

Platelet-derived growth factor receptor β activation and regulation in murine myelofibrosis

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SUPPLEMENTS

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METHODS

Blood cell counts

Blood was collected directly *post mortem* from cardiac puncture into ethylenediaminetetraacetic acid (EDTA)-coated tubes from Sarstedt (Nümbrecht, Germany). Whole blood was analyzed within 6 h from sample collection using a Sysmex K4500 hematology analyzer (Kobe, Japan).

Quantitative real-time PCR (qPCR)

Total RNA was obtained from whole murine femurs by pulverizing the frozen bone in a pestle cooled with liquid nitrogen. The pulverized tissue was dissolved in Trizol and RNA was isolated according to the manufacturer's instructions. One μ g RNA was used as template for reverse transcription. cDNA was synthesized with High Capacity RNA-to-cDNA Kit (Applied Biosystems, Darmstadt, Germany) and qPCR was performed using SybrGreen PCR Master Mix (Applied Biosystems, Darmstadt, Germany). Primers were used at a final concentration of 100 nM, sequences are listed in Supplementary Table S1. Each gene transcript was measured in triplicates. mRNA levels were calculated in relative units using the $2^{-\Delta\Delta Ct}$ method with *Hprt* as housekeeping gene. Values observed in age-matched wild type control mice served as references.

RNA sequencing (RNAseq)

Total RNA from whole femurs of 10-month-old mice ($n = 3$ Gata-1^{low} vs. $n = 3$ WT) was used for RNAseq analyses. RNAseq and bioinformatics were performed by Microsynth (Balgach, Switzerland). In brief, RNA quality was evaluated on Agilent 2100 BioAnalyzer (Santa Clara, Ca, USA), library preparation including poly(A) enrichment was done using Illumina stranded TruSeq RNA library incl. poly(A) enrichment (San Diego, CA, USA). Sequencing was conducted with 1*75bp reads and 30 Mio reads per sample. Reads were mapped to the mouse genome, counts were normalized and analyzed for differential expression. Genes of interest were plotted using GraphPad Prism 6.01 (GraphPad Software Inc., San Diego, CA). Significantly upregulated genes were further used for gene ontology (GO) enrichment analysis.¹

Tissue processing and histology

Murine femurs were fixed in 4% phosphate-buffered formaldehyde for 6 h and decalcified in 10% EDTA for 7 days with daily changes of the solution. Femurs were paraffin-embedded using standard procedures and paraffin blocks were cut into 1 μ m sections. Slides were deparaffinized in NeoClear, followed by graded ethanol and transferred to distilled water. For the visualization of reticulum fibers

in the bone marrow, Reticulum Stain Kit (Abcam, Cambridge, UK) was used according to the manufacturer's instructions.

Multiplex staining

Multiplex staining of bone marrow sections was performed using Opal™ fluorophores (PerkinElmer, Waltham, MA, USA) according to the manufacturer's instructions. Slides were deparaffinized, rehydrated and antigen retrieval was performed in citrate buffer, pH 6, by microwave treatment with power settings allowing boiling of the buffer for 20 minutes. Slides were washed and blocked using Serum-free Protein Block Solution (Dako Agilent Technologies, Santa Clara, CA, USA), followed by incubation with primary antibody for 30 min at room temperature. All antibodies used are listed in Supplementary Table S2. Slides were washed in TBS-T and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 10 min. Slides were incubated with Opal fluorophores for 10 min, followed by washing and antigen retrieval by microwave treatment to remove primary and secondary antibodies. This procedure was repeated for staining of other targets. Nuclei were counterstained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) and mounted with Prolong Diamond Antifade Mountant (Thermo Fisher Scientific, Waltham, MA, USA). Imaging was performed with the Vectra® Polaris™ system (PerkinElmer, Waltham, MA, USA). A whole slide scan was first acquired at $\times 10$ magnification to image the whole bone and region selection for multispectral imaging was performed in Phenochart™ software (PerkinElmer, Waltham, MA, USA). Ten multispectral images at $\times 40$ magnification were acquired. Spectral unmixing and quantification was performed with inForm® software (PerkinElmer, Waltham, MA, USA).

