Supplemental Materials

Materials and Methods

Cell Culture

Isolation of primary Müller cells was performed as previously described (1).

EC Migration Assay

For (inverse) cell migration, serum-starved 2×10^4/well HMECs were plated in the bottom wells of a Boyden chamber, and a fibronectin-coated polycarbonate membrane (8 μm; Neuroprobe) was placed above. The chamber was inverted and incubated for 2 hours at 37°C to allow cell attachment. 50 ml of sample containing ANGPTL4 was added to the top wells. After 6 hours, the membrane was stained with Diff-Quick stain (Dade Behring) and analyzed with ImageJ as previously described (2).

EC Proliferation Assay

Cell proliferation was evaluated by the BrdU Cell Proliferation Assay Kit (Cell Signaling Technology) according to the manufacturer's instructions as previously described (2).

EC Survival Assay

Cell survival was evaluated by the CellTiter 96 Aqueous Cell Proliferation Assay kit (Promega), following the manufacturer's instructions. Cells were grown to confluence in 96-well plates, serum-starved for 24 hours, and treated with rhANGPTL4 as previously described (2).

Supplemental Figure Legends

Figure S1. Angiogenic potential of aqueous fluid correlates with diabetic eye disease. (*A*,*B*) Aqueous fluid from 15 individual non-diabetic control patients (Con 1-15; *A*) and 10 individual diabetic patients without DR (DM no DR 1-10; *B*) does not stimulate tubule formation. (*C*) Aqueous fluid from 4 individual diabetic patients with active PDR (PDR 1-4) does stimulate tubule formation. Please see Table 1 for details for each patient. Student's t-test.

Figure S2. Aqueous fluid VEGF levels are elevated in PDR patients.

Aqueous fluid VEGF levels are elevated in PDR patients compared to non-diabetic patients (Control), diabetic patients without DR (DM no DR), diabetic patients with NPDR, and PDR patients treated with anti-VEGF therapy within 2 weeks of sample collection (PDR anti-VEGF). Student's t-test.

Figure S3. Stimulation of tubule formation by aqueous fluid from PDR patients is independent of VEGF levels. (*A*) The levels of VEGF in the aqueous fluid from PDR patients show considerable variability. NOTE: All PDR samples are included in this scatter plot; solid diamonds represent PDR aqueous fluid samples not depicted in Fig. 1D. (*B*,*C*) Aqueous fluid samples from individual low-VEGF PDR samples are able to induce tubule formation (PDR Low VEGF; *B*) at levels similar to that observed in individual high-VEGF PDR aqueous fluid samples (PDR High VEGF; *C*). (*D*) The ability of aqueous fluid from PDR patients to stimulate tubule formation is not statistically different in patients with low or high concentrations of VEGF. Please see Table 1 for details for each patient. Student's t-test.

Figure S4. Bevacizumab inhibits the ability of rhVEGF to stimulate tubule formation. (*A*) Bevacizumab completely inhibits the ability of rhVEGF (at a concentration similar to the highest levels measured in the aqueous fluid from PDR patients) to stimulate tubule formation. (*B*) Bevacizumab at doses 10 to 100 fold less than that used to neutralize VEGF in aqueous samples causes a marked reduction in "free" VEGF that can be detected by ELISA. One-way ANOVA.

Figure S5. Angiogenic potential of aqueous fluid from individual PDR patients is affected by anti-VEGF therapy

(*A*) Treatment with bevacizumab does not affect the ability of aqueous fluid from most PDR patients to stimulate tubule formation. (*B*) Aqueous fluid from 8 individual PDR patients who received a recent (within two weeks of sample collection) intravitreal injection with anti-VEGF therapy (PDR Anti-VEGF), has VEGF levels similar to those of control patients and diabetics without DR, but is still able to stimulate tubule formation. (*C*) The ability of PDR anti-VEGF aqueous fluid to stimulate tubule formation is not affected by treatment with additional bevacizumab. Wilcoxon tests.

Figure S6. Hypoxic Müller cells upregulate mRNA expression of genes previously reported to play a role in angiogenesis.

The mRNA expression of 20 known inflammatory cytokines, proteases, and angiogenic cytokines regulated (directly or indirectly) by HIF-1α and previously reported to play a role in angiogenesis in hypoxic human retinal Müller cells. All mRNA levels normalized to β-actin mRNA and reported as fold induction compared with cells exposed to 20% O_2 (control).

Student's t-test.

Figure S7. ANGPTL4 stimulates EC survival and migration. (*A*-*C*) rhANGPTL4 stimulates EC survival (*A*) and migration (*B*), but does not affect EC proliferation (*C*). One-way ANOVA and Student's t-test.

Figure S8. ANGPTL4 levels are increased in the aqueous fluid of PDR patients. ANGPTL4 protein levels in the aqueous fluid of PDR patients and PDR patients recently treated with anti-VEGF therapy (PDR + anti-VEGF) compared to non-diabetic (control) patients, diabetic patients without DR (DM no DR), and diabetic patients with NPDR. Student's t-test.

Figure S9. ANGPTL4 neutralizing antibody inhibits the stimulation of tubule formation by ANGPTL4 but not VEGF. (*A*,*B*) Addition of an ANGPTL4 neutralizing monoclonal antibody inhibits the stimulation of tubule formation by rhANGPTL4 in a dose-dependent manner *in vitro* (*A*), but has no effect on the stimulation of tubule formation by rhVEGF (*B*). (*C*) Addition of ANGPTL4 neutralizing antibody (10 μg/ml) to aqueous fluid in the tubule formation assay inhibits the stimulation of tubule formation by conditioned media from hypoxic retinal Müller cells *in vitro*. One-way ANOVA.

Figure S10. ANGPTL4 neutralizing antibody reduces the angiogenic potential of aqueous fluid from individual PDR patients.

(*A*) ANGPTL4 neutralizing antibody potently inhibits the ability of individual low-VEGF aqueous fluid to stimulate tubule formation. (*B*) Pre-treatment of aqueous fluid from individual

PDR patients who received anti-VEGF therapy within 2 weeks of sample collection (PDR anti-VEGF) with ANGPTL4 neutralizing antibody reduces their angiogenic potential. Wilcoxon test.

Figure S11. The expression of ANGPTL4 in the aqueous fluid of diabetic patients. The expression of ANGPTL4 in the aqueous fluid of PDR and NPDR patients compared to diabetic and non-diabetic control patients. NOTE: All PDR samples are included in this scatter plot; solid diamonds represent PDR aqueous fluid samples not depicted in Fig. 6A.

Figure S12. 2-dimensional scatter plot demonstrating the expression of VEGF and ANGPTL4 in the aqueous fluid of PDR patients compared to diabetic and non-diabetic control patients. (*A*) The expression of VEGF and ANGPTL4 in the aqueous fluid of PDR and NPDR patients compared to diabetic and non-diabetic control patients. NOTE: All PDR samples are included in this scatter plot. The average [VEGF] (0.425 ng/ml, orange dashed line) and [ANGPTL4] (36.7 ng/ml, blue dashed line) in the aqueous fluid from PDR patients is depicted. (*B*) As in (A), but PDR samples with [ANGPTL4] > 60 ng/ml and/or with [VEGF] > 1.2 ng/ml are not included in the plot to adequately demonstrate the variability within the PDR samples and the separation of controls, diabetics without DR, diabetics with NPDR, and diabetics with PDR.

