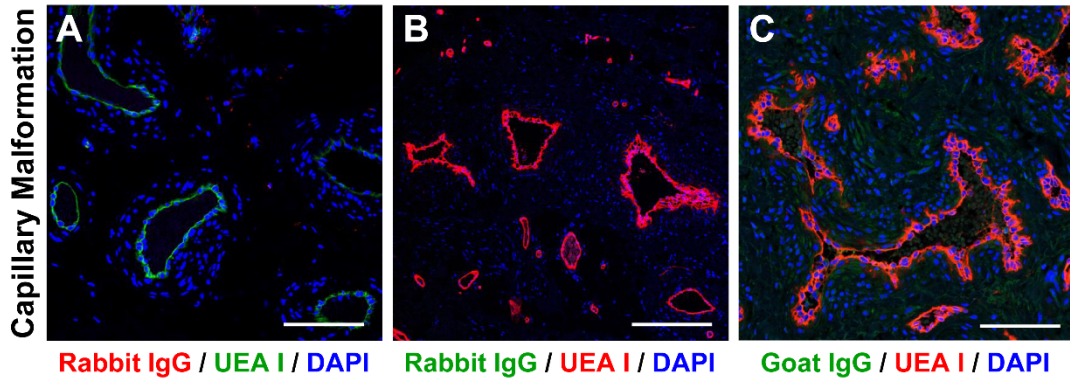
(upper panel) and nuclear (lower panel) fractions in EC-R183Q versus EC-WT. N=3. P-values

calculated by unpaired, two-tailed student t-test. (C) EC-WT and EC-R183Q were treated with

5μM proteasome inhibitor MG132 for 1 and 4 hours. A 4-hour MG132 treatment decreased

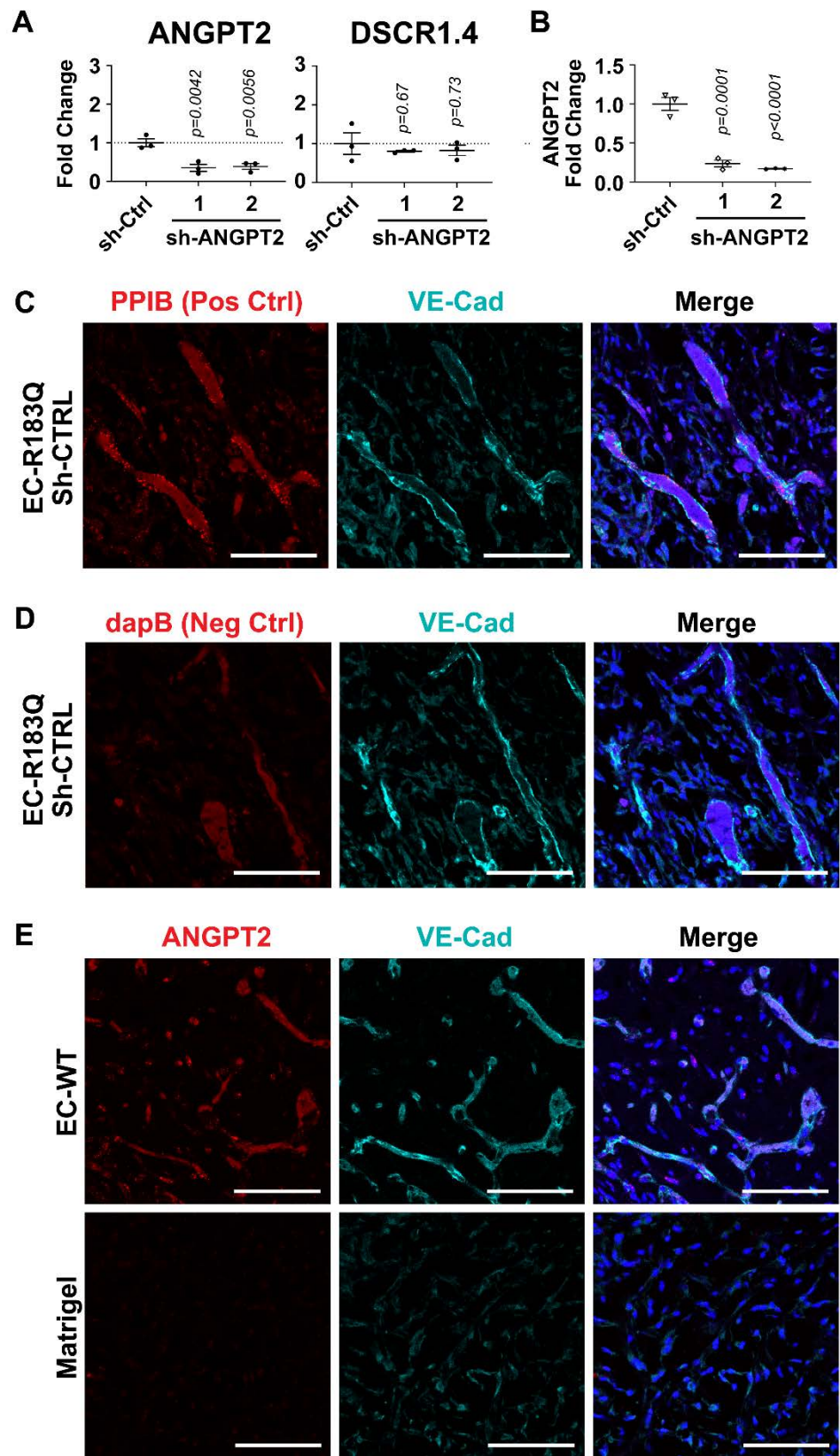
ANGPT2 mRNA levels measured by qPCR. P-values calculated by one-way ANOVA followed