Proximity Ligation Assay (PLA)

All PLA reagents, if not otherwise stated, were purchased from Duolink® (Sigma-Aldrich, Darmstadt, Germany). Tissue slides were deparaffinized and antigen retrieval was performed in citrate buffer, pH 6, by microwave treatment. Nonspecific binding sites were blocked with Blocking Solution for 60 min. Bone marrow sections were incubated with single antibodies for single recognition approaches, or with combined antibodies for the interaction and phosphorylation analyses at 4°C overnight. Primary antibodies and combinations used for the assays are specified in Supplementary Tables S2 and S3. Unbound antibodies were removed by washing in TBS-T and the slides were incubated with oligonucleotide-conjugated secondary antibodies (PLA Probes) PLUS and MINUS for 1 h at 37°C. Subsequently, slides were washed and a ligation mixture (Detection Reagent Orange) was incubated on the slides for 30 min at 37°C. Slides were washed and incubated with an amplification mixture (Detection Reagent Orange) for 100 min at 37°C. After amplification, the slides were washed and rinsed in distilled water before mounting the slides using Mounting Medium with DAPI. Cells were visualized

using a BZ-9000 Keyence epifluorescence microscope (Osaka, Japan) with filters for DAPI and TRITC and a 60x objective (CFI Plan Apo, Nikon, Düsseldorf, Germany). Rolling circle products (RCPs) were quantified using Duolink[®] Image Tool. For clear visualization of RCPs, the PLA images were processed with ImageJ 1.8.0 software using a maximum filter (3 pixels) for the PLA channel.

Cell culture and transfection

NIH-3T3 fibroblasts were purchased from American Type Culture Collection (ATCC[®], Wesel, Germany) and maintained in DMEM (Dulbecco's Modified Eagle Medium) containing 10% fetal bovine serum (FBS). Cells were transfected using 30 nM mouse *Ptpn2* siRNA (id 150390) and nontargeting (NT) Silencer Negative Control No. 1 siRNA (Thermo Fisher Scientific, Bonn, Germany) with Lipofectamine 2000 (Invitrogen, Karlsruhe, Germany) according to the manufacturer's recommendations. Before stimulation with human recombinant PDGF-BB (Peprotech, Hamburg, Germany), NT control and *Ptpn2* knockdown cells were growth-arrested for 24 h in DMEM with 100 μ g/ml bovine serum albumin (BSA). 72 h after transfection, cells were treated with 50 ng/ml PDGF-BB for 5 min.

Immunoblotting

After PDGF-BB treatment, cells were washed with ice-cold PBS and lysed in buffer supplemented with Complete Mini protease inhibitor and PhosStop phosphatase inhibitor tablets (Merck, Darmstadt, Germany). 20 μ g protein per lane was separated on a 10% gel using sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene fluoride (PVDF) membrane. Antibodies used for immunoblotting are listed in Supplementary Table S2. HRP-conjugated secondary antibodies and chemiluminescence (GE Healthcare, Freiburg, Germany) were used for visualization. Results are from one representative experiment (n = 3) which was determined in duplicates and densitometric analyses were performed using ImageJ 1.8.0 software.

Proliferation assay

NIH-3T3 fibroblasts were seeded in 96 well microplates at 1000 cells/well and growth-arrested for 24 h in DMEM with 100 μ g/ml BSA before changing to the indicated culture media. For each condition, proliferation was monitored in 5 wells of 96 well plates using IncuCyte Live-Cell Imaging Systems (Essen Bioscience, Ann Arbor, MI, USA). Four phase contrast images per well at 10x magnification were acquired in 3 h intervals for six days. Proliferation was analyzed using IncuCyte software (Essen Bioscience, Ann Arbor, MI, USA) and results are shown as occupied area in percent confluence over time with standard error.

REFERENCES

1. Ashburner M, Ball CA, Blake JA, et al. Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. Nat Genet. 2000;25(1):25-29.

Table S1: List of primer sequences used for quantitative real-time PCR (qPCR) analyses.

Primer	Sequence (5'–3')
<i>Col1a1</i> forward	ctgacgcatggccaagaaga
<i>Col1a1</i> reverse	atacctcgggtttccacgtc
<i>Col3a1</i> forward	ctggctctgctggaaggat
<i>Col3a1</i> reverse	tccattgcgtccatcaaagc
<i>Pdgfra</i> forward	agtggctacatcatcccct
<i>Pdgfra</i> reverse	ccgaagtctgtgagctgtgt
<i>Pdgfrb</i> forward	acggcatggacttcttagcc
<i>Pdgfrb</i> reverse	atcttgaccagcttgccctc
<i>Pdgfa</i> forward	ttgtaacaccagcagcgta
<i>Pdgfa</i> reverse	ctccactttggccacctga
<i>Pdgfb</i> forward	cggcctgtgactagaagtcc
<i>Pdgfb</i> reverse	gagcttgaggcgtcttgg
<i>Ptpn1</i> forward	cgggaggtcaggacctt
<i>Ptpn1</i> reverse	gggtctttcctcttgccatca
<i>Ptpn2</i> forward	gctacgacggctcagaaggt
<i>Ptpn2</i> reverse	tgtctgtcaatcttggcctttt
<i>Ptpn6</i> forward	cgtaccctcccgtgtga
<i>Ptpn6</i> reverse	tttctgtacacctcctcttg
<i>Ptpn11</i> forward	cctcaacacaactcgtatcaatgc
<i>Ptpn11</i> reverse	tgttgctggagcgtctcaaa
<i>Ptpn12</i> forward	gagtcgcctcctccttacc
<i>Ptpn12</i> reverse	tggaagttcatgccactcagg
<i>Ptprj</i> forward	gcagtgttgatgtatcttgg
<i>Ptprj</i> reverse	cttcattattcttggcatctgcctt
<i>Hprt</i> forward	tgctgacctgctggattaca
<i>Hprt</i> reverse	tatgtccccgttgactgat