Supplemental References

1. Xin X, *et al.* (2013) Hypoxic retinal Muller cells promote vascular permeability by HIF-1-dependent up-regulation of angiopoietin-like 4. *Proceedings of the National Academy of Sciences of the United States of America* 110(36):E3425-3434.

2. Ma T, *et al.* (2010) Viral G protein-coupled receptor up-regulates Angiopoietin-like 4 promoting angiogenesis and vascular permeability in Kaposi's sarcoma. *Proceedings of the National Academy of Sciences of the United States of America* 107(32):14363-14368.

fig. S1

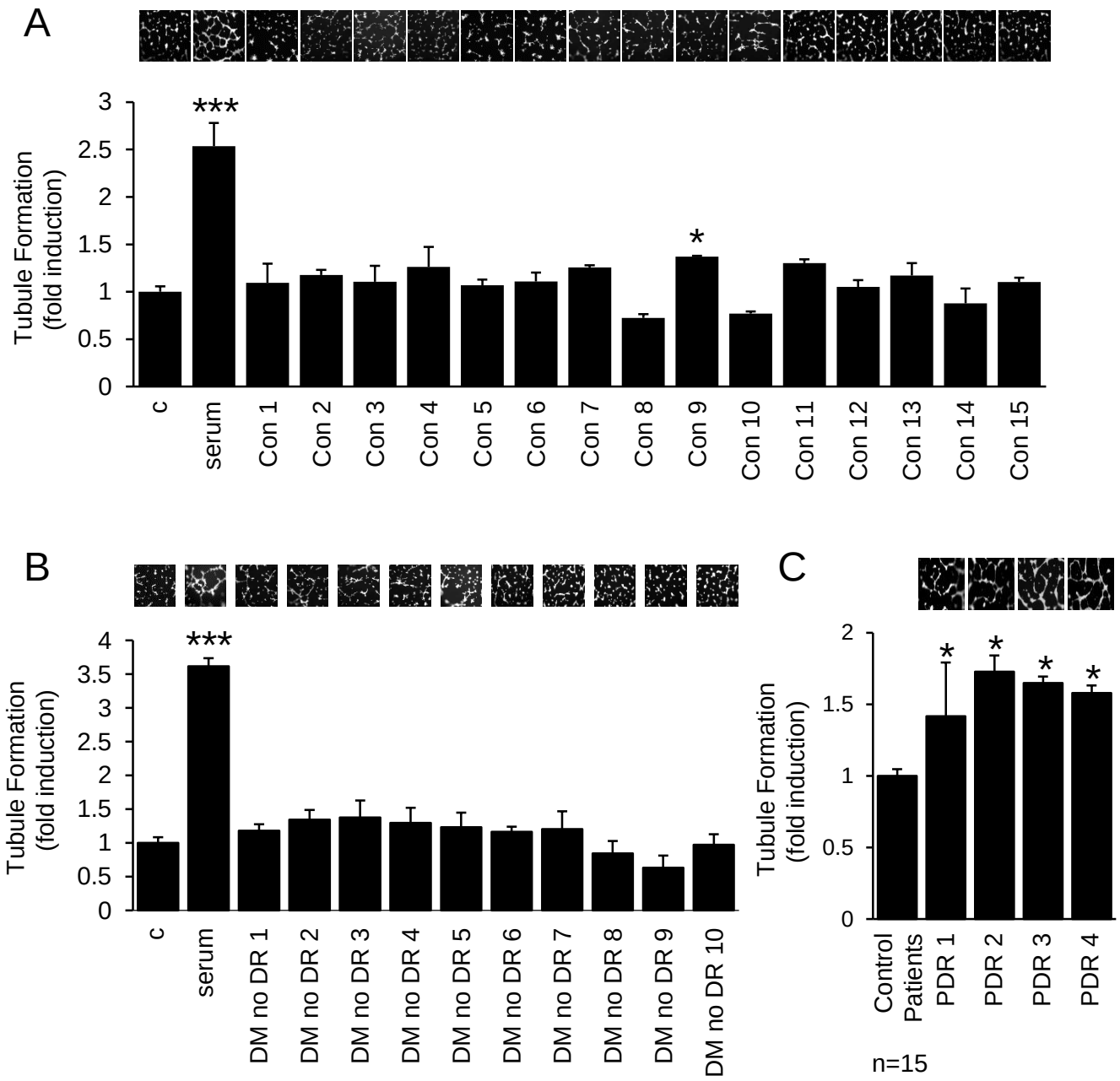
A
Tubule Formation (fold induction)
3
2.5
2
1.5
1
0.5
0
***
*
c
serum
Con 1
Con 2
Con 3
Con 4
Con 5
Con 6
Con 7
Con 8
Con 9
Con 10
Con 11
Con 12
Con 13
Con 14
Con 15
B
Tubule Formation (fold induction)
4
3.5
3
2.5
2
1.5
1
0.5
0
***
c
serum
DM no DR 1
DM no DR 2
DM no DR 3
DM no DR 4
DM no DR 5
DM no DR 6
DM no DR 7
DM no DR 8
DM no DR 9
DM no DR 10
C
Tubule Formation (fold induction)
2
1.5
1
0.5
0
*
*
*
*
Control
Patients
PDR 1
PDR 2
PDR 3
PDR 4
n=15

fig. S2

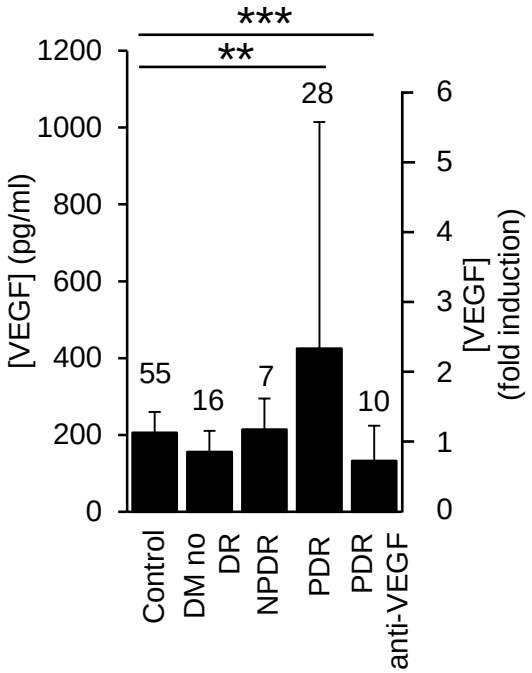
***
**
1200
1000
800
600
400
200
0
[VEGF] (pg/ml)
6
5
4
3
2
1
0
[VEGF]
(fold induction)
55
16
7
28
10
Control
DM no DR
NPDR
PDR
PDR anti-VEGF

