Supplemental Material

Supplemental Materials and Methods

Culture of stem cells and EBs

Embryonic stem cells (ESCs) were routinely grown on mouse embryonic fibroblasts in DMEM with glutaMAX (Invitrogen), 25mM HEPES, 1.2mM sodium pyruvate, 19mM monothioglycerol (Sigma), 15% fetal bovine serum, and 1000 units/ml of leukemia inhibitory factor (LIF). EBs were generated as previously described[1]. Briefly, at day 0, 1200 ESCs were aggregated in hanging drops (20 µl) without LIF and VEGFA. After 4 days, EBs were seeded either in 10 cm tissue culture dishes (for PDGFRß signaling assays), in T75 flasks (for metabolic labeling), in T225 flasks (for HS and CS compositional analysis) or into a gel of collagen I (for analysis of angiogenic sprouting by immunofluorescence), in the presence or absence of 30 ng/ml recombinant $VEGFA_{165}$ (PreproTech). In rescue experiment, PDGFB (20 ng/ml or 50 ng/ml) and TGFβ1 (2 ng/ml or 5 ng/ml) both obtained from Preprotech were added to VEGFA-induced *Ext1*$^{-/-}$ cultures between day 8 and day 12. The EBs were normally harvested day 12 unless indicated otherwise.

Isolation of metabolically labeled HS and CS

After a 20 h incubation of ESCs or EBs with ^{35}S-sulfate, the cell media was discarded, and the cells washed four times with cold phosphate-buffered saline followed by incubation in 2 ml of solubilization buffer (50 mM Tris-HCl, pH 8, 1% Triton X-100, 0.10 M NaCl), containing the protease inhibitors EDTA, phenylmethanesulfonyl fluoride, and pepstatin A at 4°C for 1 h. After centrifugation at 13000 x *g* for 10 min,

the proteoglycans were isolated from the solubilized cell lysate on a 0.3-ml column of DEAE-Sephacel (GE Healthcare Biosciences), equilibrated in 50 mM Tris-HCl pH 8, 0.1% Triton X-100, 0.1 M NaCl. The column was washed first with equilibration buffer and subsequently with 50 mM acetate buffer, pH 4.0 containing 0.1% Triton X-100 and 0.1 M NaCl. The proteoglycans were eluted with 50 mM acetate buffer, pH 4.0, containing 0.1% Triton X-100 and 2 M NaCl and alkali-treated as described[2]. After desalting in water on PD10 columns (GE Healthcare Biosciences) followed by lyophilization, the ^{35}S-labeled GAGs were subjected to enzymatic or chemical degradation, see the main text.

Isolation of HS and CS for compositional analysis by RPIP-HPLC

Cells or EBs were dissolved in 0.5 ml of Pronase buffer (50 mM Tris/HCl, pH 8.0,1 % Triton X-100, 1 mM $CaCl_2$, 0.8 mg/ml Pronase) and incubated end-over-end for 19 h at 55 °C. After heat inactivation of the enzyme, $MgCl_2$ was added to a final concentration of 2 mM. After addition of Benzonase (12 milliunits), the sample was incubated for 2 h at 37 °C followed by heat inactivation. The NaCl concentration was subsequently adjusted to 0.1 M and the sample centrifuged at 13,000 x g for 10 min. The supernatant was diluted with 0.5 ml 50 mM Tris/HCl pH 8.0, 0.1 M NaCl, and applied to a Sep-Pak® C18 cartridge (Waters) that had been primed first with methanol, then with water and finally with 50 mM Tris/HCl pH 8.0, 0.1 M NaCl. The cartridge was washed with 2 ml of the Tris/HCl buffer and the washing fraction combined with the flow through fraction and applied to a 0.2 ml DEAE-Sephacel column equilibrated in loading buffer (50 mM Tris/HCl pH 8, 0.1 % Triton X-100, 0.1 M NaCl). After washing with six column volumes of loading buffer, six volumes of low pH buffer (50 mM NaAc, pH 4.0, 0.1% TritonX-100, 0.1 M NaCl) and six

volumes of loading buffer without Triton X-100, the GAGs were eluted with 0.6 ml of elution buffer (50 mM Tris/HCl, pH 8.0, 1.5 M NaCl). The samples were next desalted using NAP-10 columns (GE Healthcare Biosciences) equilibrated in water, and dried by SpeedVac centrifugation. The GAG pool was digested with 50 milliunits of CSase ABC in 100 μl 40 mM Tris/Ac buffer pH 8.0. The CS digestion was allowed to proceed for 4 h at 37 °C and the sample was then boiled to stop the reaction. After removal of 10 μl for CS analysis by RPIP-HPLC as previously described[3], HS was recovered after a second round of DEAE-Sephacel chromatography as described for total GAG isolation. Purified HS was dissolved in 200 μl of heparinase buffer (5 mM Hepes buffer, pH 7.0, 50 mM NaCl, 1 mM $CaCl_2$) and divided into two equal aliquots. One of the aliquots was treated with 0.4 milliunits each of heparitinases I, II and III and incubated for 16 h at 37 °C. The other aliquot (control sample) was incubated under the same conditions without enzymes. After heat inactivation the samples were analyzed by RPIP-HPLC as described by Ledin and co-workers[3].