Table S2: List of antibodies used for this study, including species/clonality, Catalog#, supplier and dilutions for the different applications. mAb: monoclonal antibody, pAb: polyclonal antibody, IB: immunoblot, MSI: multispectral imaging, PLA: proximity ligation assay.

Antibody	Species/ Clonality	Catalog#	Supplier	Application/ Dilution
Akt	Rabbit pAb	9272	Cell Signaling Technology, Frankfurt a. M., Germany	IB: 1:1000
pAkt S ⁴⁷³	Rabbit mAb	4060	Cell Signaling Technology, Frankfurt a. M., Germany	IB: 1:2000
Erk1/2	Rabbit pAb	9102	Cell Signaling Technology, Frankfurt a. M., Germany	IB: 1:1000
pErk1/2 T ²⁰⁴ /Y ²⁰⁴	Rabbit mAb	4370	Cell Signaling Technology, Frankfurt a. M., Germany	IB: 1:2000
GAPDH	Mouse mAb	MAB374	Millipore, Schwalbach, Germany	IB: 1:50 000
Pan-pY pY100	Mouse mAb	9411	Cell Signaling Technology, Frankfurt a. M., Germany	PLA: 1:500
Pan-pY pY20	Mouse mAb	ab10321	Abcam, Campridge, UK	PLA: 1:900
Pan-pY 4G10	Mouse mAb	05-321	Merck Millipore, Darmstadt, Germany	PLA: 1:1000
PDGFR α D1E1E	Rabbit mAb	3174	Cell Signaling Technology, Frankfurt a. M., Germany	MSI: 1:80 PLA: 100
PDGFR β 28E1	Rabbit mAb	3169	Cell Signaling Technology, Frankfurt a. M., Germany	IB: 1:1000
PDGFR β 42G12	Mouse mAb	NBP1- 19191	Novus Biologicals, Centennial, CO, USA	PLA: 1:50
PDGFR β Y92	Rabbit mAb	ab32570	Abcam, Campridge, UK	MSI: 1:50 PLA: 1:50
pPDGFR β Y ⁷⁵¹	Mouse mAb	3166	Cell Signaling Technology, Frankfurt a. M., Germany	IB: 1:1000
pPDGFR β Y ¹⁰²¹	Rabbit pAb	ab16868	Abcam, Campridge, UK	IB: 1:2000
PDGF-A	Mouse mAb	sc-9974	Santa Cruz, CA, USA	MSI: 1:50 PLA: 1:50
PDGF-B	Rabbit pAb	ab21234	Abcam, Campridge, UK	MSI: 1:80 PLA: 1:50

PLC γ 1	Rabbit pAb	#2822	Cell Signaling Technology, Frankfurt a. M., Germany	IB: 1:1000
pPLC γ 1 Y ⁷⁸³	Rabbit pAb	#2821	Cell Signaling Technology, Frankfurt a. M., Germany	IB: 1:1000
TC-PTP F-8	Mouse mAb	sc-373835	Santa Cruz, CA, USA	IB: 1:100 MSI: 1:100 PLA 1:50
Negative control	Mouse IgG1	X0931	Dako, Hamburg, Germany	PLA: 1:50
Negative control	Rabbit IgG fraction	X0903	Dako, Hamburg, Germany	PLA: 1:10 000
Anti-Mouse-HRP	Rabbit pAb	P0260	Dako, Hamburg, Germany	IB: 1:2000
Anti-Rabbit-HRP	Swine pAb	P0217	Dako, Hamburg, Germany	IB: 1:1000
ImmPRESS [®] Anti- Mouse-HRP	Horse pAb	MP-7402	Vector Laboratories, Burlingame, CA, USA	MSI: ready-to- use
ImmPRESS [®] Anti- Rabbit-HRP	Horse pAb	MP-7401	Vector Laboratories, Burlingame, CA, USA	MSI: ready-to- use

Table S3: Antibody combinations used for proximity ligation assay (PLA).