fig. S3

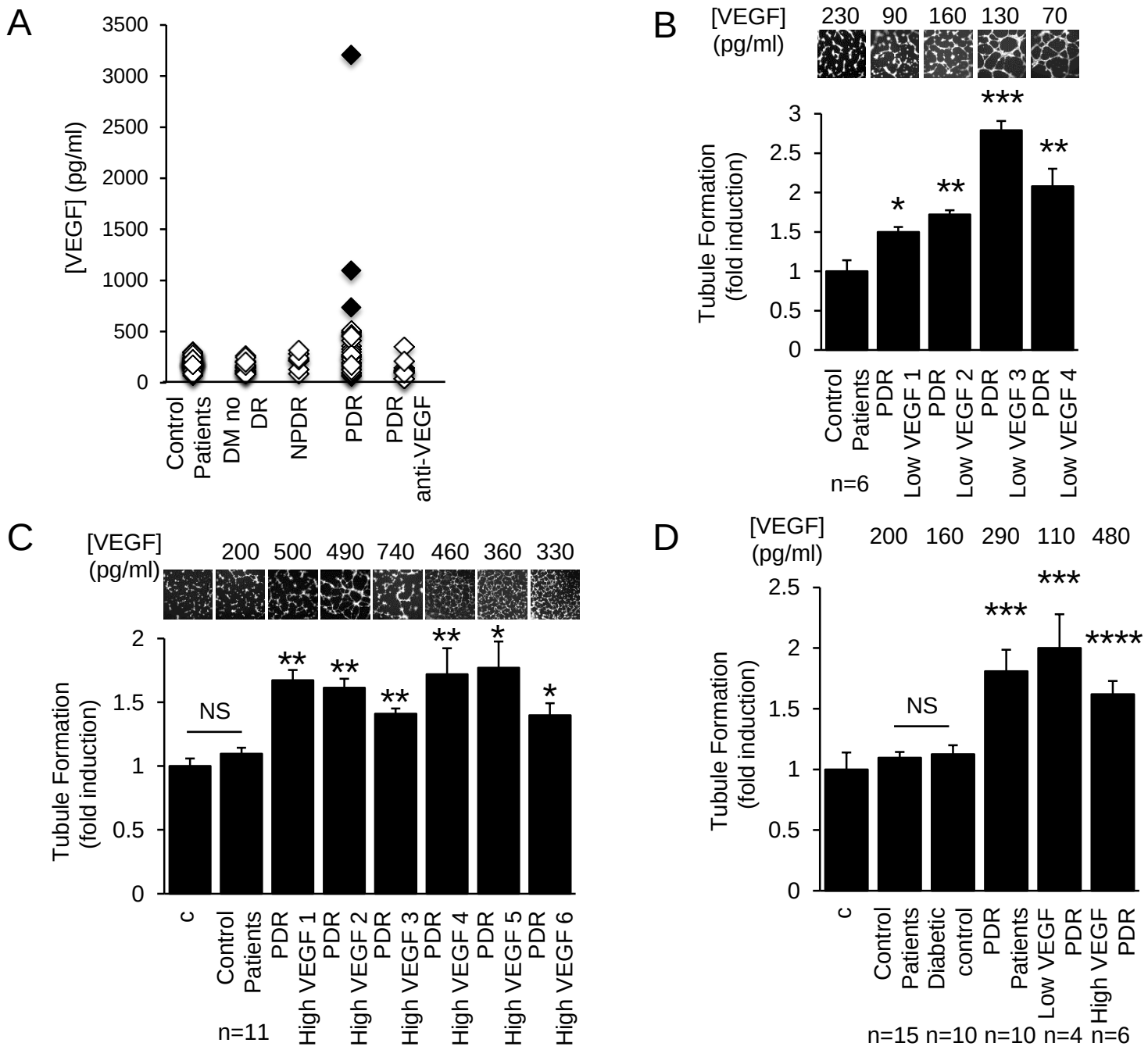
A
[VEGF] (pg/ml)
3500
3000
2500
2000
1500
1000
500
0
Control Patients
DM no DR
NPDR
PDR
PDR anti-VEGF
B
[VEGF] (pg/ml)
230 90 160 130 70
Tubule Formation (fold induction)
3
2.5
2
1.5
1
0.5
0
*
**
***
**
Control Patients
PDR Low VEGF 1
PDR Low VEGF 2
PDR Low VEGF 3
PDR Low VEGF 4
n=6
C
[VEGF] (pg/ml)
200 500 490 740 460 360 330
Tubule Formation (fold induction)
2
1.5
1
0.5
0
NS
**
**
**
**
*
*
c
Control Patients
PDR High VEGF 1
PDR High VEGF 2
PDR High VEGF 3
PDR High VEGF 4
PDR High VEGF 5
PDR High VEGF 6
n=11
D
[VEGF] (pg/ml)
200 160 290 110 480
Tubule Formation (fold induction)
2.5
2
1.5
1
0.5
0
NS
***
***
****
c
Control Patients
Diabetic control
PDR Patients
PDR Low VEGF
PDR High VEGF
n=15 n=10 n=10 n=4 n=6

fig. S4

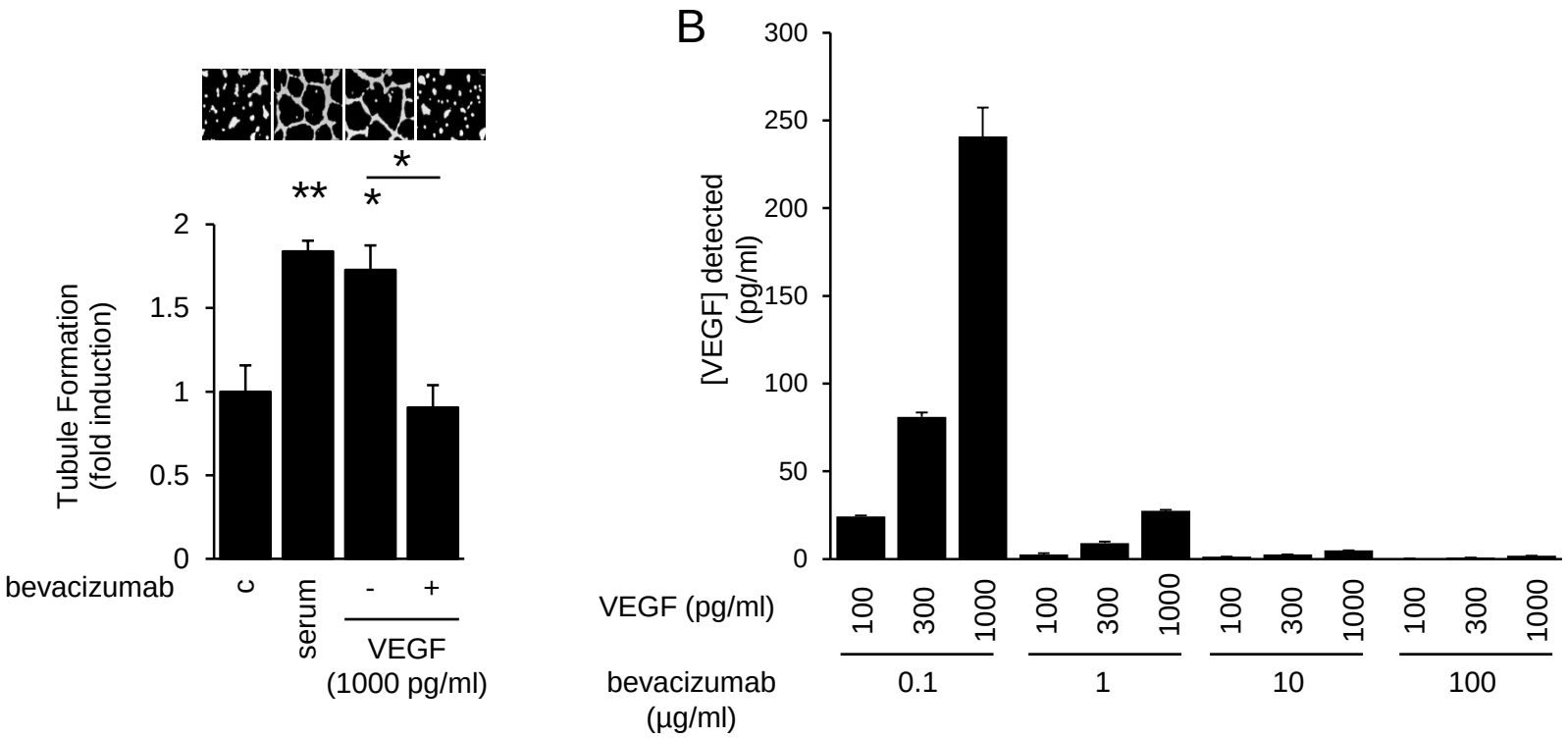
A
Tubule Formation
(fold induction)
2
1.5
1
0.5
0
**
*
*
bevacizumab
c
serum
-
+
VEGF
(1000 pg/ml)
B
[VEGF] detected
(pg/ml)
300
250
200
150
100
50
0
VEGF (pg/ml)
100
300
1000
bevacizumab
(µg/ml)
0.1
1
10
100

