Article title: Involvement of $\alpha_v\beta_3$ integrin in gremlin-induced angiogenesis

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Online Resource 1:

Figure Legends:

Fig. S1. β_3 integrin down-regulation inhibits the chemotactic activity of gremlin. **a)** HUVECs were treated for 24 h with anti-β_3 or control nontargeting (nt) siRNAs and analysed for surface β_3 integrin expression by FACS. Cells were incubated on ice with anti-β_3 integrin antibody (BV4) and with anti-mouse Alexafluor 488 IgG. The secondary Ab alone was used as a control (red line). FACS analysis was performed with a Cyflow Partec flow cytometer (Partec). β_3 integrin down-modulation caused a partial but significant reduction of β_3 integrin expression on EC surface (orange line). **b)** siRNA transfected HUVECs were assessed for their capacity to migrate in response to 50 ng/ml of gremlin or 30 ng/ml VEGF-A in a Boyden chamber assay. After 4 h, cells migrated to the lower side of the filter were counted and data were expressed as fold increase *versus* cells migrated in the absence of a chemotactic stimulus. β_3-siRNA transfection suppresses the chemotactic activity of gremlin and VEGF-A (*, $P<0.05$, Student's *t* test).

Fig. S2. Kinetics of VEGFR2/β_3 integrin complex formation. **a)** Serum-starved HUVECs were incubated for 3, 15, 30, 60 min with 50 ng/ml gremlin or 30 ng/ml VEGF-A. Next, cell lysates were tested in a sandwich ELISA for the presence of VEGFR2/β_3 integrin complexes as detailed in Materials and Methods section. **b)** Anti-β_3 integrin antibody BV4 prevents VEGFR2/$\alpha_v\beta_3$ integrin complex formation in ECs. HUVECs were incubated for 15 min at room temperature with 30 ng/ml VEGF-A in absence or presence of anti-β_3 integrin antibody BV4. Cells were then lysed and VEGFR2/β_3 integrin complexes were quantified by sandwich ELISA**.**

Fig. S3 Gremlin does not bind $\alpha_v\beta_3$ integrin. **a)** Parental and $\alpha_v\beta_3$ integrin-overexpressing HEK 293 cells were seeded on polystyrene non-tissue culture microtiter plates uncoated (control) or coated with 2 μg/ml of FG, CO, FN or gremlin. After 2 h, adherent cells were washed, fixed, stained with methylene blue/Azur II, solubilized with acetic acid and plates were read with a microplate reader at 595 nm. $\alpha_v\beta_3$ integrin overexpression caused a significant increase of HEK 293 cell adhesion to immobilized FG but not to the other immobilized proteins (*, $P<0.05$, Student's *t* test). **b)** Serum-starved HUVECs were incubated for 2 h at 4°C in the absence or in the presence of 50 ng/ml gremlin and the BS3 cross-linker. After Tris-HCl saturation, cells were lysed and cell lysates (1.0 mg of protein) were immunoprecipitated with an anti-β_3 integrin antibody (clone BV4), separated on a SDS-PAGE gel under reducing conditions, and probed in a Western blot with anti-β_3 integrin and anti-gremlin antibodies. Note the absence of any gremlin-integrin complex in the anti-β_3 IP of gremlin-treated sample

Fig. S4 Kinetics of VEGFR2 activation by gremlin in FG-adherent ECs. HUVECs were seeded on FG or on uncoated wells in M199 *plus* 5% FCS. 2 h after plating, cells were stimulated with 50 ng/ml gremlin for 0, 3, 5, 15 or 30 min. Then, 50 μg of total cell lysates were separated on SDS-

PAGE and assessed for VEGFR2 phosphorylation in a Western blot using an anti-phospho-VEGFR2 (pTyr1175) antibody (upper panel). Uniform loading of the gel was confirmed by re-probing the membrane with an anti-VEGFR2 antibody (lower panel)

