**Supplemental information**

# Histidine-Rich Glycoprotein-deficiency enhances tumor growth and metastasis by promoting pro-tumorigenic immune response and vessel abnormalization

Sònia Tugues[1], Satoshi Honjo[1], Christian König[1], Oriol Noguer[1], Marie Hedlund[1], Johan Botling[1], Sofie Deschoemaeker[2,3], Mathias Wenes[2,3], Charlotte Rolny[1], Wilhelm Jahnen-Dechent[4], Massimiliano Mazzone[2,3] and Lena Claesson-Welsh[1]

[1] Uppsala University, Department of Immunology, Genetics and Pathology, Rudbeck Laboratory, 75185 Uppsala, Sweden, [2] Vesalius Research Center, VIB, 3000 Leuven, Belgium, [3] Vesalius Research Center, K.U.Leuven, 3000 Leuven, Belgium, [4] RWTH Aachen University, Department of Biomedical Engineering, Aachen, Germany

## Materials and methods

### Lentiviral vectors and transduction

Lentivirus stocks were established using the FUGIE lentiviral vector backbone (derived from the FUGW vector, a kind gift from Dr. David Baltimore, Caltech, USA) and used to transduce T241 and Panc02 cells. The vector carries an internal ribosomal entry site (IRES) to promote translation of the downstream reporter gene enhanced green fluorescent protein (EGFP). The murine *hrg* cDNA was cloned into pFUGIE. Tumor cells ($10^5$) were infected with $10^6$ U/ml of empty virus (EV) lentivirus or mHRG-encoding lentivirus supplemented with 8 μg/ml polybrene in DMEM medium to generate T241-EV and T241-mHRG cell lines.

### Retinal neovascularization

Retinal tissue was prepared from 5 day-old mouse pups. The animals were anaesthetized by intraperitoneal injection of ketamine (20 mg/kg) and xylazine (6 mg/kg). Eyes were removed and fixed in 4% PFA-PBS at 4°C for 2-3 h and washed in PBS. Retinas were dissected, washed twice in the isolectin incubation buffer PBlec (PBS, pH 6.8/1% Triton X-100/0.1 mM $CaCl_2$/0.1 mM $MgCl_2$/0.1 mM $MnCl_2$), and incubated with biotinylated isolectin-B4 (B. simplicifolia, Sigma-Aldrich) in PBlec at 4°C overnight. After six washes of 10 min each in PBS, samples were incubated with 488-streptavidin (Molecular Probes, Invitrogen) in PBS containing 3% BSA and 0.1% Triton X-100 at RT for 2 h. After repeated washings, retinas were flat-mounted using Vectashield mounting medium (Vector Laboratories). Images of whole-mount preparations were taken with a Nikon Eclipse E100 microscope. Vascular density was quantified by measuring IB4-positive vessels in a fixed area photographed using a 10x objective. The radius of vascular networks was quantified by measuring the length between the center of the optic nerve and the edge of leading vessels. These measurements

were performed in four quadrants and then averaged for each retina. Littermate pups (+/+ and -/-) were compared.

**Differential leukocyte counts**

Wild type and *hrg*$^{-/-}$ mice were used for comparison of peripheral leukocyte counts in adult naïve mice. Mice were anaesthesized by isofluoran inhalation and blood samples were collected by cardiac puncture. Differential counting of peripheral leukocytes was performed at the clinical chemistry unit of the University Veterinary Hospital (UDS), Uppsala (http://www.slu.se/sv/universitetsdjursjukhuset/klinisk-kemiska-laboratoriet/).

**Chemotaxis assay**

The chemotaxis assay was performed using a modified Boyden chamber with 8 μm micropore polycarbonate filters (Neuro Probe Inc., Gaithersburg, MD). Panc02 cells were starved overnight with RPMI + 0.5% FBS, trypsinized and resuspended at $4x10^5$ cells/ml in RPMI media with 10 μM $ZnCl_2$, 0.25% BSA and Trasylol at 1000 KIE/ml. The cell suspension was added in the upper chamber and 2.5% FBS in the lower chamber. After 5 h at 37ºC, cells that had migrated through the filter were stained with Giemsa and counted and analyzed using ImageJ (NIH, Bethesda, MD). Data is presented as the number of cells that migrated through the filter.

**Tumor studies**

Mice were anaesthesized by isoflurane inhalation (Forene, Abbott Laboratories, Abbott, IL) and inoculated with T241-EV or T241-mHRG subcutaneously into the left flank. Tumors were measured with a caliper once every 2 days, in a blind procedure, and volumes were calculated by the formula Tumor volume = 0,52 x ($D \times d^2$), where d is the minor tumor axis and D is the major tumor axis. On day 21 after injection, mice were sacrificed and tumors were harvested. For orthotopic pancreatic tumor growth, mice were anesthetized with isoflurane, the stomach exteriorized via abdominal midline incision, and $10^6$ Panc02 tumor cells in 30 μl PBS were injected as described (1). Metastases to the mesenteric lymph nodes were recorded 11 days after injection.

