Deregulation of tumor angiogenesis and blockade of tumor growth in PPARβ deficient mice

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Supplemental Methods

Tumor growth experiments. Three-month old mice were inoculated subcutaneously with $7x10^5$ LLC1 cells or $1x10^6$ B16F1 cells. Tumor dimensions were measured using a caliper and tumor volumes were calculated by the following formula assuming an ellipsoid shape: W^2 x c x $\pi/6$, where W=measured tumor width and L=measured tumor length (Tomayko and Reynolds, 1989). Statistical analysis was performed by Student's t-test (two-tailed, equal variance). Mice were killed when the calculated tumor volume exceeded 1000 mm^3 or when tumors became necrotic.

Mouse endothelial cell culture. Aortic ECs were isolated from *Pparb*$^{+/+}$ and *Pparb*$^{-/-}$ mice as described (Chen et al., 2004) and plated as primary cells on matrigel-coated 24-well plates in EGM-2 medium (Cambrex, Copenhagen, Denmark) at a density of 5,000 cells per well essentially as described. The purified preparations contained >96% ECs as determined by isolectin B4 lectin staining (Kawamoto et al., 2003). When confluent, cells were reseeded in 24-well plates (2500 cells/well) and cultured for up to 6 days in EGM-2 medium supplemented with growth factors provided with the medium. Cell growth was determined using a Z1 Coulter counter (100 μm aperture, 10 μm threshold).

Establishment of fibroblasts from *Pparb*$^{+/+}$, *Pparb*$^{-/-}$ and *Flox-Pparb* (PPARδ^{ck}) mice. Primary cultures of mouse lung fibroblasts were established from fetal lungs removed aseptically from day 18-19 embryos. Lungs were cut into small pieces and incubated for 30 minutes at 37°C in cell culture grade trypsin solution. Following centrifugation, cells and remaining tissue pieces were cultivated in DMEM supplemented with antibiotics, fungicide, and 20 % fetal calf serum. Cell lines were established after ~15 passages and grown in DMEM plus 20 % fetal calf serum. These lung fibroblast lines were named LF-wt, LF-PPARb-null and LF-Flox-PPARb cells.

Retroviral transduction experiments. Murine *Pparb* with an N-terminal triple-FLAG-Tag (3xFLAG-mPPARb) was cloned into the retroviral vector pLPCX (Clontech). Phoenix cells expressing ecotropic *env* were transfected with 3xFLAG-mPPARb-pLPCX or with the empty pLPCX vector as described (*http://www.stanford.edu/group/nolan/retroviral_systems/retsys.html*). Culture supernatant was used to infect *Pparb*$^{-/-}$ fibroblasts (LF-PPARb-null; see above), and cells were selected with puromycin (2 μg/ml; Sigma, Munich, Germany). A clone

expressing 3xFLAG-mPPARb (3Fb cells) and a control line harboring empty pLPCX vector (Lpcx cells) were used for all further studies (see Fig. S1 for immunoblot analysis of PPARβ expression). All infections were performed in the presence of 4-8 μg/ml polybrene using standard protocols. For *in vivo* matrigel plug assays, 3Fb and control Lpcx cells were infected with Mo-MuLV helper virus to generate 3Fb-p and control Lpcx-p producer cells. 3Fb-1 were also infected with a replication defective virus (pBABE-puro) to generate a second control cell line, non-producer 3Fb-np cells. *Pparb* virus production was verified by infection of murine fibroblasts with 3Fb-p supernatant followed by a FLAG-Tag immunostaining two days later. Culture supernatants from phoenix cells transfected with 3xFLAG-mPPARb-pLPCX or with the empty pLPCX vector were also used to infect AO2-PPARb-null cells. The medium was replaced after 24 hours with normal growth medium plus 1μM of the PPARβ agonist GW501516, and cells were harvested after another 48 hrs without selection. The retroviral Cre vector construct (Li et al., 1997) was kindly provided by Dr. T. Blankenstein (Berlin). Supernatants from transfected phoenix cells were used to infect Flox-PPARb fibroblasts, and cells harvested without selection as described for AO2-PPARb-null cells above.