fig. S5

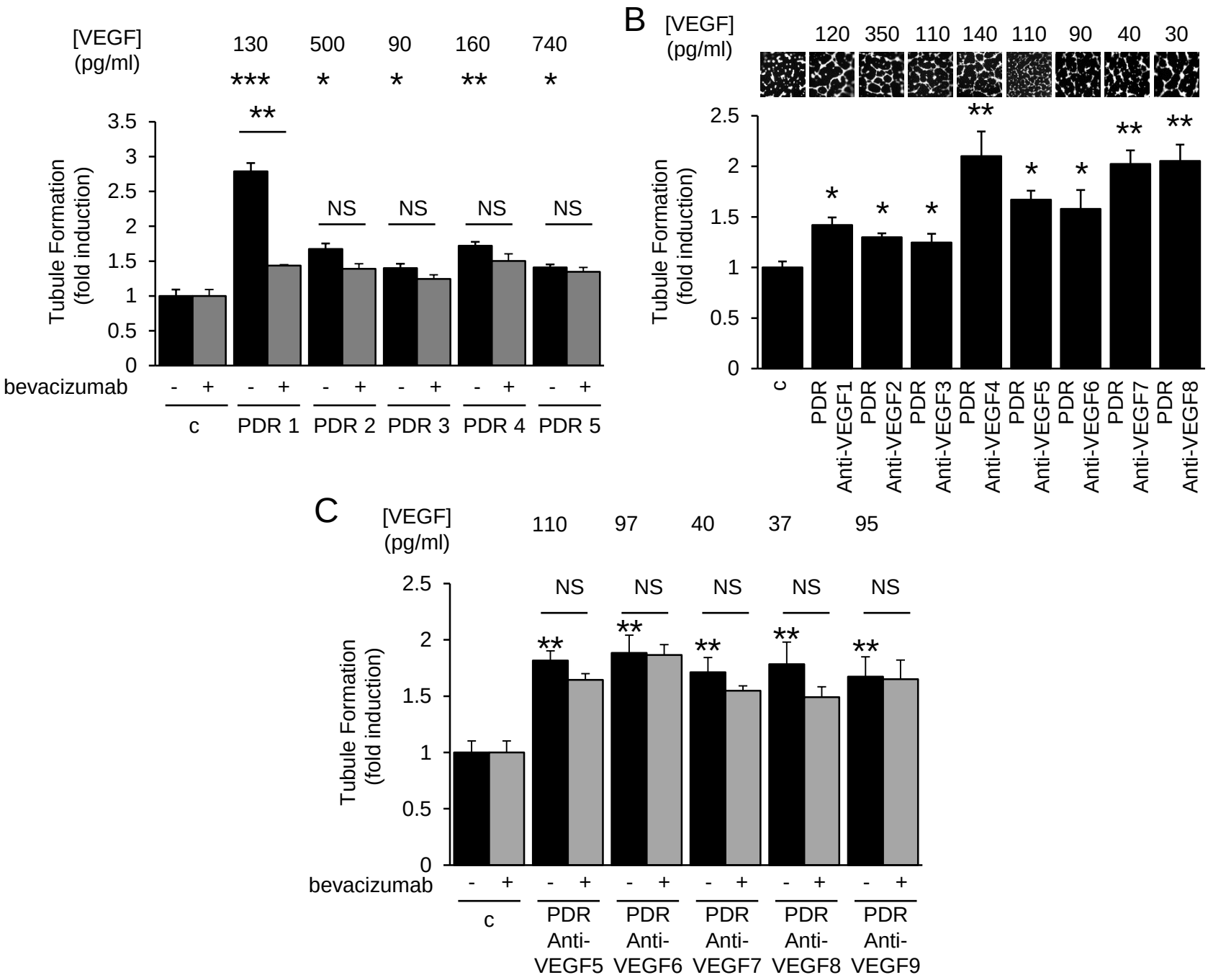
A
[VEGF] (pg/ml)
130
500
90
160
740
***
*
*
**
*
**
NS
NS
NS
NS
Tubule Formation (fold induction)
3.5
3
2.5
2
1.5
1
0.5
0
bevacizumab
- + - + - + - + - + - +
c
PDR 1
PDR 2
PDR 3
PDR 4
PDR 5
B
[VEGF] (pg/ml)
120 350 110 140 110 90 40 30
Tubule Formation (fold induction)
2.5
2
1.5
1
0.5
0
c
PDR Anti-VEGF1
PDR Anti-VEGF2
PDR Anti-VEGF3
PDR Anti-VEGF4
PDR Anti-VEGF5
PDR Anti-VEGF6
PDR Anti-VEGF7
PDR Anti-VEGF8
C
[VEGF] (pg/ml)
110 97 40 37 95
NS
Tubule Formation (fold induction)
2.5
2
1.5
1
0.5
0
bevacizumab
- + - + - + - + - + - +
c
PDR Anti-VEGF5
PDR Anti-VEGF6
PDR Anti-VEGF7
PDR Anti-VEGF8
PDR Anti-VEGF9