**Immunofluorescence and Immunohistochemistry**

For immunofluorescence stainings, 8 μm sections of snap frozen tissue embedded in OCT (Tissue-Tek Sakura, Zoeterwoude, The Netherlands) were fixed in methanol (Sigma-Aldrich) for 10 minutes and equilibrated in PBS. Slides were blocked in 3% bovine serum albumin/ 0.1 % Triton X-100/ 5% FCS in PBS (blocking solution) for one hour before incubation with primary antibodies diluted in blocking solution over night at 4°C. The following primary

antibodies were used: rat anti-CD31 (BD Pharmingen), rat-anti-mouse F4/80 (Serotec), rat anti-mouse CD68 (Serotec), goat anti-mouse CD45 (Serotec), hamster anti-mouse CD11c (BD), FITC-conjugated rabbit anti-$\alpha$-smooth muscle actin (Sigma), goat anti-VE-cadherin (R&D Systems), rabbit anti-cleaved caspase 3 (Cell Signaling), rabbit anti-human phospho-histone H3 (Cell Signaling), rat anti-mouse CD86 (BD), rat anti-mouse CD8 (BD), goat anti-mouse MCR1 (R&D Systems). Sections were then incubated with the appropriate fluorescently conjugated secondary antibodies (Alexa 488 or 555, Molecular Probes). Samples were mounted using Fluoromount-G (Southern Biotech) and analyzed using a Nikon Eclipse E100 microscope or an LSM 510 META confocal laser-scanning inverted microscope (Carl Zeiss International, Oberkochen, Germany). Immunohistochemistry to detect Pimonidazole adducts was performed following the manufacturer's instructions (Hypoxyprobe kit, Chemicon). For all the studies, 20-40 optical fields (10x or 20x) per tumor section were randomly chosen and analyzed using ImageJ (NIH, Bethesda, MD).

**Hypoxia assessment, tumor perfusion, hemorrhage and necrosis**

Tumor hypoxia was detected 2 h after injection of 60 mg/kg pimonidazole hydrochloride in tumor-bearing mice. Mice were sacrificed and tumors harvested. To detect the formation of pimonidazole adducts, tumor paraffin sections were immunostained with Hypoxyprobe-1-Mab1 (Hypoxyprobe kit, Chemicon). Vessel perfusion was detected by intravenous injection of 0.05 mg FITC-labelled lectin (Lycopersicon esculentum; Vector Laboratories). Ten minutes later, mice were perfused by intracardiac injection of saline (5 min) and 2% PFA (7 min). Tumors were then harvested and frozen in OCT medium. Tumor perfusion was analyzed by counting the number of FITC-lectin and CD31 positive vessels. Tumor hemorrhage and necrosis were scored by an independent pathologist on H&E stained tumor sections.

**FACS analysis and flow sorting of tumor-associated cells**

Lentivirus-transduced T241 fibrosarcoma were used for flow sorting. Briefly, mice were sacrificed by cervical dislocation and tumors were harvested. Tumors were minced in DMEM medium containing 0.1% collagenase type I and incubated in the same solution for 1h at 37°C with gentle agitation. The digested tissue was passed 10 times through a 19G needle, filtered using a 70 µm pore sized mesh and cells were centrifuged 5 min at 1000 rpm. Red blood cell lysis was performed by using ACK buffer (10mM $KHCO_3$, 150 mM $NH_4Cl$, 0.1 mM EDTA, pH 7.4). Cells were resuspended in FACS buffer (1% FCS in PBS) and passed again through a 70 µm cell strainer before labeling with PE conjugated F4/80 (Serotec) for 45 min at 4°C. Cells were subsequently washed and resuspended in cold RPMI media. GFP+ tumor cells and F4/80+TAMs were separated using a FACS Vantage Cell Sorter (BD).

U-937 cells were washed in FACS buffer and incubated with mouse anti-human CD14 (BD) or mouse control IgG1 for 1 h at 4°C, washed twice and incubated with goat anti-mouse IgG conjugated to Alexa488 (Invitrogen). DAPI was added to discriminate living and dead cells. All directly conjugated antibodies and corresponding isotype controls (BioLegend, BD) were used at a concentration of 2 µg/ml.

**Western Blotting**

T241 fibrosarcoma tumors were lysed in NP40 lysis buffer (1% NP40, 150 mM NaCl, 10% glycerol, 20 mM HEPES, 1 mM phenylmethylsulfonyl fluoride, 2.5 mM EDTA, 100 µM $Na_3VO_4$, and 1% aprotinin). The samples were separated in NuPage 4-12% Bis-Tris Gels using MOPS buffer (Invitrogen) and transferred to a nitrocellulose membrane. Membranes were blocked in 5% non-fat dry milk in TBS + 0.01% Tween (blocking solution) for 1 h, incubated with rabbit-anti mouse HRG antibody (produced in-house) and detected using horseradish peroxidase-conjugated secondary antibody. Immune complexes were visualized by enhanced chemiluminescence (Amersham Biosciences).

Immunoblotting on lysates from peritoneal macrophages was performed in whole cell extracts using antibodies specific for phosphotyrosine pY641 STAT6 (Cell Signaling), STAT6 (Santa Cruz Biotechnology), pT180/Y182-p38 MAPK (Cell Signaling) and p38-MAPK (Cell Signaling).