siRNA transfections. For siRNA transfection cells were seeded at a density of $4x10^5$ cells per 6cm dish in 4 ml antibiotic-free DMEM with 10% FCS and cultured overnight. 400pmol siRNA in 500μl OptiMEM (Invitrogen) and 10μl Lipofectamin 2000 (Invitrogen) in 500μl OptiMEM (Invitrogen) were separately incubated for 5 min at room temperature, mixed and incubated for another 20 min. The siRNA-lipid complex was added to the culture medium (time = 0 hrs), and the medium was changed to normal growth medium after 4-6 hrs. Cells were passaged and replated 24 hrs post-transfection at a density of $4x10^5$ cells per 6cm dish. Transfection was repeated 72 and 144 hrs after start of the experiment, and each time passaged after another 24 hrs. RNA was isolated at 0, 48 and 192 h. The following siRNAs were used: p57 siRNA (Santa Cruz Biotechnology, Santa Cruz, California, USA; pool of 37621A-C); control siRNA (Qiagen, Hilden, Germany; negative control siRNA 1022563).

Immunohistology. Paraffin-embedded sections were deparaffinized, and antigen retrieval was performed by either microwave treatment in PBS, pH 6.0, containing

200 mM EDTA, or by proteinase K digest as necessary. The following antibodies were used at the indicated concentrations: mouse monoclonal antibodies against SMA (peroxidase-conjugated, 1:5; Sigma, Munich, Germany), FLAG-tag (1:100; Becton Dickinson, Heidelberg, Germany), and neutrophils (clone 7/4; 1:500; Cedarlane, Hornby, Canada); rat monoclonal antibodies against CD34 (1:40; Abcam, Cambridge, UK), F4/80 antigen (1:600; Serotec, Düsseldorf, Germany), and CD45 (1:200; Becton Dickenson, Heidelberg, Germany); polyclonal rabbit antibodies against PCNA (1:200; Santa Cruz Biotechnology), von Willebrand Factor (Dako, Germany), and AQP1(1:20; kind gift of Dr. S. Nielsen, Aarhus, Denmark) (Gresz et al., 2001). Signals were visualized using biotinylated secondary antibodies and either avidin-conjugated peroxidase with diaminobenzidine as substrate (DAB; Sigma, Munich, Germany) or avidin-conjugated alkaline phosphatase and Vector® Red (Vector Lab, California, USA) as substrate. For double staining sections were microwave-treated after DAB staining and then incubated with the second primary antibody. Double immunofluorescence was performed using a FITC-labeled α-SMA antibody and the polyclonal α-rabbit anti-AQP1 antibody (see above) visualized by a Cy5-labeled secondary antibody. Staining with fluorescently labeled isolectin B4, a selective label for mouse blood vessels, was performed as described (Kawamoto et al., 2003). Slides were evaluated with a Leitz RMB 3 microscope using either bright field or fluorescence optics. Digital microphotographs were used for histomorphometric analysis (Spot-Cam, Diagnostic Instruments, Munich, Germany) and color composites were generated with Adobe Photoshop v6.0. For quantitative analysis at least ten 40x high power fields were analyzed. Statistical analysis was performed by Student's t-test (two-tailed, equal variance).

Isolation and amplification of RNA

RNA was isolated using the Nucleospin RNA II kit (Macherey-Nagel, Düren, Germany). For microarray studies, mRNA samples were amplified using the MessageAmp™ II RNA Amplification Kit (Ambion, Huntingdon, UK) according to the manufacturer's instructions. The amplification procedure consists of reverse transcription with an oligo(dT) primer bearing a T7 promoter followed by *in vitro* transcription of the resulting cDNA with T7 RNA polymerase to generate multiple copies of each mRNA. After one round of *in vitro* transcription starting with 1 μg of total RNA, the yield of amplified mRNA ranged from 50 -100 μg. Prior to

amplification RNA samples from two *Pparb*$^{+/+}$ and two *Pparb*$^{-/-}$ mice, respectively, were pooled.