fig. S6

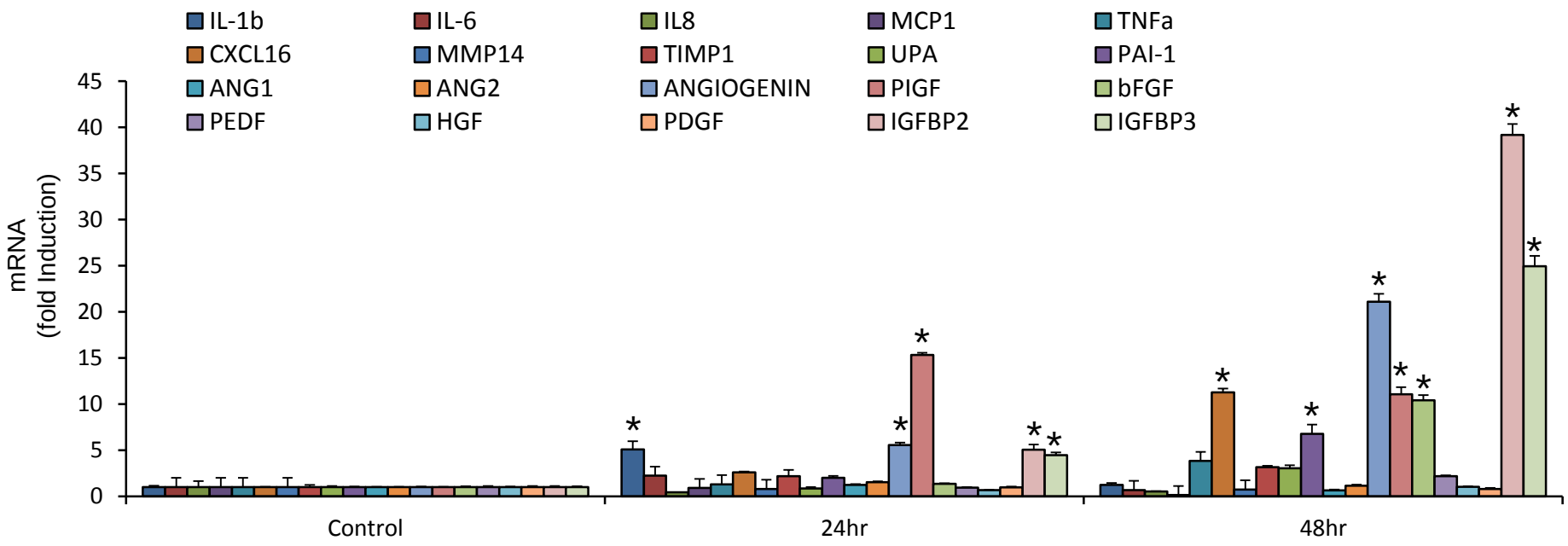
IL-1b
IL-6
IL8
MCP1
TNFa
CXCL16
MMP14
TIMP1
UPA
PAI-1
ANG1
ANG2
ANGIOGENIN
PIGF
bFGF
PEDF
HGF
PDGF
IGFBP2
IGFBP3
mRNA (fold Induction)
45
40
35
30
25
20
15
10
5
0
Control
24hr
48hr

fig. S7

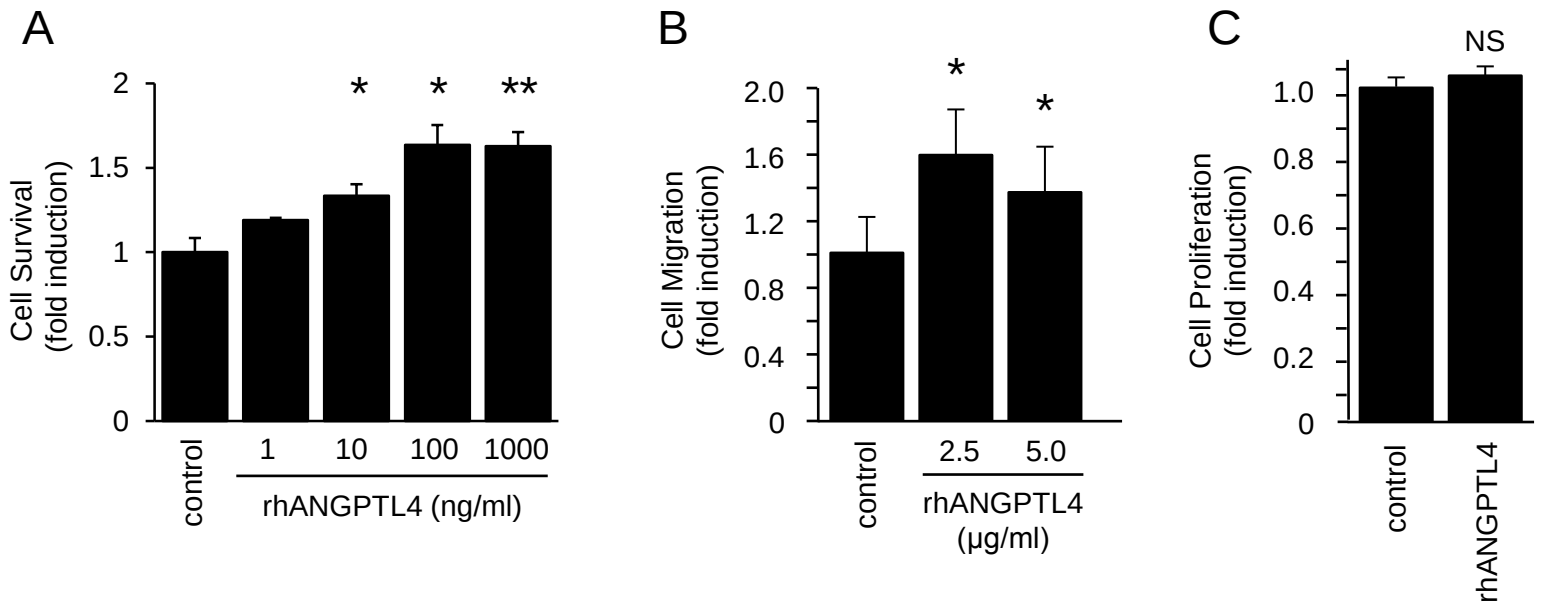
A
Cell Survival
(fold induction)
2
1.5
1
0.5
0
*
*
**
control
1
10
100
1000
rhANGPTL4 (ng/ml)
B
Cell Migration
(fold induction)
2.0
1.6
1.2
0.8
0.4
0
*
*
control
2.5
5.0
rhANGPTL4
(μg/ml)
C
Cell Proliferation
(fold induction)
1.0
0.8
0.6
0.4
0.2
0
NS
control
rhANGPTL4

fig. S8

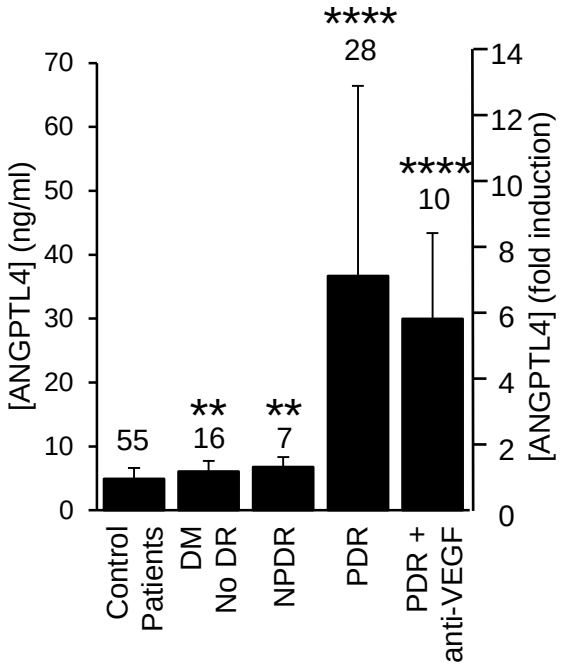
[ANGPTL4] (ng/ml)
[ANGPTL4] (fold induction)
0
10
20
30
40
50
60
70
0
2
4
6
8
10
12
14
55
16
7
28
10
**
**
****
****
Control Patients
DM No DR
NPDR
PDR
PDR + anti-VEGF