**Microarray expression analysis**

RNA concentration was measured using a ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) and RNA quality was determined with the Agilent 2100 Bioanalyzer system (Agilent Technologies Inc, Palo Alto, CA). Two hundred and fifty nanograms of total RNA/sample were used to generate amplified and biotinylated sense-strand cDNA from the entire expressed genome according to the Ambion WT Expression Kit (P/N 4425209 Rev B 05/2009) and Affymetrix GeneChip® WT Terminal Labeling and Hybridization User Manual (P/N 702808 Rev. 1, Affymetrix Inc., Santa Clara, CA). GeneChip® ST Arrays (GeneChip® Mouse Gene 1.0 ST Array) were hybridized for 16 hours in a 45°C incubator, rotated at 60 rpm. According to the GeneChip® Expression Wash, Stain and Scan Manual (PN 702731 Rev 2, Affymetrix Inc., Santa Clara, CA) the arrays were then washed and stained using the Fluidics Station 450 and finally scanned using the GeneChip® Scanner 3000 7G. Subsequent analysis of the gene expression data was carried out in the freely available statistical computing language R (2) using packages available from the Bioconductor project (3). The raw data was normalized in the Affymetrix provided software Expression Console using the robust multi-array average (RMA) method. To search for

differentially expressed genes between the *hrg*$^{-/-}$ and wt groups, an empirical Bayes moderated t-test was applied, using the 'limma' package. To address the problem with multiple testing, the p-values were adjusted using the method of Benjamini and Hochberg (4). Functional annotation of genes was done using the DAVID functional annotation tool (5). Only GO biologic process terms (GO_BP FAT) were used. P-Values were calculated using modified Fisher's exact test and terms with a p-value < 0.05 were considered to be significantly enriched. The results were submitted to the Gene Expression Omnibus (6) with the accession number (GSE34164). Prediction of transcription factors potentially engaged downstream of HRG was done through TFactS (7).

**Primers used**

The following primers and Taqman probes were used: hprt fwd: CAAACTTTGCTTTCCCTGGT rev: TTCGAGAGGTCCTTTTCACC; Ccl12 fwd: GCCTCCTGCTCATAGCTACCACCA; Ccl12 rev: GRGACTGGGGTGCTCACCGC; Mmp8 fwd: CCACACACAGCTTGCCAATGCC, Mmp8 rev: CGTGGCATTCCTCGAAGACCGG, Ang2 fwd: GGACGTGGGTGGAGGAGGGT, Ang2 rev: ACTCTCCCAGAGGGCTCCCGA, CD80 fwd: GGGGCAGGATTCGGCGCAGTA, CD80 rev: GGCCCGAAGGTAAGGCTGTTGTT, Cxcl10 fwd: AAGTGCTGCCGTCATTTTCT, Cxcl10 rev: GGACCGTCCTTGCGAGAG; Cxcl11 fwd: CATAGCCCTGGCTGCAATA, Cxcl11rev: CGCCCCTGTTTGAACATAAG, E-Cadh fwd: GACTGTGAAGGGACGGTCAAC, E-Cadh rev: CCACCGTTCTCCTCCGTAGA, N-Cadh fwd: AGGCGGAGACCTGTGAAACTC, N-Cadh rev: CCATTAAGCCGGTTGATGGT, Snail fwd: CCACTGCAACCGTGCTTTT, Snail rev: CACATCCGAGTGGGTTTGG; Il1f9 fwd: TGACTTGGACCAGCAGGTGTGGA, il1f9 rev: TGCATGGGAGGATAGTCACGCTG, Ccr5 fwd: TGGGGTGGAGGAGCAGGGAG, Ccr5 rev: CAGCATCGGCCCTGTGTCCG; CD74 fwd: TGAGCAAGAACTCCCTGGAGGAGAA, CD74 rev: CCCGGGTAGACGGCAGGGAT; Ccr2 fwd: AGGAGCCTCTTTGCCTTGTGGC, Ccr2 rev: TGGCAGCCTCATGCCCTCCT; Ccl6 fwd: GGGTCCCAGGCTGGCCTCAT, Ccl6 rev: TGTGTGGCATAAGAGAAGCAGCAGT; Ifitm6 fwd: TGGCTGCTGCCTGGGTTTCA, Ifitm6 rev: TGGCGGTTGAAGCATGGGATTGG; mFcgr1 fwd: GCCAGTTTTCAGGACAGTGGCGA, mFcgr1 rev: GACTCTGCGGGAGGCCTGGA; hFcgr1 fwd: AGGGGAATCTGGTCACCCTGAGC; hFcgr1 rev: CCTCGCAGGGTCTTGCTGCC hprt: Mm01324427_m1; arg1: Mm00475991_m1; Ccl22: Mm00436438_m1; Il10: Mm00439616_m1; Plgf: Mm00435611_m1; Vegfa: Mm03015193_m1; hrg: Mm00504391_m1; CD11c: Mm01015070_m1.

**Supplemental Table 1**

Peripheral blood cells in wt and *hrg*$^{-/-}$ mice

| | wt | *hrg* $^{-/-}$ |
|---|---|---|
| Concentration leukocytes ($10^9$ cells/l) | 4.6 ± 1.8 | 2.8 ± 0.9 |
| Concentration thrombocytes ($10^9$ cells/l) | 948 ± 137 | 920 ± 63 |
| % neutrophils | 9 ± 0.8 | 8.4 ± 1.4 |
| % lymphocytes | 85 ± 2.8 | 87 ± 5.3 |
| % monocytes | 2.9 ± 1 | 3.3 ± 0.5 |