Assay	Rabbit primary antibodies	Mouse primary antibodies
PDGFR β –PDGF-B interaction	PDGF-B	PDGFR β 42G12
PDGFR β tyrosine phosphorylation	PDGFR β Y92	Pan-pY pY100 Pan-pY pY20 Pan-pY 4G10
PDGFR β –TC-PTP interaction	PDGFR β Y92	TC-PTP F-8

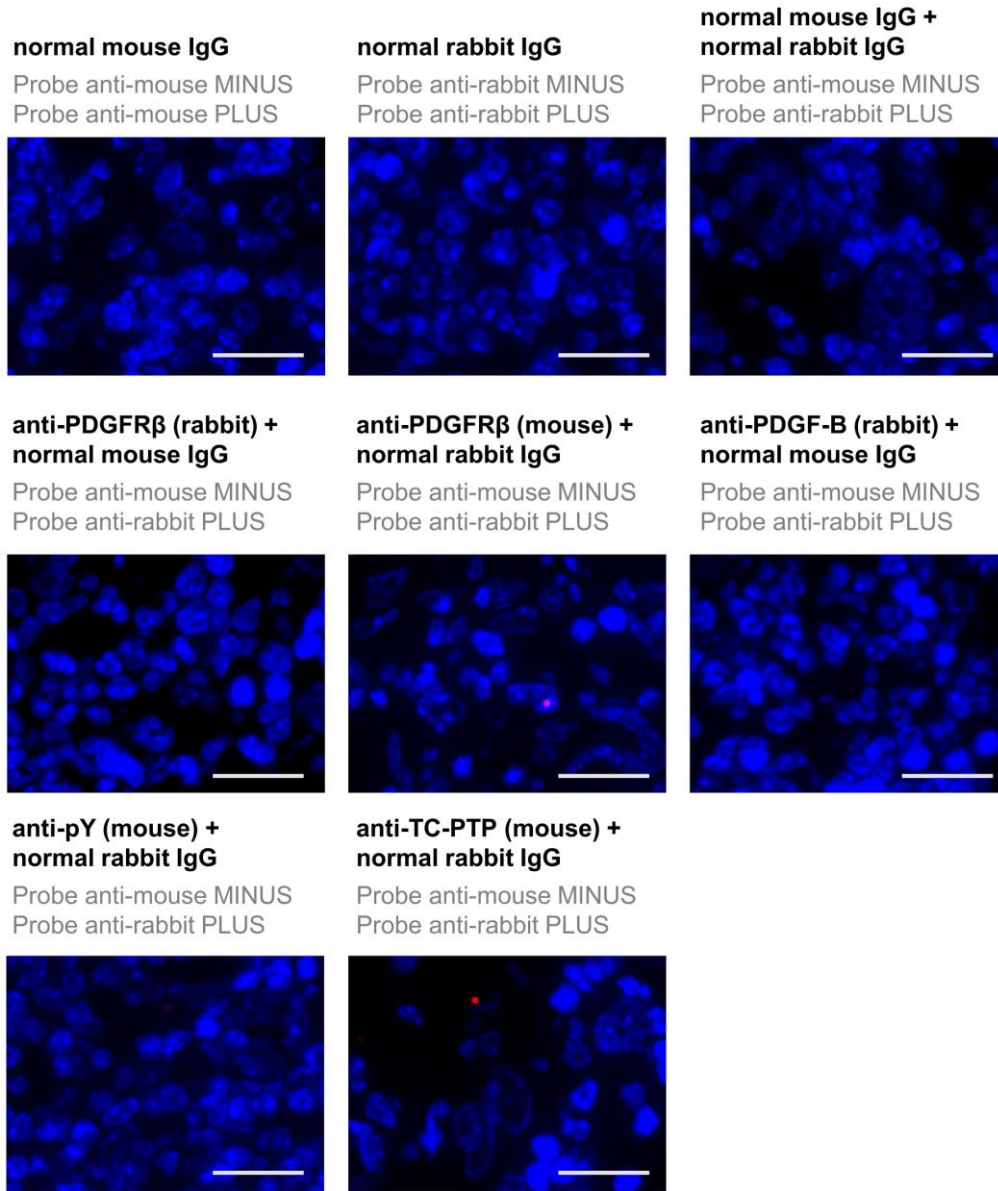


Figure S1: Technical negative controls for the different proximity ligation assay (PLA) approaches. Bone marrow from Gata-1^{low} mice at 15 months of age was incubated with normal mouse, normal rabbit immunoglobulin G (IgG), a combination of both, and in combination with the stated specific primary antibodies used within this study. For clear visualization of rolling circle products (RCPs), the PLA images were processed with a maximum filter (3 pixels) for the PLA channel. Scale bars = 20 μ m.