by Dunnett's multiple comparisons. One-way ANOVA showed $P=0.0004$ for EC-WT comparison and $P<0.0001$ for EC-R183Q comparison. (D) Western blot detection of p-AKT(Ser473) in EC-R183Q and EC-WT with densitometric analysis shown in (E), expressed as the fold change to EC-WT. $N=3$. P -value calculated by unpaired, two-tailed Student t -test.



Online Figure V

Online Figure V. Negative controls of immunostaining, MFI of ANGPT2 and DSCR1, and CM vessel circumference distribution. (A-B): UEA I and rabbit IgG co-staining, and (C) UEA I and goat IgG co-staining of CM skin tissue sections, followed by secondary antibodies, shows no background staining with species-matched IgG for rabbit anti-p-PLC β 3 (Ser537), rabbit anti-DSCR1 and goat anti-ANGPT2. Scale bar, 100 μ m. (D-E): The MFI of ANGPT2 (D) and DSCR1 (E) measured in Figure 4A-B was plotted versus vessel circumference. 50-100 vessels from 4-5 sections of skin CM2 (Table 1) and 2 sections of the normal skin were measured. (F): The vessel circumferences measured in four skin CMs and one normal skin was categorized and plotted. *P* values calculated using the Mann-Whitney U test. (G-H): The immunostaining of a Matrigel only implant with goat IgG (G) and goat anti-human ANGPT2 antibody (H), followed by the secondary antibody, shows background staining due to anti-ANGPT2. Nuclei counterstained with DAPI (blue). Scale bar, 100 μ m.



Online Figure VI

Online Figure VI. ANGPT2 shRNA knockdown in EC-R183Q and ANGPT2 RNAscope. (A-

B): EC-R183Q transduced with two ANGPT2 shRNA lentiviral vectors and the control vector were assayed for gene expression by qPCR (A) and ELISA (B). ANGPT2 was significantly

decreased by ANGPT2-shRNA at both mRNA level and protein level while DSCR1.4 was not affected. *P*-values calculated by one-way ANOVA followed by Dunnett's multiple comparisons.

(C-D): EC-R183Q sh-CTRL implant sections were co-stained with the positive (PPIB) or

negative (dapB) RNAscope probes (red) and anti-human VE-Cad (cyan). (E): Sections from EC-

WT implants (upper panel) and acellular Matrigel implants (lower panel) were stained

with human ANGPT2 RNAscope probe (red) and anti-human VE-Cad (cyan). Nuclei

counterstained with DAPI (blue). Scale bar, 100 μ m.