Fig. S1

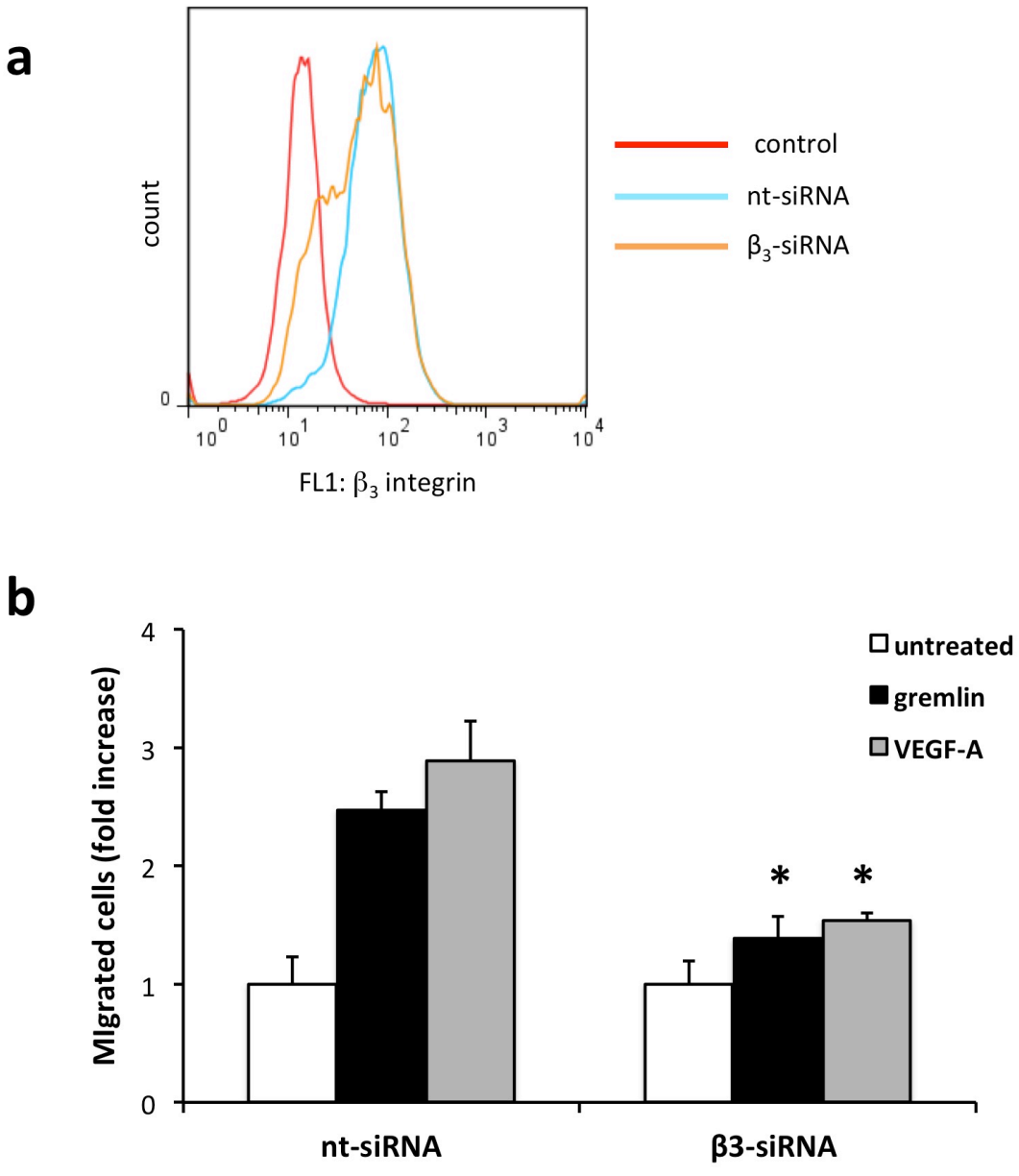

a
count
0
10^0
10^1
10^2
10^3
10^4
FL1: β_3 integrin
control
nt-siRNA
β_3-siRNA
b
Migrated cells (fold increase)
0
1
2
3
4
untreated
gremlin
VEGF-A
*
*
nt-siRNA
β3-siRNA