Isolation of endothelial cells from EBs and quantitative RT-PCR

Sheep anti-rat IgG Dynabeads (Dynal, Invitrogen) were coated with rat anti-mouse CD31 (BD Pharmingen) and incubated with collagenase-treated wild type or *Ext1*$^{-/-}$ EBs for 30 min at 4°C. The CD31+ and CD31- cell populations were collected according to the manufacturers instructions for RNA isolation using Qiazol lysis buffer (Qiagen). Samples were subjected to reverse transcription (Superscript III, Invitrogen) and amplified using intron spanning primers to CSPGs and CS biosynthesis enzymes. Quantitation was done by the 2ΔΔCT method, using β-actin as control RNA[4]. CT values from triplicate assays were used to calculate fold-expression as compared to β-actin. The results shown in Fig. 3 are based on three independent

assays, and the mean values ± s.d. are shown. The statistical significance was determined using ANOVA. See Supplemental Table 1 for information about the primers used to detect PG core protein transcripts.

Immunofluorescence

At day 12 of differentiation, whole EBs in collagen gel were cut out and fixed in 4% p-formaldehyde in phosphate-buffered saline (PBS) for 30 min at room temperature, and thereafter blocked and permeabilized in 3% BSA, 0.1% Tween20 in PBS for one h. EBs were incubated overnight at 4°C with primary antibodies: rat anti-mouse CD31 antibody (Pharmingen) and rabbit polyclonal anti-NG2 antibody (AB5320, Chemicon International). Secondary antibodies used were: Alexa 488 goat anti-rat IgG and Alexa 555 goat anti-rabbit IgG (Molecular Probes). Samples were analyzed in a Zeiss Axiovert 200M microscope equipped with a Zeiss AxioCam MRm camera and pictures were taken using Axiovision software (Zeiss). Quantification of the morphology and localization of NG2+ cells in the different cultures was scored in a blind fashion. Pictures were taken at 20X magnification (11 EBs for each genotype; in total 420 cells were scored in wt and 1100 cells in *Ext1*$^{-/-}$EBs). Results are presented as mean ± s.d. and the statistical significance was determined using the ANOVA test. hAoSMCs were stained for CS using mouse monoclonal anti-CS antibody (Sigma; clone CS-56), and HUVECs were stained for degraded HS or sulfated HS using the 3G10 or HepSS1 antibodies respectively (both from Seigakaku).

Western blotting and signaling assays

Smad2, PDGFRβ and VEGFR2 phosphorylation and downstream target activation of PDGFRβ in response to TGFβ1, PDFGB or VEGFA165 stimulation respectively

were analyzed in *Ext1*$^{-/-}$ and wt EBs cultured in regular cell culture dishes from day 4 to 11 in complete medium. At day 11, the cultures were starved in 0.5% serum for 16 hs before TGFβ1, PDGFB or VEGFA165 (PeproTech) was added to give the final concentration of 5, 100 or 50 ng/ml respectively. All EBs were harvested and corresponding cell lysates prepared as previously described [1].

HUVECs and hAoSMCs were starved in 1% serum for 16 hours, and thereafter treated with heparitinase III (1U/ml) or CSase ABC (25mU/ml) for 1.5 h, thereafter rinsed 3 times with starvation media and stimulated with VEGFA165 (100 ng/ml; 5 min), TGFβ1 (5 ng/ml; 1h) or PDGFB (50 ng/ml; 5 min) prior to harvest for analysis using Western blotting. For immunoprecipitation experiments, the cell lysates were incubated with polyclonal anti-PDGFRß (sc-432, Santa Cruz Biotechnologies) or anti-VEGFR2 (R&D, AF644 and AF357) antibodies for 2 hs at 4°C, followed by incubation with Immunosorb A beads (Medicago) at 4°C for 45 min. Proteins were released by boiling the beads in sample buffer (Tris-HCl pH 6.8, 1.5% SDS, 4.35% glycerol, 4% ß-mercaptoethanol, 0.0025% bromophenol blue). Immunoprecipitates or total cell lysates were separated on SDS polyacrylamide gel and transferred to a Hybond-ECL nitrocellulose membrane (GE Healthcare Biosciences). The membranes were incubated with the following primary antibodies: rabbit anti-phospho Smad2 (antibody kindly provided by Dr Aris Moustakas, the Ludwig Institute for Cancer Research, Uppsala, Sweden), mouse anti-Smad1/2/3 (sc-7960; Santa Cruz), mouse anti-phosphotyrosine (4G10; Upstate, Lake Placid, NY), rabbit polyclonal anti-PDGFRß antibody (sc-432; Santa Cruz), goat anti-human VEGFR2 (AF357; R&D Systems;), goat anti-mouse VEGFR2 (AF644; R&D Systems), rabbit anti-phospho Akt, rabbit anti-phospho-ERK1/2, rabbit anti-total Akt or rabbit anti-total ERK1/2 (all from Cell Signaling Technology). Immuno-reactivity