Online Table I

Analysis Type:	PANTHER Overrepresentation Test (Released 20200407)						
Annotation Version and Release Date:	GO Ontology database DOI: 10.5281/zenodo.3873405 Released 2020-06-01						
Analyzed List:	upload_1 (Homo sapiens)						
Reference List:	Homo sapiens (all genes in database)						
Test Type:	FISHER						
Correction:	FDR						
GO biological process complete	Homo sapiens - REFLIST (20851)	upload_1 (288)	upload_1 (expected)	upload_1 (over/under)	upload_1 (fold Enrichment)	upload_1 (raw P-value)	upload_1 (FDR)
ciliary neurotrophic factor-mediated signaling pathway (GO:0070120)	5	3	0.07	+	43.44	1.33E-04	4.09E-02
maintenance of protein location in nucleus (GO:0051457)	24	5	0.33	+	15.08	4.12E-05	1.88E-02
maintenance of protein localization in organelle (GO:0072595)	43	6	0.59	+	10.1	5.19E-05	2.12E-02
type I interferon signaling pathway (GO:0060337)	69	8	0.95	+	8.39	1.00E-05	1.07E-02
cellular response to type I interferon (GO:0071357)	69	8	0.95	+	8.39	1.00E-05	1.00E-02
response to type I interferon (GO:0034340)	74	8	1.02	+	7.83	1.60E-05	1.28E-02
maintenance of protein location (GO:0045185)	101	9	1.4	+	6.45	1.99E-05	1.45E-02
protein localization to nucleus (GO:0034504)	168	11	2.32	+	4.74	3.63E-05	1.87E-02
positive regulation of I-kappaB kinase/NF-kappaB signaling (GO:0043189)	189	12	2.61	+	4.6	2.15E-05	1.49E-02
defense response to virus (GO:0051607)	204	11	2.82	+	3.9	1.87E-04	4.98E-02
viral process (GO:0016032)	792	31	10.94	+	2.83	3.37E-07	2.69E-03
cytokine-mediated signaling pathway (GO:0019221)	696	27	9.61	+	2.81	2.37E-06	6.31E-03
positive regulation of response to external stimulus (GO:0032103)	518	19	7.15	+	2.66	1.56E-04	4.53E-02
symbiotic process (GO:0044403)	884	31	12.21	+	2.54	3.11E-06	7.10E-03
protein localization to organelle (GO:0033365)	774	26	10.69	+	2.43	5.47E-05	2.13E-02
cellular response to cytokine stimulus (GO:0071345)	1034	34	14.28	+	2.38	4.22E-06	7.49E-03
response to cytokine (GO:0034097)	1124	36	15.53	+	2.32	4.94E-06	7.17E-03
apoptotic process (GO:0006915)	910	29	12.57	+	2.31	3.86E-05	1.87E-02
cell death (GO:0008219)	1079	34	14.9	+	2.28	1.21E-05	1.14E-02
programmed cell death (GO:0012501)	1042	31	14.39	+	2.15	1.06E-04	3.61E-02
defense response (GO:0006952)	1424	38	19.67	+	1.93	1.42E-04	4.21E-02
negative regulation of biosynthetic process (GO:0009890)	1589	42	21.95	+	1.91	7.69E-05	2.73E-02
negative regulation of cellular biosynthetic process (GO:0031327)	1559	40	21.53	+	1.86	1.80E-04	4.95E-02
cellular response to organic substance (GO:0071310)	2378	60	32.85	+	1.83	6.19E-06	8.24E-03
regulation of cell population proliferation (GO:0042127)	1668	42	23.04	+	1.82	1.84E-04	4.97E-02
interspecies interaction between organisms (GO:0044419)	2073	52	28.63	+	1.82	2.81E-05	1.73E-02
cell surface receptor signaling pathway (GO:0007166)	2519	63	34.79	+	1.81	4.41E-06	7.04E-03