**References**


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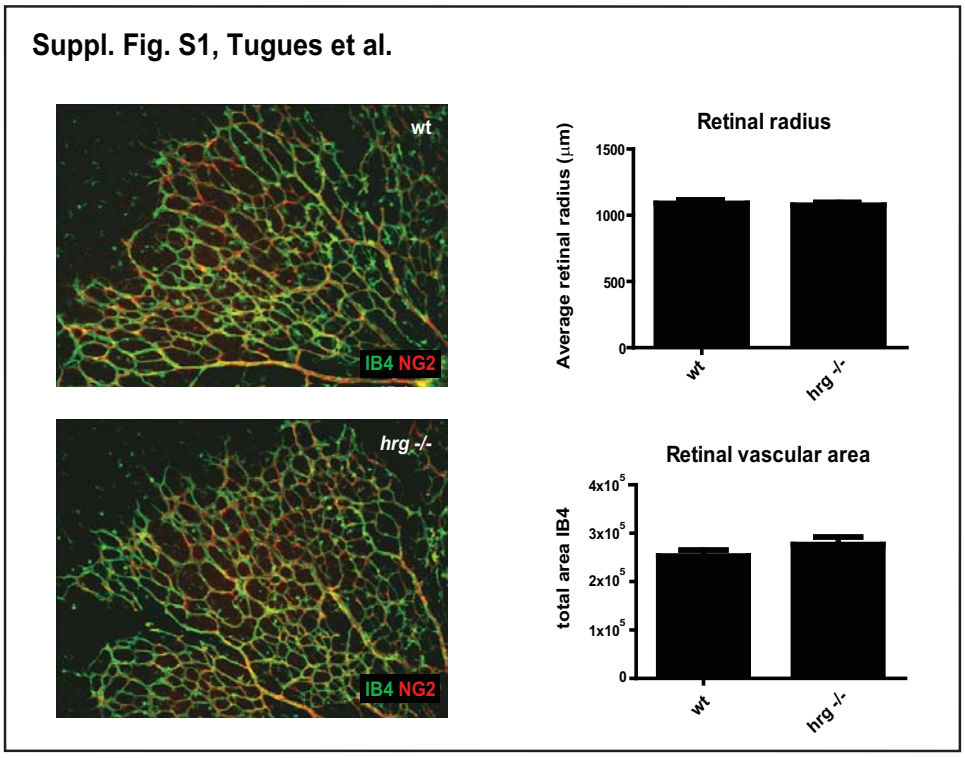
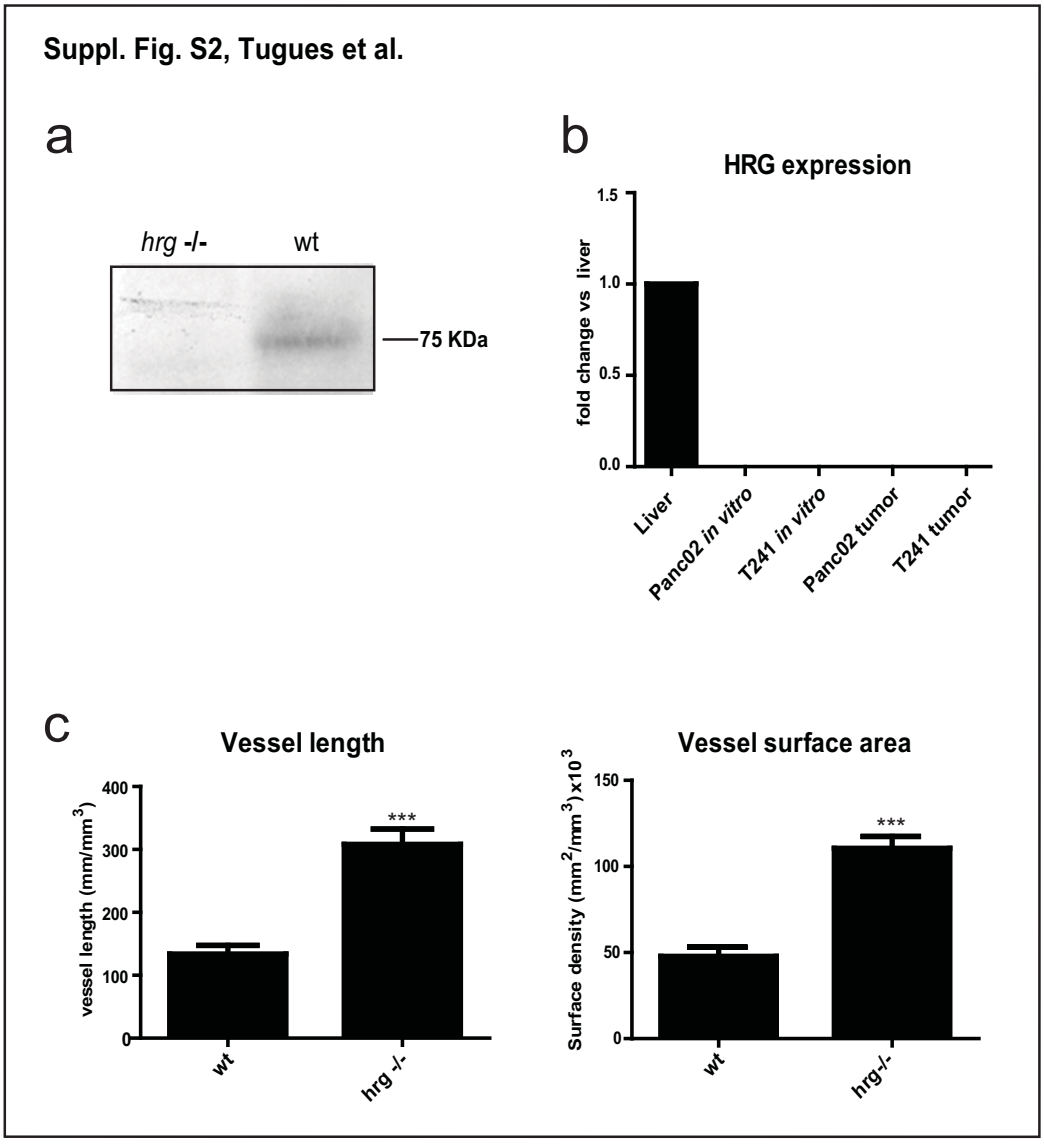
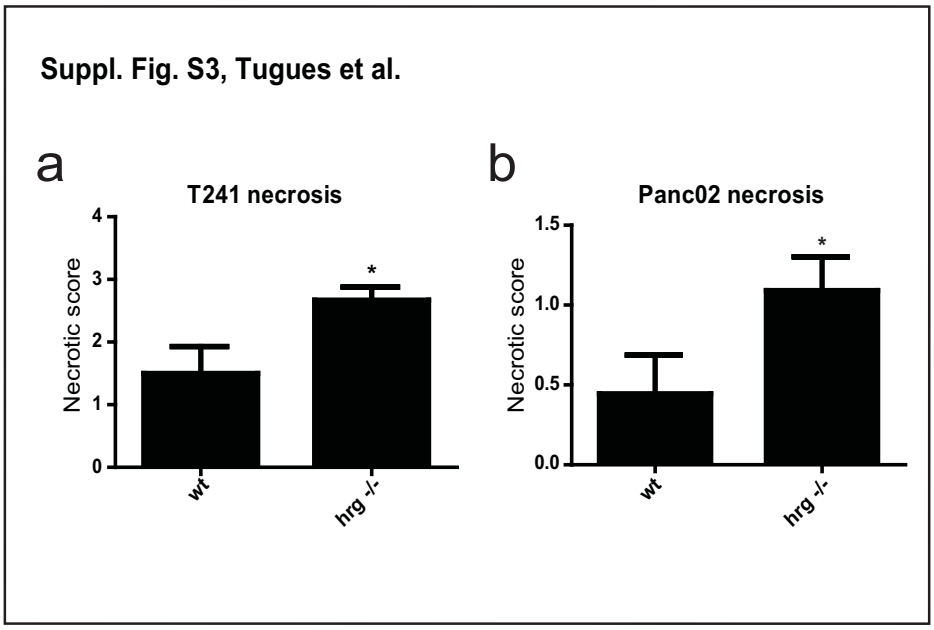