was visualized by enhanced chemiluminescence (ECL or ECL Plus Western Blotting System, GE Healthcare Biosciences). *Ndst1*$^{-/-}$ MEFs [5] were grown in high-glucose DMEM with 10% FBS, starved for 16 hrs in DMEM supplemented with 0.2 BSA and treated with CSase (75 mU/ml, 3 x 1 h incubations) prior to stimulation with PDGFB (100 ng/ml, 10 min) followed by analysis of cell lysates by Western blotting. All cell signaling experiments were repeated at least 3 times, representative blots are shown. The total levels of PDGFRβ in total EB lysates were normalizatied to the level of β-actin (detected by rabbit anti-β-actin antibody (968227; Abcam) in each sample. Quantified results obtained by Western blotting are presented as the mean value ± s.d. for the indicated number of experiments. The statistical significance was determined using the Student's *t*-test.

In Situ PLA Analysis of phospho-PDGFRβ in EBs

wt and *Ext1*$^{-/-}$ EBs were cultured for 14 days on glass slides in presence of VEGF (30 ng/ml). At day 14, the EBs were starved for 20 hs and then stimulated or not with PDGFB (100 ng/ml) for 1 h on ice. The samples were fixed using ethanol and thereafter blocked in 20% goat serum (Invitrogen) containing 2.5 ng/µl sonicated salmon sperm DNA (GE Healthcare Biosciences), 2.5 mM L-cysteine (Sigma), 0.1% Tween 20, 5 mM EDTA in PBS for 2 h at 37 °C. The following primary antibodies were applied and incubated overnight at 4 °C: mouse anti-phosphotyrosine (anti-pY100; #9411, Cell Signaling; 1: 250 final concentration) and rabbit anti-PDGFRβ (#3169, Cell Signaling; 2 ng/µl final concentration) dissolved in blocking buffer. Incubation with secondary proximity probes (4.9 ng/µl anti-mouse proximity probe and 1.6 ng/µl anti-rabbit proximity probe; Olink Biosciences AB, Uppsala, Sweden), hybridization, ligation, rolling circle amplification and detection were performed as

previously described [6] with one major modification; prior to the detection step, the samples were incubated overnight at 4°C with rat anti-CD31 and rabbit anti-NG2 antibodies. In the detection step secondary antibody anti-rat Alexa-633 (Invitrogen) and anti-rabbit FITC Fab_2-fragment (Jackson ImmunoResearch) were added. Z-stack images were collected using a LSM-500 confocal microscope (Zeiss) and converted to maximum intensity projections using ImageJ software (NIH). For better visualization, the signals (so called "blobs") were enhanced with a maximum filter (0.5 pixel radius) and the red channel subtracted from the green and blue channels before merging.

Supplemental figure legends

Supplemental Figure I. Phosphorylation of PDGFRß *in situ.* (A) Phosphorylation of PDGFRβ was analyzed *in situ* on wt and *Ext1*$^{-/-}$ EBs using the proximity ligation assay. Co-staining for CD31 (blue) and NG2 (green) was used to identify ECs and PCs respectively. Each red dot corresponds to one detected molecule of phosphorylated PDGFRβ. Red dots located outside NG2+ cells correspond to activated PDGFRβ in fibroblasts (counterstaining for fibroblasts not shown). (B) Addition of PDGFB or TGFβ1 to *Ext1*$^{-/-}$ cultures from day 8 to day 12 did not rescue the pericyte attachment phenotypes, but instead increased both the pericyte numbers and the extent of pericyte detachment.

Supplemental Figure II. PDGFB signaling in *Ext1*$^{-/-}$ EBs. (A) wt and *Ext1*$^{-/-}$ EBs were at day 12 stimulated with PDGFB for the indicated time periods. PDGFRβ

protein was immunoprecipitated (IP), followed by immunodetection of phosphotyrosine residues (P-Tyr), and for PDGFRβ. See the graph in Fig. 1O for a quantification of tyrosine-phosphorylated PDGFRβ relative to total PDGFRβ protein (n = 2). (B) Total cell lysates from wt and *Ext1*$^{-/-}$ EBs were analyzed for PDGFRβ expression by immunoblotting. The levels of PDGFRβ was normalized to the levels of β-actin in each sample (n = 3), a representative blot is shown in the top panel. (C) The ability of PDGFB to induce PDGFRβ downstream signaling was studied in wt and *Ext1*$^{-/-}$ EBs by immunoblotting of total lysates as indicated. Quantifications of the results are shown in the two lower panels (n = 4).