Hybridization probes for microarrays. Hybridization probes were generated by indirect labeling with Cy3 and Cy5 dyes, using the CyScribe cDNA Post Labeling Kit (GE Healthcare, Munich, Germany). Four micrograms of amplified RNA, generated from mRNA of ECs in matrigel were reverse transcribed with nonamer primers in the presence of modified amino-allyl-dUTP. After purification the cDNAs were labeled with Cy5 and Cy3 respectively.

Quantitative PCR (qPCR). cDNA was synthesized from 1 μg of RNA using oligo(dT) primers and the Omniscript kit (Qiagen, Hilden, Germany). qPCR was performed in a Mx3000P Real-Time PCR system (Stratagene, La Jolla, CA) for 45 cycles at an annealing temperature of 58-60°C. PCR reactions were carried out using the Absolute QPCR SYBR Green Mix (Abgene, Hamburg, Germany) and a primer concentration of 0.2 μM following the manufacturer's instructions. *Arp0* and *L27* were used as normalizers. Comparative expression analyses were statistically analyzed by Student's t-test (two-tailed, equal variance). The following primers were used in the present study:

PPARb_for: 5´-CTCCATCGTCAACAAAGACG
PPARb_rev: 5´-TCTTCTTTAGCCACTGCATC
PPARa_for: 5´-TGCTGTCCTC CTTGATGAAC
PPARa_rev: 5´-GCTTAAGCAC GTGCACAATC
PPARg_for: 5´-GACCAAGTGA CTCTGCTCAAG
PPARg_rev: 5´-CTCTGTGACG ATCTGCCTGA
Htatip2 (Tip30)_for: 5´-TCATGATGTTGGATTCTGTTGC
Htatip2 (Tip30)_rev: 5´-GTCAACACGAACAAACCCTTC
Cd36_for: 5´-TTGAAAAGTCTCGGACATTGAG
Cd36_rev: 5´-TCAGATCCGAACACAGCGTA
Padi4_for: 5´-TGACCCTACAGGTGAAAGCA
Padi4_rev: 5´-GGGTCCATAGTATGAAACTCGAA
Gdf3_for: 5´-TGTTCGTGGGAACCTGCT
Gdf3_rev: 5´-CCATCTTGGAAAGGTTTCTGTG
Fabp4_for: 5´-CGCAGACGACAGGAAGGT

Fabp4_rev: 5´-TTCCATCCCACTTCTGCAC

Igfbp3_for: 5´-CATCTGAAGTTCCTCAATGTGC

Igfbp3_rev: 5´-CGCTTTCTGCCTTTGGAA

Cdkn1c_for: 5´-CGCAAACGTCTGAGATGAGT

Cdkn1c_rev: 5´-CCCAGAGTTCTTCCATCGTC

Aof2_for: 5´-ATGAAAGTGAGCCGGAAGAG

Aof2_rev: 5´-GCTTCCTGAGAGGTCATTCG

Thbs2_for: 5´-GAACCAACCCTTCGGTGTT

Thbs2_rev: 5´-TGGATTCTCTGGCTCACACA

L27_for: 5´-AAAGCCGTCATCGTGAAGAAC

L27_rev: 5´-GCTGTCACTTTCCGGGGATAG

Arp0_for: 5´-AGATGCAGCAGATCCGCAT

Arp0_rev: 5´-GTGGTGATACCTAAAGCCTG

Accession numbers. The accession number for the *PPARb* gene is NM_011145, the accession number for PPARβ protein is NP_035275. Microarray data have been deposited with the ArrayExpress Database (accession number E-MEXP-983).

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