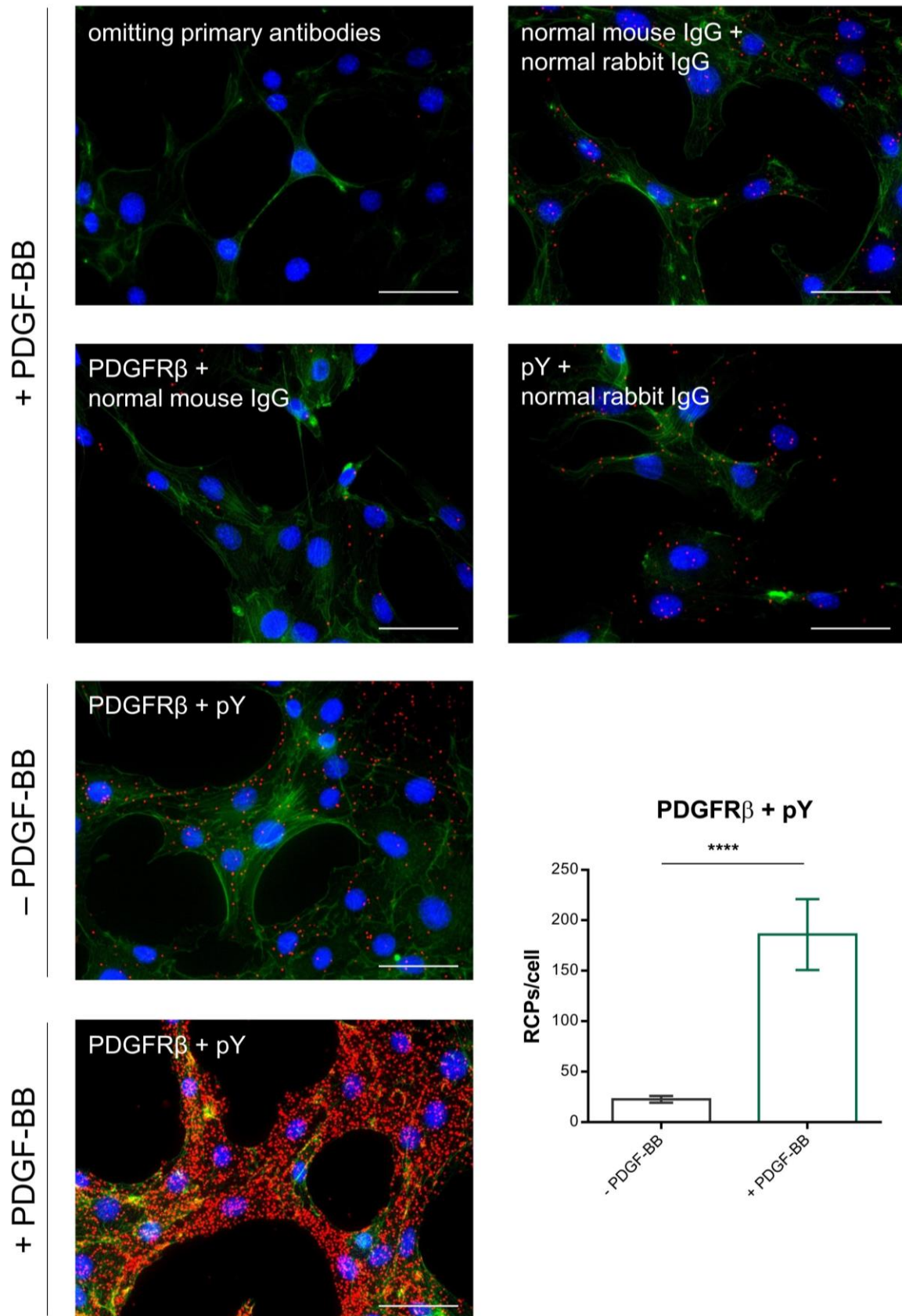


Figure S2: Representative images of a proximity ligation assay (PLA) showing negative controls and PDGFR β tyrosine phosphorylation (pY) levels in NIH-3T3 fibroblasts after treatment with 50 ng/ml PDGF-BB for 5 minutes. Cells were co-stained with phalloidin (green) and DAPI (blue) to visualize cytoskeleton and nuclei. Rolling circle products (RCPs) are shown as red dots; for clear visualization of RCPs, the PLA images were processed with a maximum filter (3 pixels) for the PLA channel. Scale bars = 50 μ m, **** p \leq 0.0001 by Student's t test.