Fig. S2

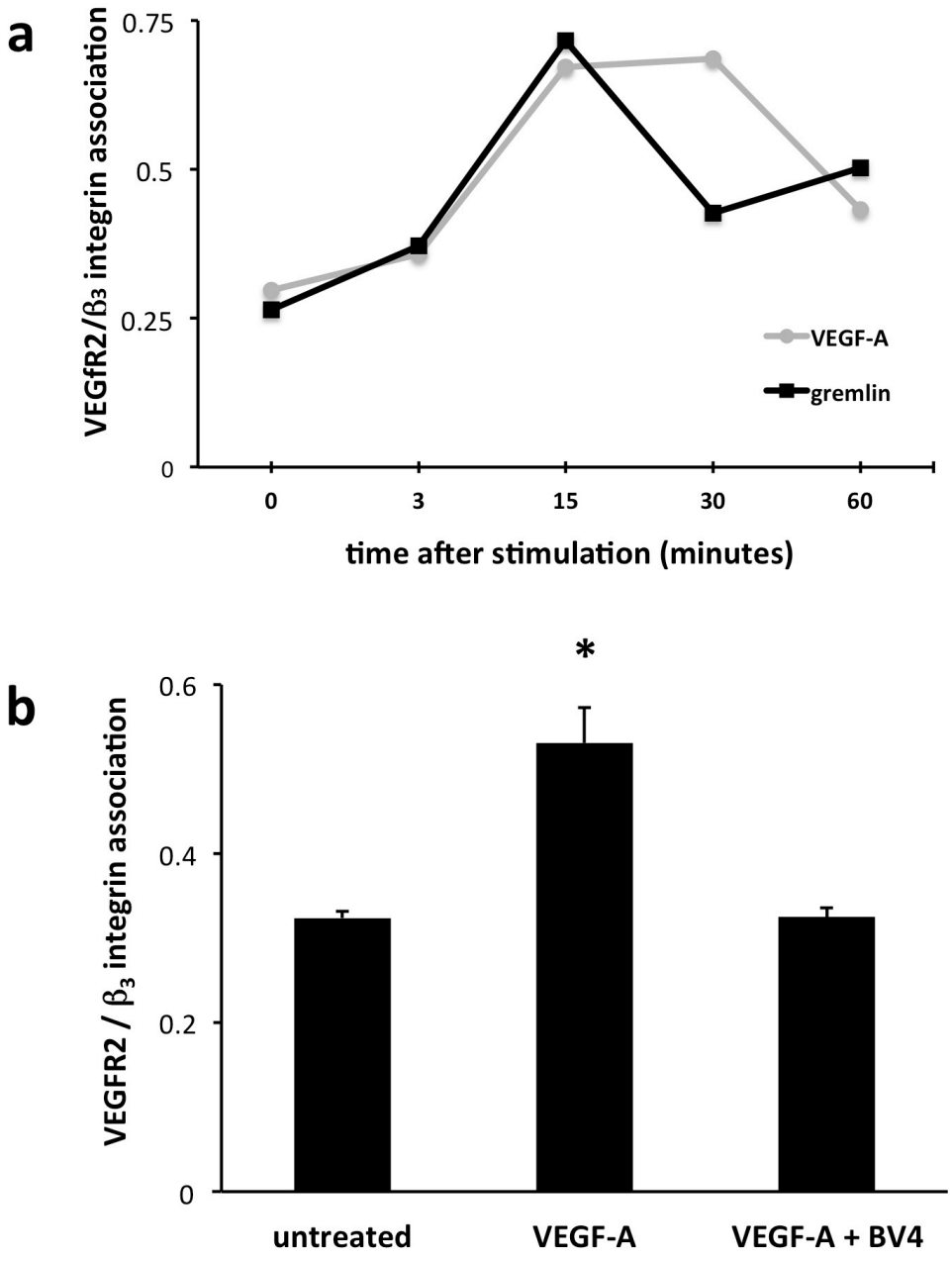

a
VEGfR2/β3 integrin association
0.75
0.5
0.25
0
VEGF-A
gremlin
0
3
15
30
60
time after stimulation (minutes)
b
*
VEGFR2 / β3 integrin association
0.6
0.4
0.2
0
untreated
VEGF-A
VEGF-A + BV4