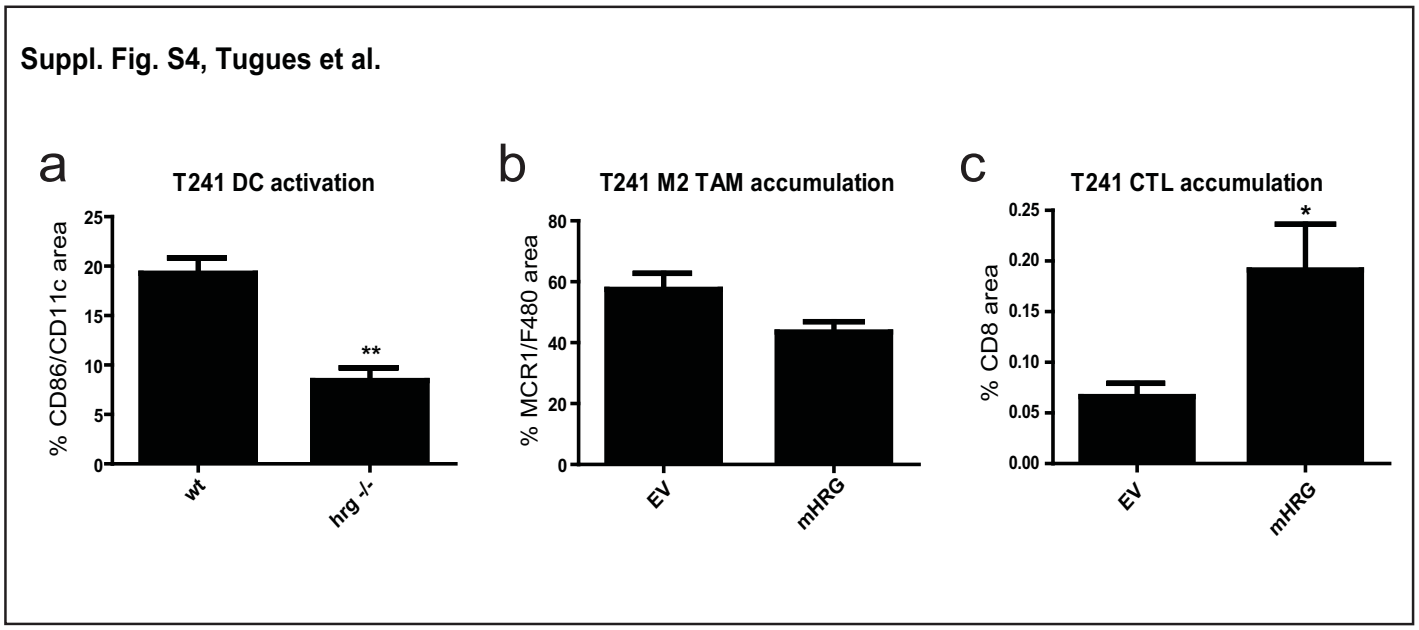

**Supplemental Figure S1**. Developmental retinal angiogenesis in wt and *hrg-/-* mice. Quantification of retinal radius and vascular area (panels to the right) shows that postnatal development of the retinal vasculature was similar in wt and *hrg-/-* eyes. n=9/group. Data are mean values ± SEM.