fig. S9

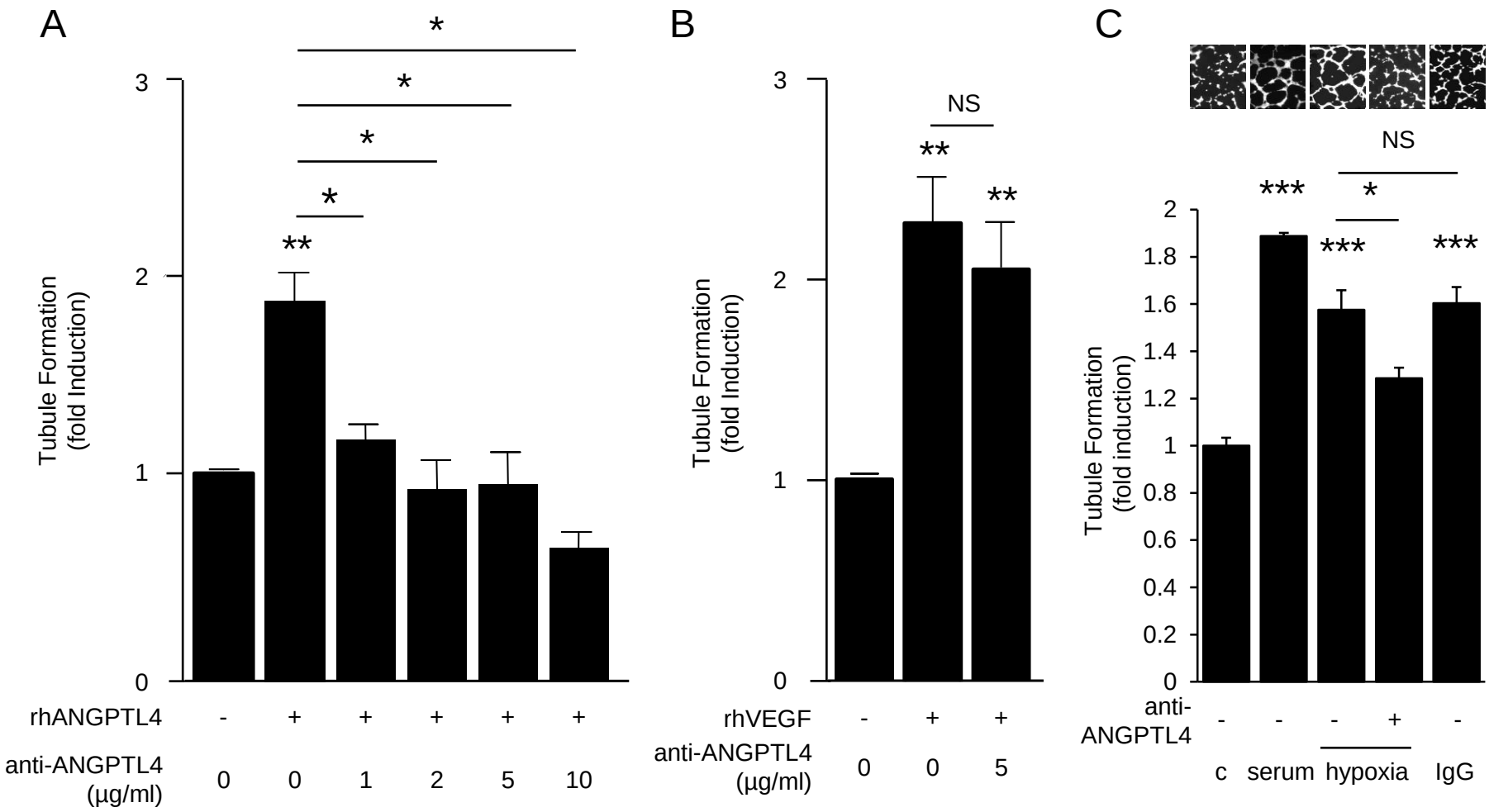
A
B
C
Tubule Formation (fold Induction)
Tubule Formation (fold induction)
rhANGPTL4
anti-ANGPTL4 (µg/ml)
rhVEGF
anti-ANGPTL4 (µg/ml)
anti-ANGPTL4
c
serum
hypoxia
IgG
NS
*
**
***

fig. S10

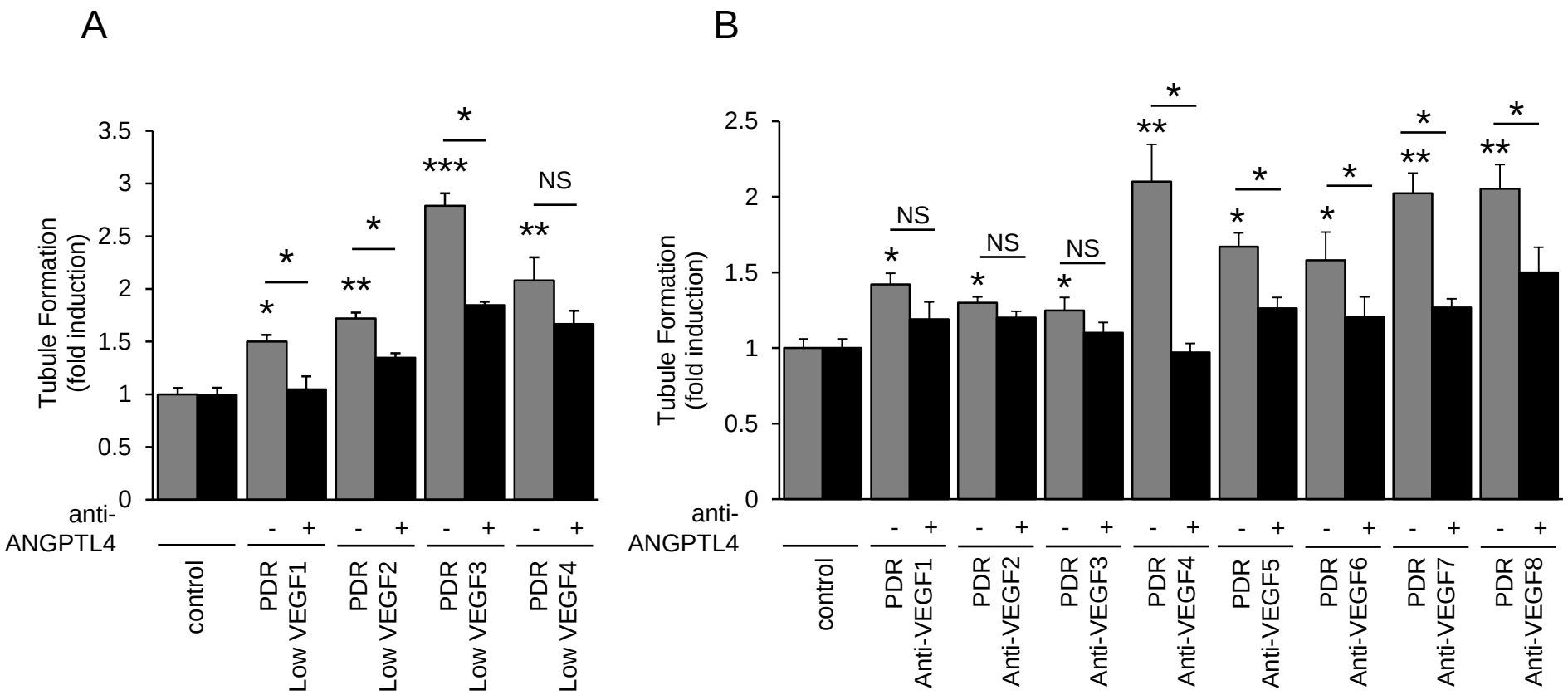
A
B
Tubule Formation
(fold induction)
anti-
ANGPTL4
control
PDR
Low VEGF1
PDR
Low VEGF2
PDR
Low VEGF3
PDR
Low VEGF4
PDR
Anti-VEGF1
PDR
Anti-VEGF2
PDR
Anti-VEGF3
PDR
Anti-VEGF4
PDR
Anti-VEGF5
PDR
Anti-VEGF6
PDR
Anti-VEGF7
PDR
Anti-VEGF8
NS