negative regulation of gene expression (GO:0010629)	1966	49	27.15	+	1.8	6.63E-05	2.41E-02
positive regulation of cell communication (GO:0010647)	1900	47	26.24	+	1.79	1.21E-04	4.04E-02
positive regulation of signaling (GO:0023056)	1908	47	26.35	+	1.78	1.27E-04	4.04E-02
response to organic substance (GO:0010033)	3040	74	41.99	+	1.76	9.22E-07	4.91E-03
negative regulation of nitrogen compound metabolic process (GO:0051309)	2409	57	33.27	+	1.71	6.00E-05	2.28E-02
regulation of molecular function (GO:0065009)	3076	71	42.49	+	1.67	1.21E-05	1.08E-02
response to stress (GO:0006950)	3654	84	50.47	+	1.66	1.64E-06	6.53E-03
negative regulation of macromolecule metabolic process (GO:0010605)	2846	65	39.31	+	1.65	4.50E-05	1.99E-02
cellular response to chemical stimulus (GO:0070887)	2941	67	40.62	+	1.65	3.88E-05	1.82E-02
regulation of cell communication (GO:0010646)	3625	79	50.07	+	1.58	3.05E-05	1.74E-02
negative regulation of metabolic process (GO:0009892)	3098	67	42.79	+	1.57	1.69E-04	4.73E-02
regulation of signaling (GO:0023051)	3664	79	50.61	+	1.56	3.48E-05	1.85E-02
positive regulation of biological process (GO:0048518)	6306	129	87.1	+	1.48	2.82E-07	4.50E-03
positive regulation of cellular process (GO:0048522)	5737	117	79.24	+	1.48	2.12E-06	6.78E-03
regulation of response to stimulus (GO:0048583)	4371	89	60.37	+	1.47	8.06E-05	2.80E-02
signal transduction (GO:0007165)	4950	97	68.37	+	1.42	1.60E-04	4.55E-02
negative regulation of biological process (GO:0048519)	5530	108	76.38	+	1.41	5.27E-05	2.10E-02
signaling (GO:0023052)	5322	103	73.51	+	1.4	1.34E-04	4.03E-02
cellular response to stimulus (GO:0051716)	6610	127	91.3	+	1.39	1.34E-05	1.13E-02
cell communication (GO:0007154)	5413	104	74.77	+	1.39	1.89E-04	4.95E-02
regulation of cellular metabolic process (GO:0031323)	6299	121	87	+	1.39	2.54E-05	1.69E-02
regulation of metabolic process (GO:0019222)	7021	134	96.98	+	1.38	7.71E-06	9.47E-03
regulation of nitrogen compound metabolic process (GO:0051171)	5895	112	81.42	+	1.38	1.27E-04	3.97E-02
regulation of macromolecule metabolic process (GO:0060255)	6474	123	89.42	+	1.38	3.83E-05	1.91E-02
cellular metabolic process (GO:0044237)	7796	143	107.68	+	1.33	2.86E-05	1.69E-02
primary metabolic process (GO:0044238)	7588	137	104.81	+	1.31	1.26E-04	4.11E-02
metabolic process (GO:0008152)	8585	154	118.58	+	1.3	3.05E-05	1.68E-02
response to stimulus (GO:0050896)	8523	152	117.72	+	1.29	6.40E-05	2.37E-02
regulation of cellular process (GO:0050794)	11160	193	154.15	+	1.25	4.21E-06	8.40E-03
regulation of biological process (GO:0050789)	11714	199	161.8	+	1.23	8.84E-06	1.01E-02
biological regulation (GO:0065007)	12390	206	171.13	+	1.2	2.78E-05	1.77E-02
cellular process (GO:0009987)	15430	244	213.12	+	1.14	1.77E-05	1.35E-02
biological_process (GO:0008150)	17912	270	247.41	+	1.09	4.97E-05	2.15E-02
Unclassified (UNCLASSIFIED)	2939	18	40.59	-	0.44	4.97E-05	2.09E-02