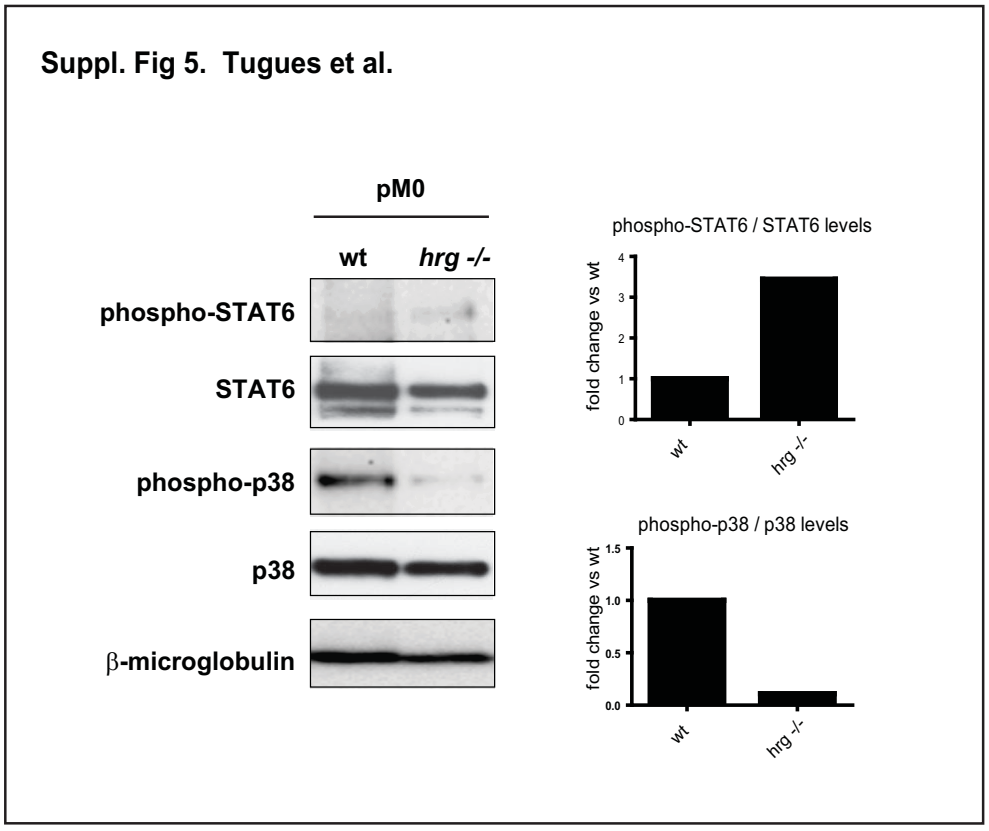

**Supplemental Figure S2**. a. Expression of HRG and effects on tumor vessels in T241 tumors from wt and *hrg-/-* mice. Samples were analyzed by immunoblotting using an anti-mouse HRG antibody produced in-house. b. *hrg* mRNA expression in T241 fibrosarcoma and Panc02 cells, *in vitro* cultures and tumor lysates. c. Stereological quantification of vessels in T241 tumors. Sections immunohistochemically stained using anti-VE-cadherin antibodies (n=15/group) were quantified using a grid, and vessel length and surface area quantified as described (1). ***p<0.001; Data are mean values ± SEM.