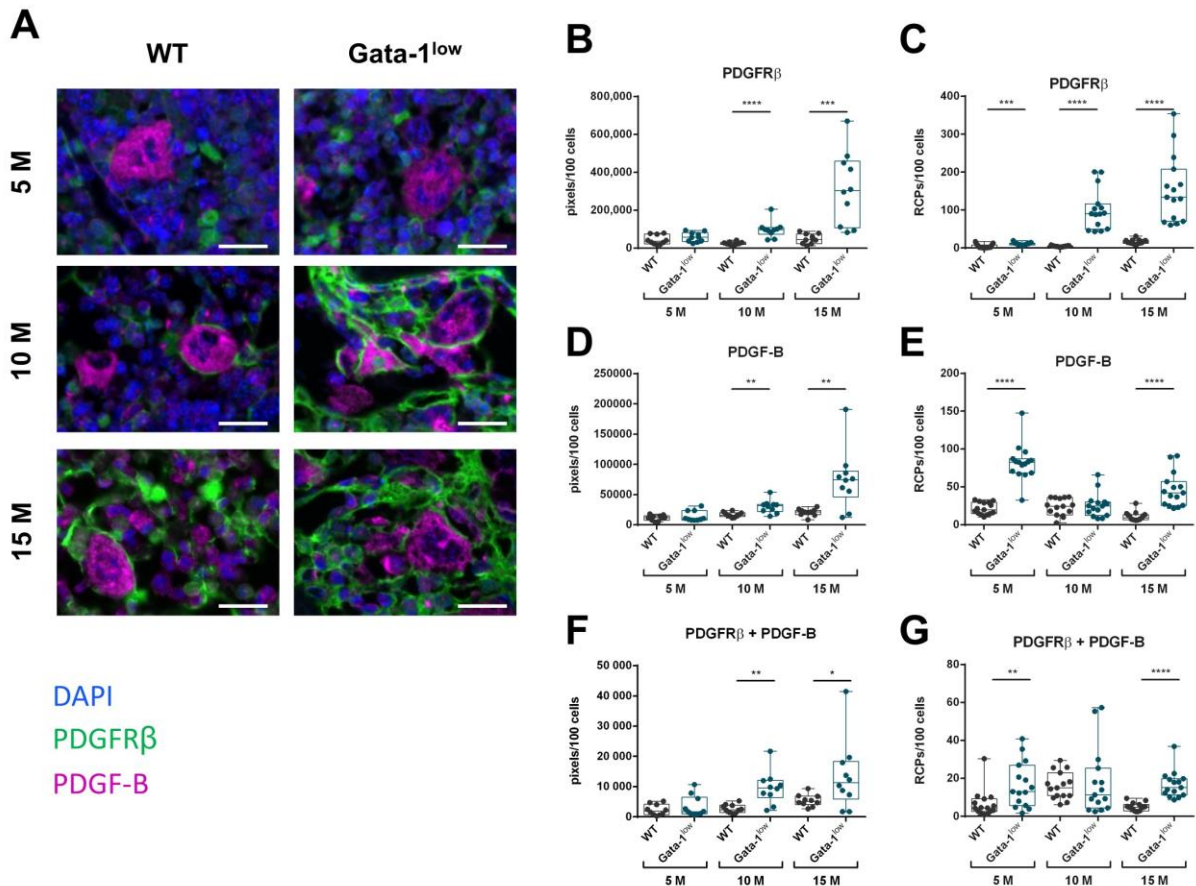


Figure S3: Multiplex staining of platelet-derived growth factor B (PDGF-B) and PDGF receptor β (PDGFR β), and *in situ* protein quantification by imaging and by single recognition proximity ligation assay (PLA) in the bone marrow of Gata-1^{low} mice at 5 months (5 M), 10 months (10 M) and 15 months (15 M) of age. A Representative images showing femoral bone marrow of Gata-1^{low} mice and wild type (WT) control mice stained for PDGFR β (green) and PDGF-B (magenta). Nuclei were counterstained with DAPI (blue), scale bars = 20 μ m. Images show the same sections which are depicted in Figure 4. **B** Quantification of PDGFR β protein expression using the acquired imaging data. **C** Quantification of PDGFR β protein expression using single recognition PLA. **D** Quantification of PDGF-B protein expression using the acquired imaging data. **E** Quantification of PDGF-B protein expression using single recognition PLA. **F** Quantification of PDGFR β –PDGF-B colocalization using the acquired imaging data. **G** Quantification of PDGFR β –PDGF-B interaction using PLA. All quantification plots show exemplary data from one and the same mouse, respectively, per group; 3335 – 8632 nucleated cells per mouse were analyzed. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$ versus the control group by Student's t test.