Supplemental Figure III. Composition of chondroitin sulfate from wt and *Ext1*$^{-/-}$ stem cells and EBs. CS was isolated and digested with CSase and the disaccharide products analyzed by RPIP-HPLC. 0S, *ΔHexuronic acid (HexA)*-GalNAc; 4S, *ΔHexA*-GalNAc4S; 6S, *ΔHexA*-GalNAc6S; 2S, *ΔHexA*2S-GalNAc; 6S4S, *ΔHexA*-GalNAc4S6S; 4S2S, *ΔHexA2S*-GalNAc4S; 6S4S2S, *ΔHexA2S*-GalNAc4S6S. Note that the 0S disaccharide pool also may contain disaccharides derived from hyaluronan (Hya) that was not completely removed during purification.

Supplemental Figure IV. qPCR screen to identify altered PG core protein mRNA expression in *Ext1*$^{-/-}$ EBs as compared to wt EBs. VEGFA-stimulated EBs of the respective genotype were harvested at day 12 of culture. Mechanical and enzymatic digestion of EBs was followed by isolation of CD31+ endothelial cells using magnetic Dynabeads coated with anti-CD31 antibodies. Thereafter total RNA from the endothelial cell fraction (A) and the left over cell fraction (B) was isolated and analyzed by qPCR to detect the mRNA levels of a set of PG core proteins known

to carry CS. See Supplemental Table I for the full gene names corresponding to the gene symbols shown here.

Supplemental Figure V. Verification of HEPase and CSase activity. The levels of HS in HUVECs (A) and CS in hAoSMCs (B) are reduced by heparitinase III (HEPase) and Chondroitinase ABC (CSase) treatments respectively. HUVECs were stained for neoepitopes generated by HEPase digestion using the 3G10 antibody, or stained for the presence of sulfated HS using the HepSS1 antibody. hAoSMCs were stained for CS using the CS-56 antibody.

Supplemental Figure VI. Effects of HEPase and CSase treatment on VEGFA and PDGFB signaling. (A) Activation of VEGFR2 in HUVECs as judged by phosphorylation of the receptor tyrosine residue 1175 and ERK1/2 phosphorylation was not significantly affected by treatment with HEPase or CSase. (B) Western blot analysis showing the activation of PDGFRβ by PDGFB in *Ndst1*$^{-/-}$ MEFs with impaired HS production. Here, treatment with CSase leads to reduced receptor phosphorylation (n = 3, representative blots are shown).

Supplemental Table

Supplemental Table I. List of primers used to detect the expression levels of PG core proteins in *Ext1*$^{-/-}$ and wt EB cultures.