Major Resources Table

Animals (in vivo studies)

Species	Vendor or Source	Background Strain	Sex
Mouse	Massachusetts General Hospital	Nude	M

Antibodies

Antibodies for immuno-staining			
Target antigen	Vendor or Source	Catalog #	Working dilution
phospho-PLC β 3, 537	Abcam	Ab73998	20 μ g/ml
VE-cadherin	R&D Systems	AF938	4 μ g/ml
Angiopoietin-2	R&D Systems	AF623	8 μ g/ml
DSCR1	Sigma-Aldrich	D-6694	15 μ g/ml
phospho-ERK1/2	CST*	4370	5 μ g/ml
Ki67	Abcam	Ab15580	10 μ g/ml
PIP2	Echelon Biosciences	Z-B045	10 μ g/ml
Ulex Europaeus Agglutinin I (UEA I)	Vector Laboratories	FL-1061/DL-1067/1068	25 μ g/ml
Antibodies for western blot			
Target antigen	Vendor or Source	Catalog #	Working dilution
G α (q)	CST	14373	30 - 50 ng/ml
phospho-PLC β 3, 537	CST	2481	200 - 250 ng/ml
phospho-PLC β 3, 1105	CST	2484	100 - 150 ng/ml
PLC β 3	CST	14247	60 -100 ng/ml
phospho-PLC γ 1, 1248	CST	8713	125 ng/ml
PLC γ 1	CST	5690	165 ng/ml
DSCR1	Sigma-Aldrich	D-6694	1 μ g/ml
phospho-VEGFR2, 1175	CST	3770	500 - 800 ng/ml
VEGFR2	CST	2479	30 - 50 ng/ml
phospho-ERK1/2	CST	4370	500 - 650 ng/ml
ERK1/2	CST	4696	100 - 125 ng/ml
NF- κ B1, p105/p50	CST	12540	480 ng/ml
NF- κ B2, p100/p52	CST	3017	50 - 100 ng/ml
NF κ B, p65	CST	8242 and 6956	50 - 100 ng/ml
RELB	CST	10544	100 ng/ml
Histone H3	CST	4499	20 ng/ml
Tubulin	Sigma-Aldrich	T9026	1:4000
GAPDH	CST	2118	50 ng/ml
*CST, Cell Signaling Technology			

Inhibitors

Inhibitor Name	Vendor or Source	Catalog #	Working concentration
YM-254890	Wako Chemicals	257-00631	10 -100nM
AEB071	Medchem Express	HY-10343	0.5 -5 μ M
Cyclosporin A	Sigma-Aldrich	30024-25MG	1 μ M
MG-132	Sigma-Aldrich	M8699	5 μ M

Growth Factors

Name	Vendor or Source	Catalog #	Working concentration
Human FGF2	ProSpec-TechnoGene	CYT-557	1 µg/ml
Human EPO	ProSpec-TechnoGene	CYT-201	1 µg/ml

SiRNA

Name	Vendor or Source	Catalog #	Working concentration
AllStars Neg. Control siRNA	Qiagen	1027281	10 nM
Hs_PLCB1_6	Qiagen	SI02781184	10 nM
Hs_PLCB1_9	Qiagen	SI04439106	10 nM
Hs_PLCB1_10	Qiagen	SI04439113	10 nM
Hs_PLCB3_3	Qiagen	SI00018781	10 nM
Hs_PLCB3_4	Qiagen	SI00018788	10 nM
Hs_PLCB3_5	Qiagen	SI03095358	10 nM

ShRNA

Name	Vendor or Source	Clone #
Empty Vector Control	Sigma	SHC001V
ShANGPT2	Sigma	TRCN0000059223
ShANGPT2	Sigma	TRCN0000059225

RNAscope

Name	Vendor or Source	Catalog #
RNAscope Probe Hs-ANGPT2-No-XMm-C1	Advanced Cell Diagnostics	Made-to-order
RNAscope 3-plex negative control probe	Advanced Cell Diagnostics	320871
RNAscope 3-plex positive control probe_Hs	Advanced Cell Diagnostics	320861
RNAscope co-detection ancillary kit	Advanced Cell Diagnostics	323180
Opal 570 reagent kit	Akoya Biosciences	FP1488001KT

Other

Name	Vendor or Source	Catalog #
Human Angiopoietin-2 ELISA Kit	R&D Systems	BAF385
Matrigel	Fisher Scientific	356237