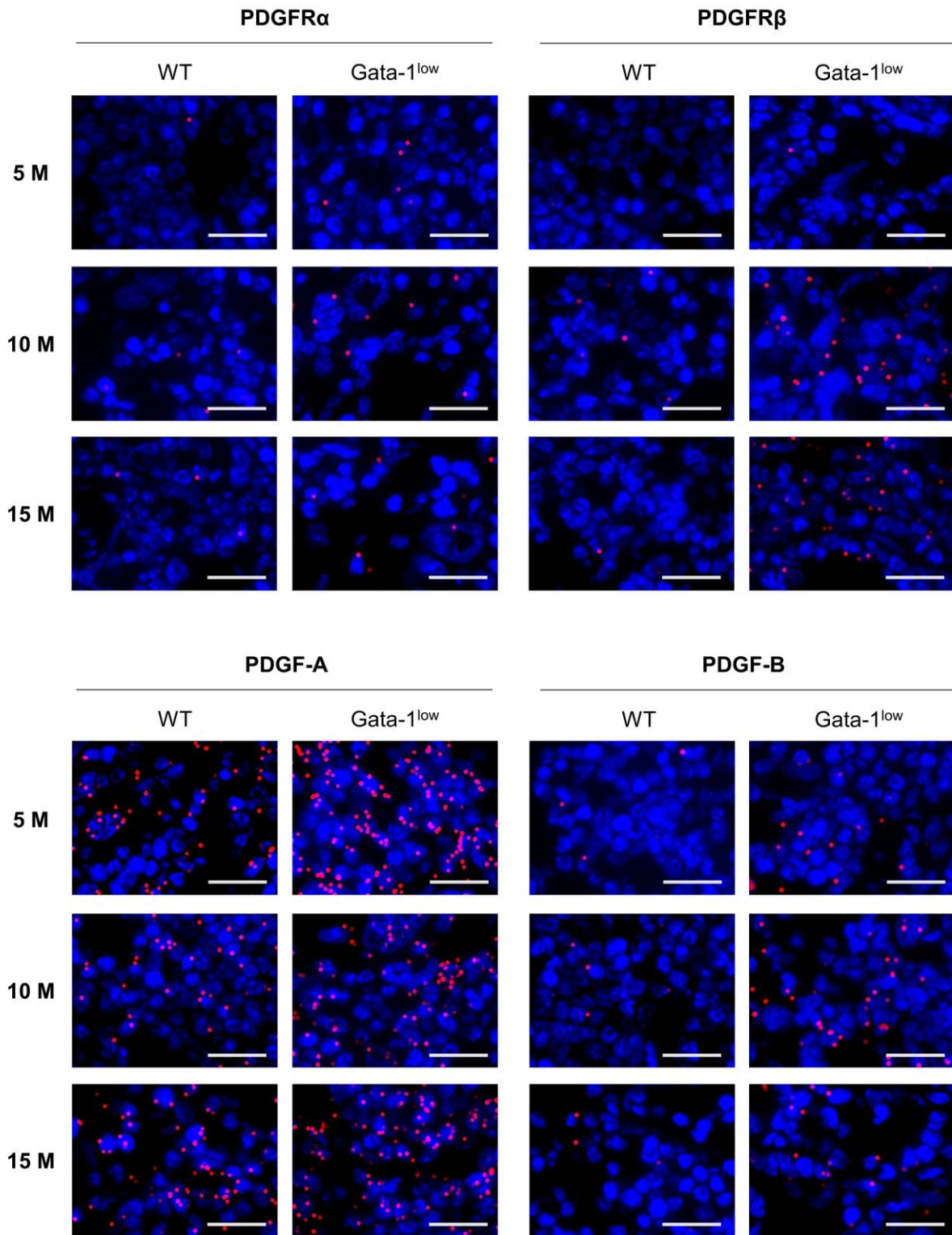


Figure S4: Representative images showing a single recognition proximity ligation assay (PLA) in the bone marrow of *Gata-1^{low}* mice and wild type (WT) controls at 5 months (5 M), 10 months (10 M) and 15 months (15 M) of age for analyses of PDGFR α , PDGFR β , PDGF-A and PDGF-B protein expression. Rolling circle products (RCPs) are shown as red dots, nuclei were counterstained with DAPI (blue). For clear visualization of RCPs, the PLA images were processed with a maximum filter (3 pixels) for the PLA channel. Scale bars = 20 μ m.