References

1. Jakobsson L, Kreuger J, Holmborn K, Lundin L, Eriksson I, Kjellen L, Claesson-Welsh L. Heparan sulfate in trans potentiates VEGFR-mediated angiogenesis. *Dev Cell.* 2006;10:625-634.
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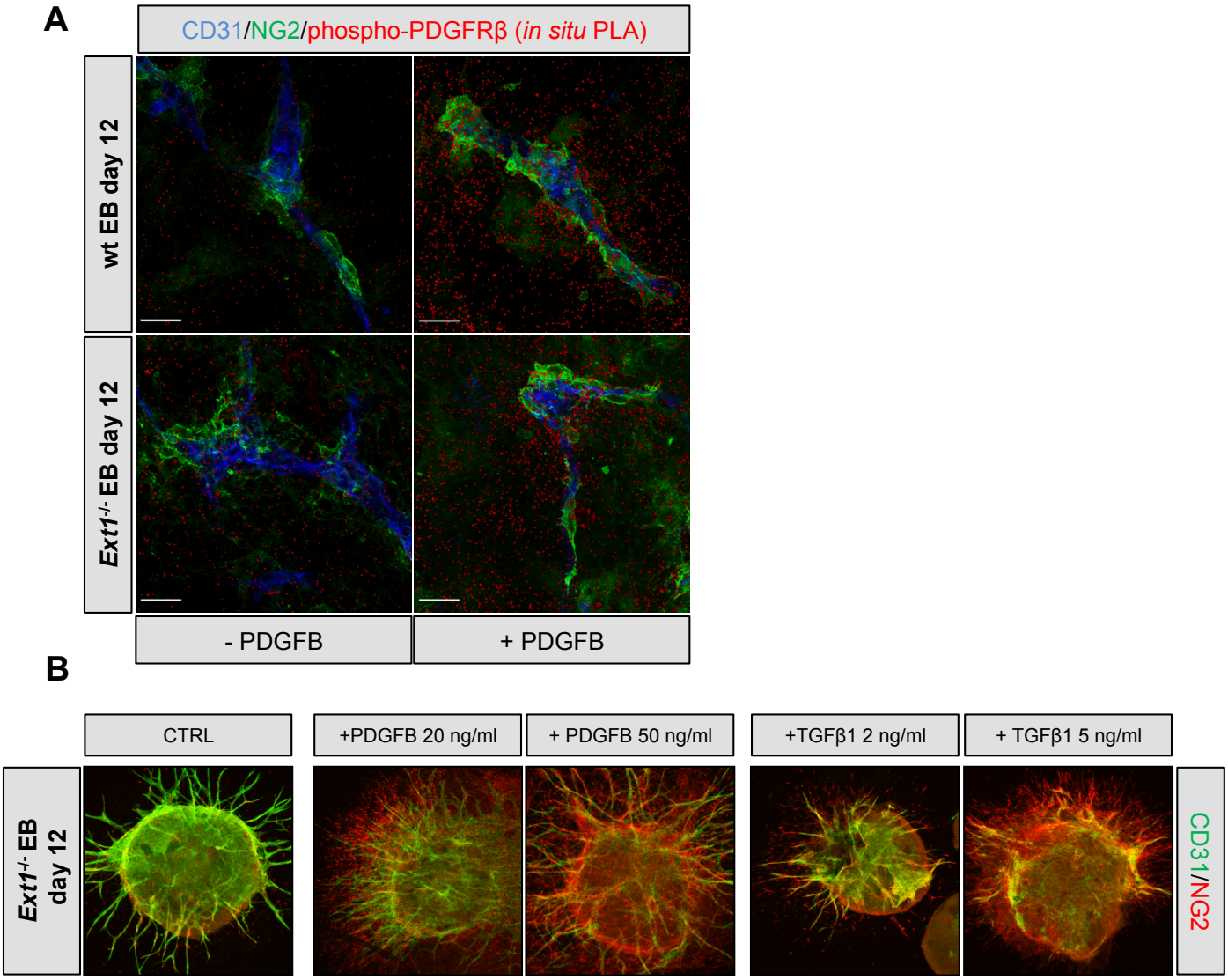
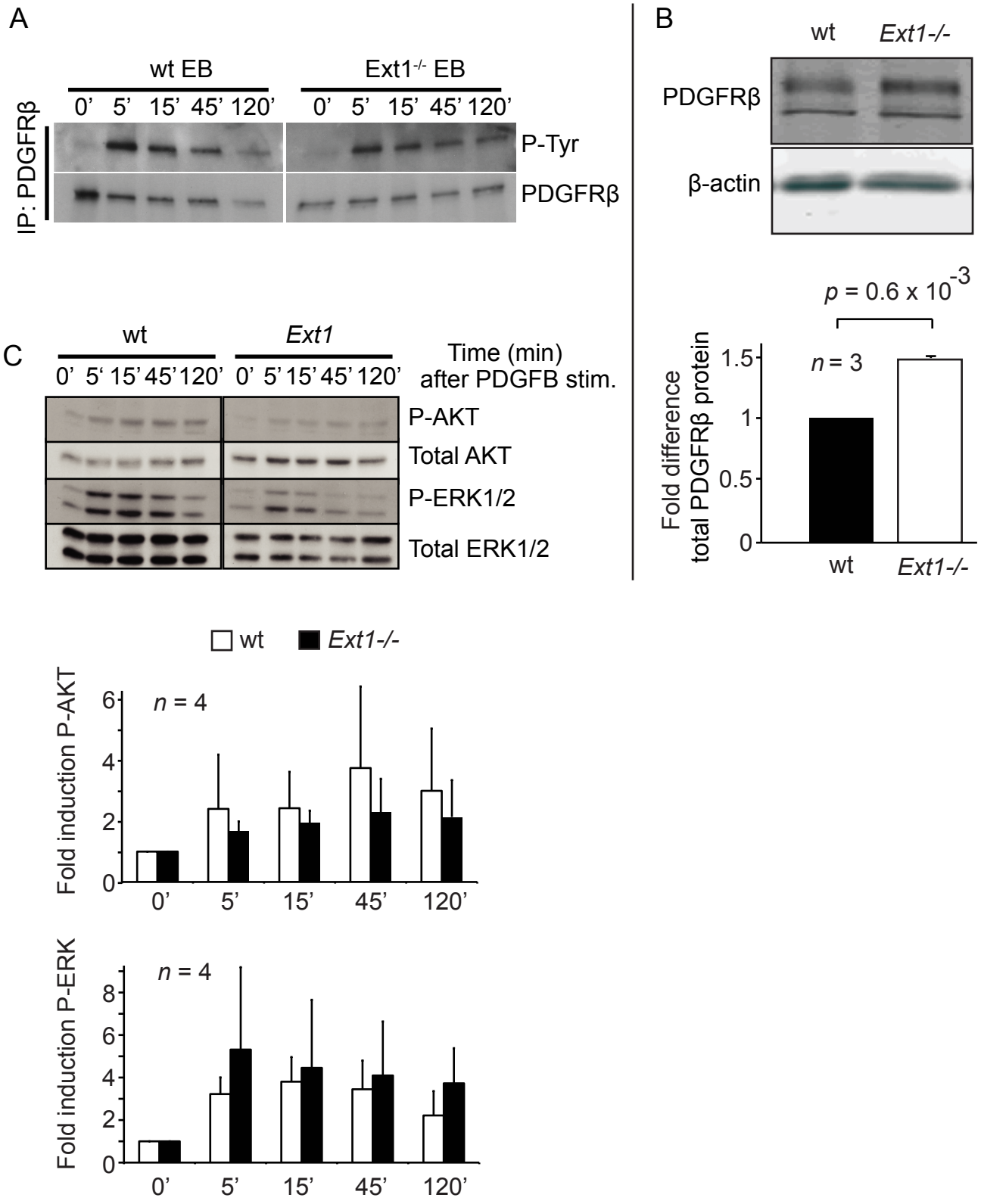
4. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods.* 2001;25:402-408.
5. Stenzel D, Nye E, Nisancioglu M, Adams RH, Yamaguchi Y, Gerhardt H. Peripheral mural cell recruitment requires cell-autonomous heparan sulfate. *Blood.* 2009;114:915-924.
6. Jarvius M, Paulsson J, Weibrecht I, Leuchowius KJ, Andersson AC, Wahlby C, Gullberg M, Botling J, Sjoblom T, Markova B, Ostman A, Landegren U, Soderberg O. In situ detection of phosphorylated platelet-derived growth factor receptor beta using a generalized proximity ligation method. *Mol Cell Proteomics.* 2007;6:1500-1509.