fig. S11

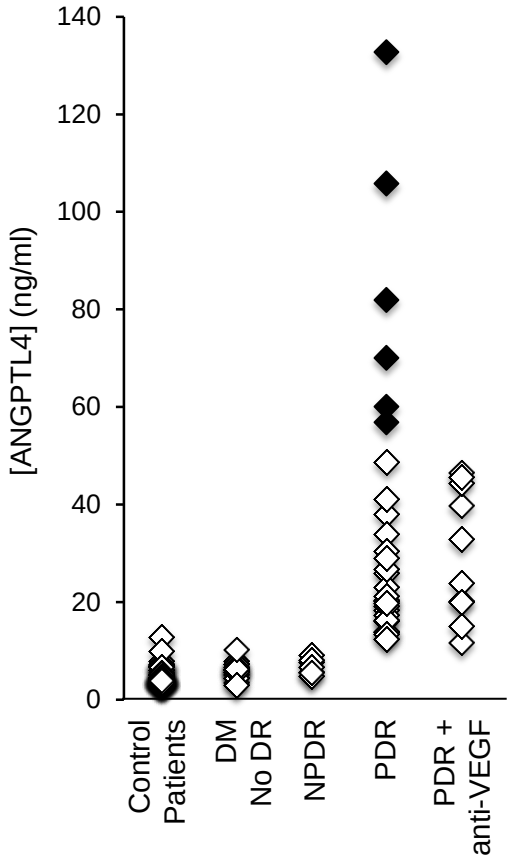
140
120
100
80
60
40
20
0
[ANGPTL4] (ng/ml)
Control Patients
DM No DR
NPDR
PDR
PDR + anti-VEGF

fig. S12

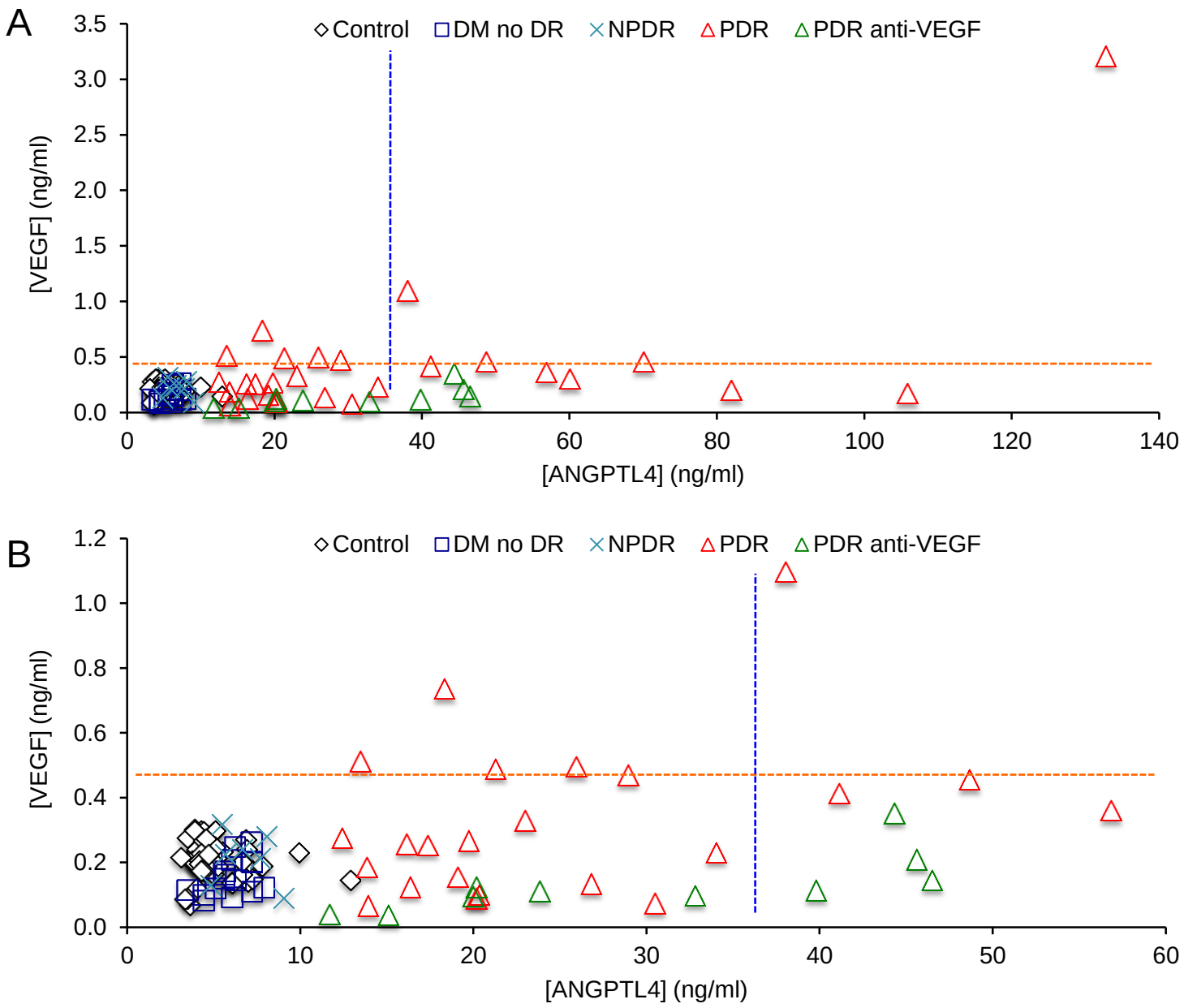
A
Control
DM no DR
NPDR
PDR
PDR anti-VEGF
[VEGF] (ng/ml)
[ANGPTL4] (ng/ml)
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
0
20
40
60
80
100
120
140
B
Control
DM no DR
NPDR
PDR
PDR anti-VEGF
[VEGF] (ng/ml)
[ANGPTL4] (ng/ml)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
0
10
20
30
40
50
60

Table S1: VEGF Patient Demographics

Characteristic	Control N=55[a]	DM (no DR) N=16	DM (NPDR) N=7	PDR N=28[b]	PDR, anti-VEGF within 2 weeks N=10[c]
Age – mean years ± SD	67.3 ± 9.8	69.1 ± 10.7	73.0 ± 9.8	51.1 ± 13.2	47.9 ± 12.2
Female sex – no. (%)	38 (69.1)	11 (68.8)	6 (85.7)	11 (37.9)	3 (30)
CVD[d] – no. (%)	28 (50.9)	14 (87.5)	7 (100.0)	23 (82.1)	9 (90)
Prior vitrectomy – no. (%)	0 (0.0)	1 (6.3)	0 (0.0)	5 (17.9)	1 (10)

DM, diabetes mellitus. DR, diabetic retinopathy. NPDR, nonproliferative diabetic retinopathy. PDR, proliferative diabetic retinopathy. VEGF, vascular endothelial growth factor. SD, standard deviation. CVD, cardiovascular disease.