Fig. S3

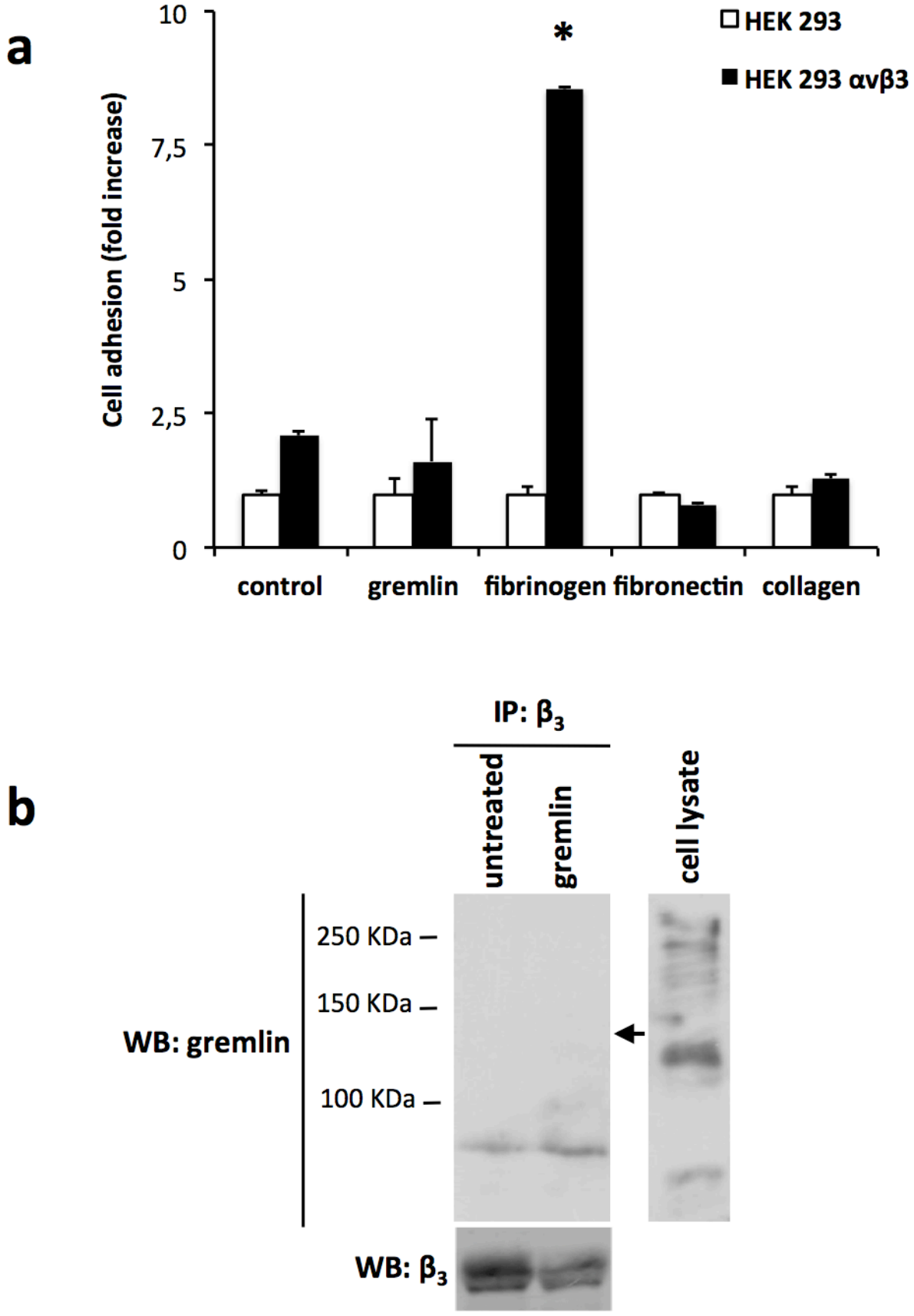
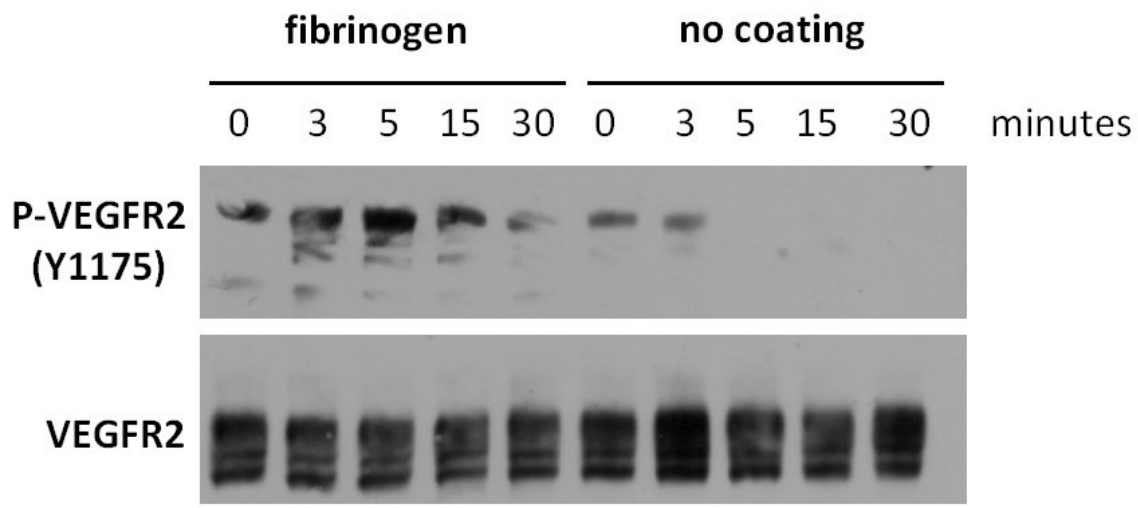

a
10
7,5
5
2,5
0
Cell adhesion (fold increase)
*
HEK 293
HEK 293 αvβ3
control
gremlin
fibrinogen
fibronectin
collagen
b
IP: β3
untreated
gremlin
cell lysate
250 KDa
150 KDa
100 KDa
WB: gremlin
WB: β3

Fig. S4


fibrinogen
no coating
0 3 5 15 30 0 3 5 15 30 minutes
P-VEGFR2 (Y1175)
VEGFR2