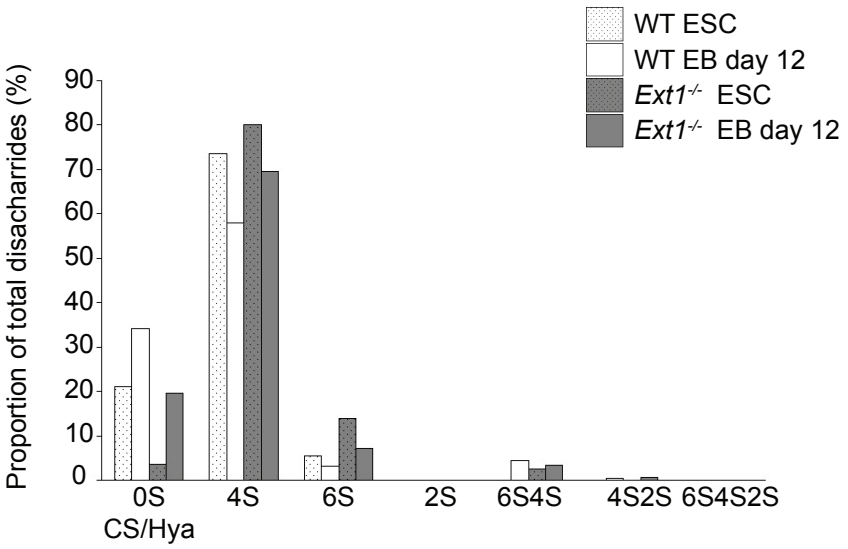
Supplemental Figure I, Le Jan et al.



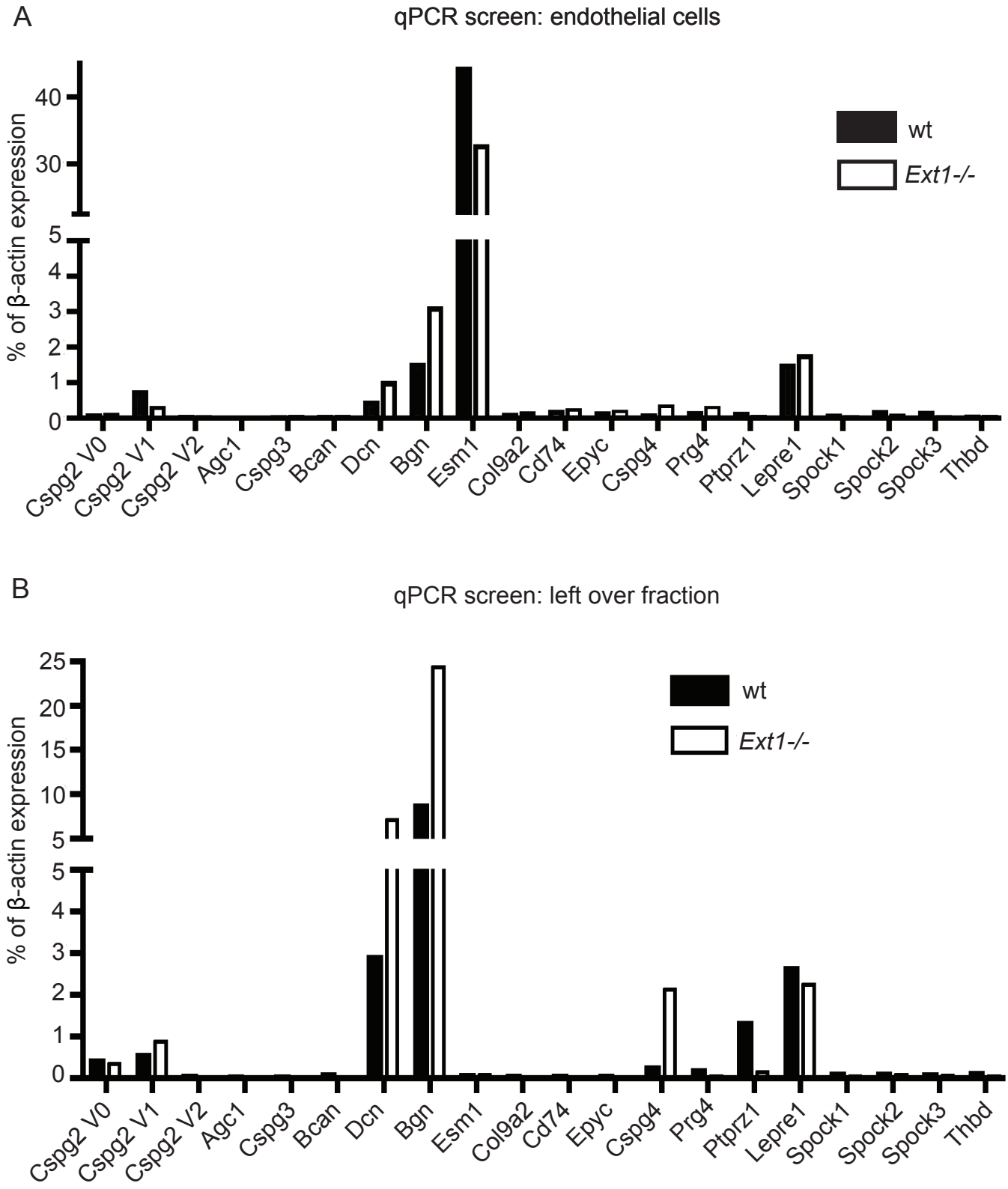
Supplemental Figure II, Le Jan et al.