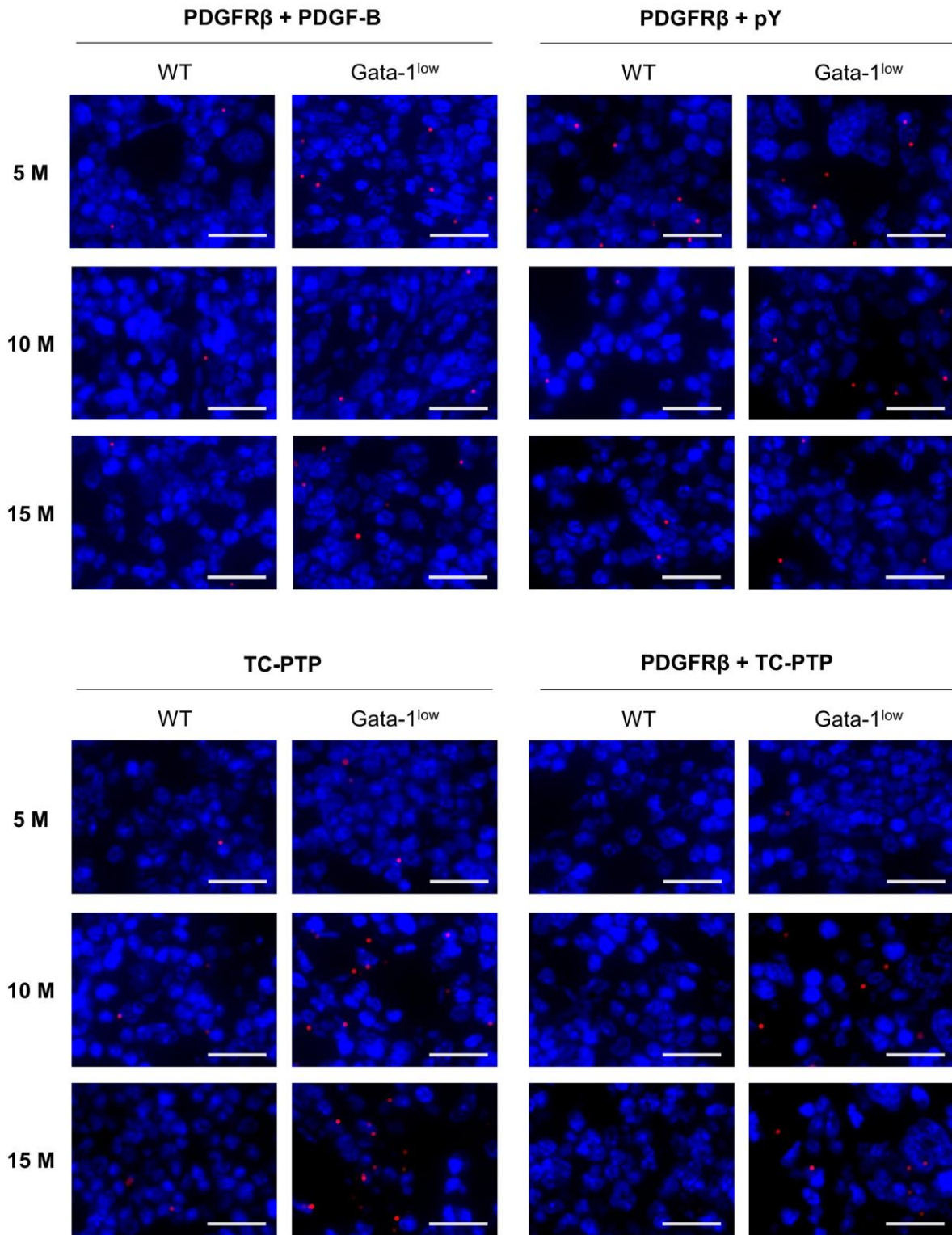


Figure S5: Representative images showing a proximity ligation assay (PLA) in the bone marrow of Gata-1^{low} mice and wild type (WT) controls at 5 months (5 M), 10 months (10 M) and 15 months (15 M) of age for analyses of PDGFR β –PDGF-B interaction, PDGFR β tyrosine phosphorylation (pY), TC-PTP protein expression and PDGFR β –TC-PTP interaction. Rolling circle products (RCPs) are shown as red dots, nuclei were counterstained with DAPI (blue). For clear visualization of RCPs, the PLA images were processed with a maximum filter (3 pixels) for the PLA channel. Scale bars = 20 μ m.