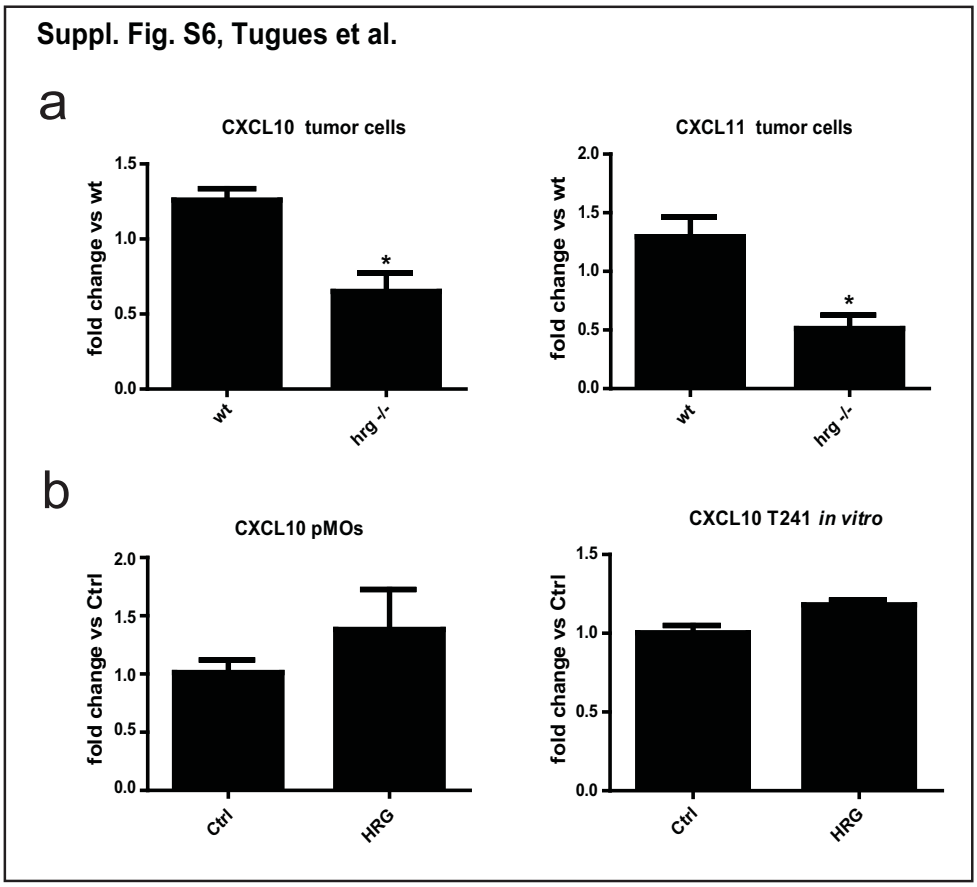

**Supplemental Figure S3**. Scoring of degree of necrosis according to clinical pathology routine in T241 fibrosarcoma and Panc02 tumors from wt vs *hrg-/-* mice; n=20/group.
a. H&E staining of T241 fibrosarcoma shows increased necrosis in tumors from *hrg-/-* mice.
b. H&E staining of Panc02 tumors shows increased necrosis in tumors from *hrg-/-* mice.
*p<0.05; Data are mean values ± SEM.



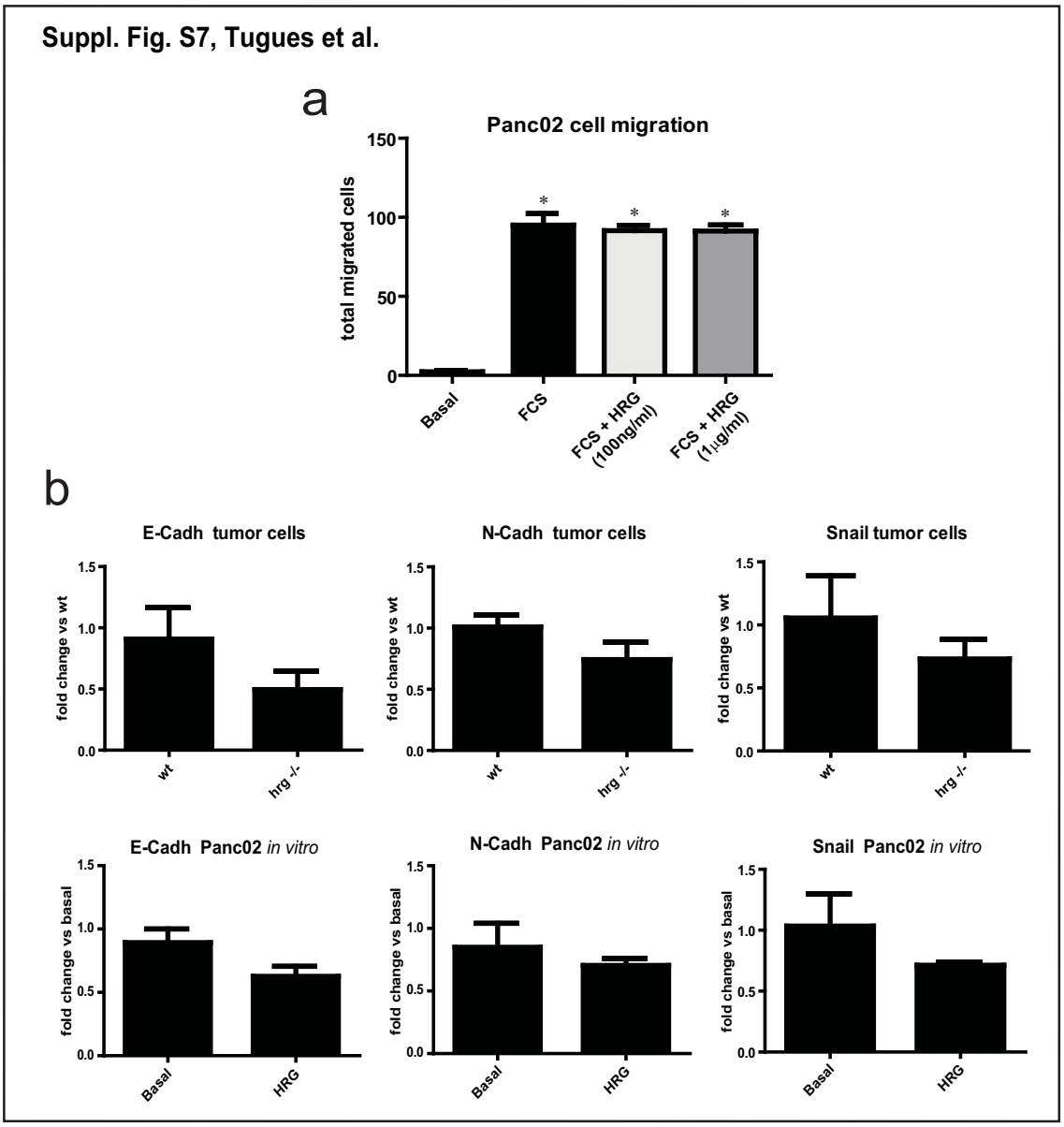

**Supplemental Figure S4.** Tumor-infiltrating immune cells in T241 and Panc02 tumors. a. % of CD86+ area/total CD11c+ area in T241 fibrosarcoma from wt and hrg-/- mice. b. % MRC1+ area/total F4/80+ area in mHRG-expressing and empty virus (EV) T241 tumors; n=10/group. c. % CD8 area/total tumor area in mHRG-expressing T241 tumors and EV T241 tumors; n=10/group. *p<0.05, **p<0.01; Data are mean values ± SEM.