Supplemental Figure III, Le Jan et al.

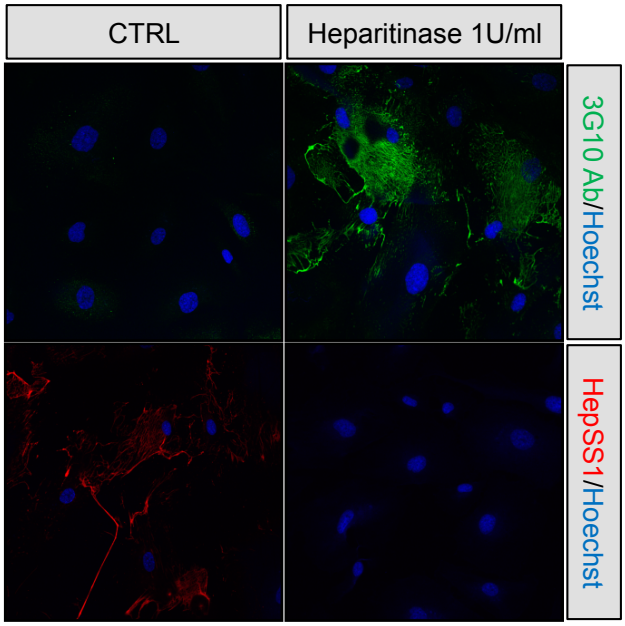


Supplemental Figure IV, Le Jan et al.



Supplemental Figure V, Le Jan et al.

A



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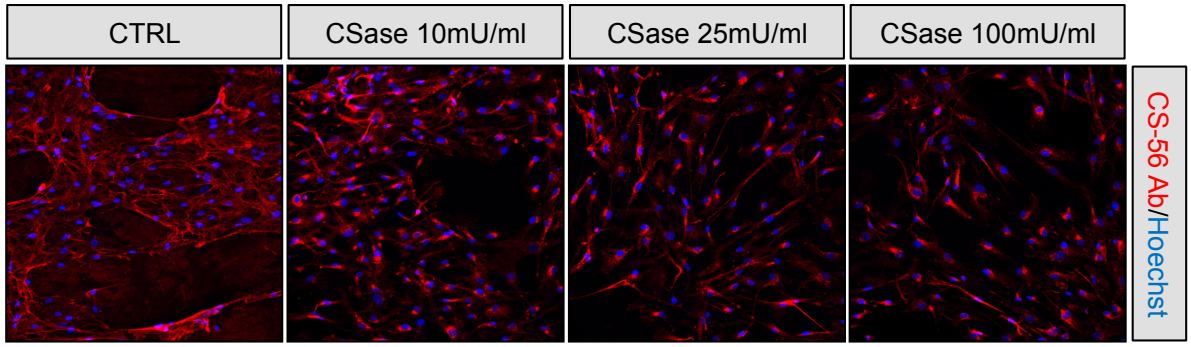