[a] Includes 1 patient who had an aqueous sample taken from the same eye twice and 4 patients who had an aqueous sample taken from each eye

[b] Includes 3 patients who had an aqueous sample taken from the same eye twice and 1 patient who had an aqueous sample taken from each eye

[c] Includes 1 patient who had an aqueous sample taken from each eye

[d] Includes any patient with a history of hypertension, hypercholesterolemia, coronary artery disease, or cerebral vascular accident

Table S2: ANGPTL4 Patient Demographics (Aqueous)

Characteristic	Control N=55[a]	DM (no DR) N=16	DM (NPDR) N=7	PDR N=28[b]	PDR, anti-VEGF within 2 weeks N=10[c]
Age – mean years ± SD	68.0 ± 9.8	68.8 ± 9.8	73.0 ± 9.8	51.1 ± 13.2	48.9 ± 12.2
Female sex – no. (%)	41 (68.3)	15 (75.0)	6 (85.7)	11 (37.9)	3 (30)
CVD[d] – no. (%)	31 (51.7)	18 (90.0)	7 (100.0)	23 (82.1)	9 (90)
Prior vitrectomy – no. (%)	0 (0.0)	1 (5.0)	0 (0.0)	5 (17.9)	1 (10)

DM, diabetes mellitus. DR, diabetic retinopathy. NPDR, nonproliferative diabetic retinopathy. PDR, proliferative diabetic retinopathy. VEGF, vascular endothelial growth factor. SD, standard deviation. CVD, cardiovascular disease.

[a] Includes 1 patient who had an aqueous sample taken from the same eye twice and 4 patients who had an aqueous sample taken from each eye

[b] Includes 1 patient who had an aqueous sample taken from each eye

[c] Includes 3 patients who had an aqueous sample taken from the same eye twice and 1 patient who had an aqueous sample taken from each eye

[d] Includes 1 patient who had an aqueous sample taken from each eye

[e] Includes any patient with a history of hypertension, hypercholesterolemia, coronary artery disease, or cerebral vascular accident

Table S3: ANGPTL4 Patient Demographics (Vitreous)

Characteristic	Control N=10	PDR N=10
Age – mean years ± SD	63.8 ± 8.7	55.0 ± 7.6
Female sex – no. (%)	3 (30)	5 (50)
CVD[a] – no. (%)	7 (70)	8 (80)

PDR, proliferative diabetic retinopathy. SD, standard deviation. CVD, cardiovascular disease.

[a] Includes any patient with a history of hypertension, hypercholesterolemia, coronary artery disease, or cerebral vascular accident

Table S4: ANGPTL4 and VEGF levels in PDR Patients (Aqueous)

Group	PDR N=28
High VEGF, low ANGPTL4 – no. (%)	5 (18)
Low VEGF, high ANGPTL4 – no. (%)	5 (18)
Low VEGF, low ANGPTL4 – no. (%)	14 (50)
High VEGF, high ANGPTL4 – no. (%)	4 (14)

PDR, proliferative diabetic retinopathy.
VEGF, vascular endothelial growth factor.
ANGPTL4, angiopoietin-like 4.

Table S5: Primers (human)

mRNA	Primer
IL-1b	Forward: 5’ AGCTACGAATCTCCGACCAC 3’ Reverse: 5’ CGTTATCCCATGTGTCGAAGAA 3’
MMP14	Forward: 5’ GGCTACAGCAATATGGCTACC 3’ Reverse: 5’ GATGGCCGCTGAGAGTGAC 3’
Angiogenin	Forward: 5’ CGTCCGTGTACACACACTCA 3’ Reverse: 5’ GCACGAAGACCAACAACAAA 3’
IGFBP2	Forward: 5’ CCTCTACTCCCTGCACATCC 3’ Reverse: 5’ AGGTTGTACAGGCCATGCTT 3’
IL-6	Forward: 5’ GATGAGTACAAAAGTCCTGATCCA 3’ Reverse: 5’ CTGCAGCCACTGGTTCTG T 3’
TIMP1	Forward: 5’ CTGTTGTTGCTGTGGCTGATA 3’ Reverse: 5’ CCGTCCACAAGCAATGAGT 3’
PIGF	Forward: 5’ GTTCAGCCCATCCTGTGTCT 3’ Reverse: 5’ CTTCATCTTCTCCCGCAGAG 3’
IGFBP3	Forward: 5’ TCTGCGTCAACGCTAGTGC 3’ Reverse: 5’ GCTCTGAGACTCGTAGTCAACT 3’
IL-8	Forward: 5’ AGACAGCAGAGCACACAAGC 3’ Reverse: 5’ ATGGTTCCT TCCGGTGGT 3’
UPA	Forward: 5’ GGGAATGGTCACTTTTACCGAG 3’ Reverse: 5’ GGGCATGGTACGTTTGCTG 3’
bFGF	Forward: 5’ CCACTTCAAGGACCCCAAG 3’ Reverse: 5’ TGAGGGTCGCTCTTCTCC 3’

mRNA	Primer
ANGPTL4	Forward: 5’ GGACACGGCCTATAGCCTG 3’ Reverse: 5’ CTCTTGGCGCAGTTCTTGTC 3’
MCP1	Forward: 5’ TGCAGAGGCTCGCGAGCTA 3’ Reverse: 5’ CAGGTGGTCCATGGAATCCTGA 3’
PAI-1	Forward: 5’ AGCTCCTTGTACAGATGCCG 3’ Reverse: 5’ ACAACAGGAGGAGAAACCCA 3’
PEDF	Forward: 5’ TGAGAAGAAGCTGCGCATAA 3’ Reverse: 5’ ACCGAGAAGGAGAATGCTGA 3’
VEGF	Forward: 5’ GGGCAGAATCATCACGAAGT 3’ Reverse: 5’ TGGTGATGTTGGACTCCTCA 3’
TNFa	Forward: 5’ GGCTCAGACATGTTTTCCGTGA 3’ Reverse: 5’ CTCAGCAATGAGTGACAGTTGG 3’
ANG1	Forward: 5’ GAAGGGAACCGAGCCTATTC 3’ Reverse: 5’ GCTCTGTTTTCCTGCTGTCC 3’
HGF	Forward: 5’ ATCAAATGTCAGCCCTGGAG 3’ Reverse: 5’ TCGATAACTCTCCCCATTGC 3’
CXCL16	Forward: 5’ ACTACACGAGGTTCCAGCTCC 3’ Reverse: 5’ CTTTGTCCGAGGACAGTGATC 3’
ANG2	Forward: 5’ GCAAGTGCTGGAGAACATCA 3’ Reverse: 5’ GTTAACTTCCGCGTTTGCTC 3’
PDGF	Forward: 5’ TTCCCTGACCATTGCTGA 3’ Reverse: 5’ AGGAAGTTGGCGTTGGTG 3’

Table S6: Primers (mouse)

mRNA	Primer
VEGF	Forward: 5’ TTACTGCTGTACCTCCACC 3’ Reverse: 3’ ACAGGACGGCTTGAAGATG 3’
ANGPTL4	Forward: 5’ TTGGTACCTGTAGCCATTCC 3’ Reverse: 3’ GAGGCTAAGAGGCTGCTGTA 3’
Cyclophilin A	Forward: 5’ AGCATACAGGTCCTGGCATC 3’ Reverse: 3’ TTCACCTTCCCAAAGACCAC 3’