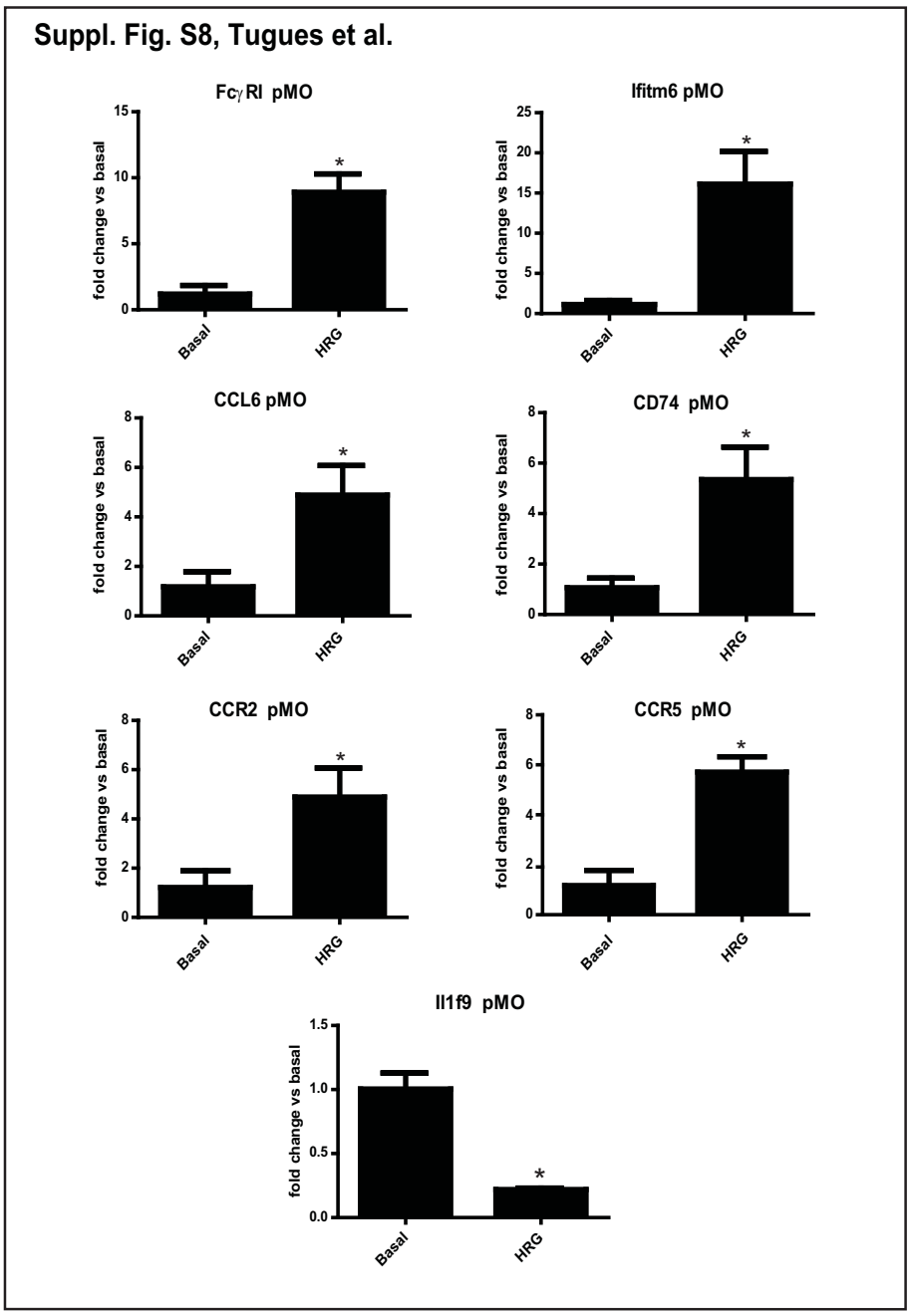

**Supplemental Figure S5**. Analysis of pSTAT6, total STAT6, phospho-p38 MAPK and total p38 MAPK levels in pMOs isolated from wt and *hrg-/-* mice. Lysates of pMO isolated from wt and *hrg-/-* mice were analyzed by immunoblotting (left panels). Quantifications of pSTAT6 vs total STAT6 and phospho-p38 MAPK vs total p38 are shown to the right.




**Supplemental Figure S6**. Transcript expression in wt and hrg-/- T241 tumor cells and pMOs treated with HRG. a. Expression of *cxcl10* and *cxcl11* in isolated tumor cells from wt and hrg-/- mice. b. Expression of *cxcl10* and *cxcl11* in pMOs and T241 fibrosarcoma cells treated with 1 μg/ml recombinant hHRG for 24 hours. *$p<0.05$; Data are mean values ± SEM.




**Supplemental Figure S7**. Effects of HRG on migration and EMT. a. Migration of Panc02 cells in the absence and presence of recombinant hHRG (1μg/ml) for 5 hours. b. Transcript expression of E-Cadherin, N-Cadherin and Snail in isolated tumor cells from wt and hrg-/- mice (upper panels) or Panc02 cells treated with recombinant hHRG (1 μg/ml) (lower panels), for 24 hours.




**Suppl. Figure S8**. Transcript expression in pMOs treated with recombinant hHRG (1 μg/ml) for 24 hours. Fold-change in expression of *FcγRI*, *Ifitm6, CCL6, CD74, CCR2, CCR5* and *Il1f9* in wt pMOs, HRG-treatment vs basal condition. *p<0.05; Data are mean values ± SEM.