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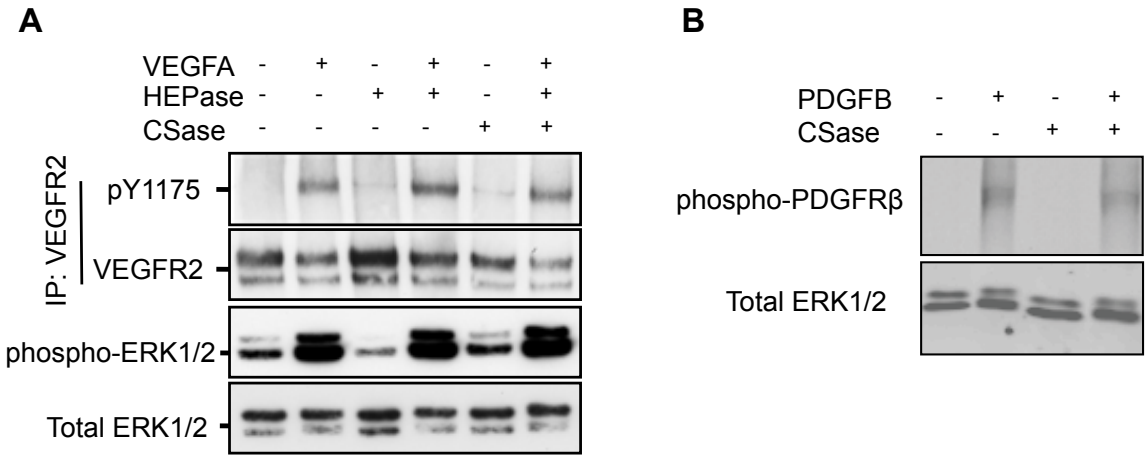

Symbol	Gene Name	Primer set	Ref. Sequence
Cspg2	Versican V0	5’ CACAGCCAACAAGACCATCA 3’ GCAAACAGATCATGCAGTGG	NM_001081249
	Versican V1	5’ CTTGGGGTGAGAACCCTGTA 3’ GCAAACAGATCATGCAGTGG	
	Versican V2	5’ ACCAAGTTCCACCCTGACAT 3’ TCACACTGGTCTCCGCTGTA	
Agc1	Aggrecan	5’ TTGGAGATCCAGAACCTTCG 3’ TGTGCTCGATCAAAGTCCAG	NM_007424
Cspg3	Neurocan	5’ GTATCCTTGGGCTTGGATGA 3’GAGGCAGAGGCAGATGAAAC	X84727
Bcan	Brevican	5’ CCTCTTCCTCTTTCCCAACC 3’ CTGCAGTTCCTCCAGCTTTT	BC052032
Dcn	Decorin	5’ GATTTTCCACCCGACACAAC 3’ GAGGTTTGAATGCCTCTGGA	NM_007833
Bgn	Biglycan	5’ ACGAACTTCACCTGGACCAC 3’ GAAGTCATTGATGCCCACCT	BC052857
Esm1	Endothelial cell-specific molecule 1, Endocan	5’ CGAGGAGGATGATTTTGGTG 3’ ATGCTGAGTCACGCTCTGTG	NM_023612
Epyc	Epiphycan	5’ GCTTGGACCACATCCCTCTA 3’ AGGCTTGAGGAGTTCTGCTG	NM_007884
Col9a2	Procollagen, type IX, alpha 2	5’ GTTCTCCTCCAGGTGCTGTG 3’ CCGGAGGACCAACTTTACCT	NM_007741
Ptprz1	DSD-1-proteoglycan, Phosphacan	5’ ATTGGCTGGTCCTACACAGG 3’ CCCAGTGTTGTGAATGAACG	NM_001081306
Spock1	Testican-1	5’ TCCCAGTATGACCGTGACAA 3’ TCCAAGCCAGTGTTTGTGAG	NM_009262
Spock2	Testican-2	5’ CTGTCGTCCATCTCGCAGTA 3’ CAGAGAAGGCTGCTTGATCC	NM_052994
Spock3	Testican-3	5’ ACAAGGAAGTCGGACAGTGG 3’ TCTGAGCATCCTGCGTAATG	NM_023689
Thbd	Thrombomodulin	5’ TGCCAGGCTCTTACTCCTGT 3’ GCACTCTCCATCCACCAACT	BC019154
Cd74	CD74	5’ GATGGCTACTCCCTTGCTGA 3’ ATCTTCCAGTTCACGCCATC	NM_001042605
Cspg4	Chondroitin sulfate proteoglycan 4, NG2	5’ TTACCTTGGCCTTGTTGGTC 3’AGTGGAGCTGGAGCAAGAGA	NM_139001
Prg4	Proteoglycan-4, Lubricin	5’TGAATCCATTCAGACCACCA 3’ACACAGATTCAGGCCTGTCC	BC130021
Lepre1	Leprecan-1	5’ CTACAGCGCTGACCTCTTCC 3’ GTGCATCACCTCATCATTGG	NM_019783