Genetic deficiency in plasma protein HRG enhances tumor growth and metastasis by exacerbating immune escape and vessel abnormalization.

by

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Abstract

Histidine-rich glycoprotein (HRG) is a 75 kDa heparin-binding plasma protein implicated in the regulation of tumor growth and vascularization. In this study, we show that hrg−/− mice challenged with fibrosarcoma or pancreatic carcinomas grow larger tumors with increased metastatic properties. Compared with wild type mice, fibrosarcomas in hrg−/− mice were more hypoxic, necrotic and less perfused, indicating enhanced vessel abnormalization. HRG-deficiency was associated with a suppressed anti-tumor immune response, with both increased infiltration of M2-marker-expressing macrophages and decreased infiltration of dendritic cells and cytotoxic T cells. Analysis of transcript expression in tumor-associated as well as peritoneal macrophages from hrg−/− mice revealed an increased expression of genes associated with a pro-angiogenic and immunoinhibitory phenotype. In accordance, expression arrays performed on HRG-treated peritoneal macrophages showed induction of genes involved in extracellular matrix biology and immune responsiveness. In conclusion, our findings demonstrate that macrophages are a direct target of HRG. HRG loss influences macrophage gene regulation, leading to excess stimulation of tumor angiogenesis, suppression of tumor immune response, and increased tumor growth and metastatic spread.

Précis

Findings establish an important link between deficiency of a highly expressed plasma protein and tumor progression via activation of pro-tumoral macrophages and immune suppression.
INTRODUCTION

Cancer progression is influenced by the tumor microenvironment, in which tumor cells and different stromal constituents exchange regulatory cues that steer tumor growth and invasion. Tumor angiogenesis and tumor-promoting inflammation are hallmarks of cancer (1). Tumor vessels are morphologically and functionally abnormal, with deficient perivascular support and partially or completely obliterated lumens, leading to poor perfusion, high interstitial pressure and hypoxia (2). Hypoxia regulates the production of cytokines that promote infiltration and activation of various populations of myeloid cells, which serve to further sustain the proangiogenic environment and to suppress anti-tumor immune responses (3). Thereby, the vicious circle of uncontrolled tumor growth, invasion and metastatic dissemination is propagated.

Thus, inflammation is a critical factor in every step of tumorigenesis, from initiation to tumor progression and metastatic dissemination (4-6). The tumor-associated macrophage (TAM), a highly adaptive myeloid-derived cell that exhibits different activation states dependent on the tumor microenvironment (7, 8), has emerged as an attractive target in cancer therapy. Two main TAM phenotypes denoted M1 and M2 coexist in tumors. The M1 (classically activated) TAMs secrete tumoricidal agents that promote T-helper-1 responses, whereas the more abundant M2 (alternatively activated) TAMs promote exaggerated, dysfunctional angiogenesis and suppress anti-tumor immunity by preventing infiltration and activation of dendritic cells (DCs), cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells (5, 9). TAMs and their released factors thereby influence different steps of invasion and metastasis (6).

Histidine-rich glycoprotein (HRG) is a 75 KDa heparin-binding plasma protein exclusively synthesized by hepatocytes (10, 11). HRG is stored in platelet α-granules but is also present free in plasma at concentrations of 100-150 μg/ml in the human (see (12) for a review). Tumor angiogenesis is inhibited by HRG and peptides derived from its central His/Pro-rich domain, leading to reduced vascularization and growth of different syngeneic tumor models (13-16). HRG inhibits adhesion and chemotaxis of primary endothelial cells in vitro (17), providing a mechanism for HRG’s effect in attenuation of tumor angiogenesis. Furthermore, HRG inhibits tumor growth and metastasis by inducing TAM skewing away from M2 to M1 leading to anti-tumor immunity and vessel normalization (11).

Through its many interactions, HRG has been implicated in diverse functions. Thus, HRG binds to different components of both the coagulation and fibrinolytic systems (18), components of the immune system such as complement proteins and Fcγ receptors (19), immunoglobulins (IgGs) (18), immunocomplexes (20) and other types
of ligands such as heme, divalent cations (21) and glycosaminoglycans (22). Through
certain of these interactions, HRG modulates immune responsiveness (12). For
example, HRG facilitates the clearance of immune complexes and dying/dead cells
via an Fc\(\gamma\)R-I dependent mechanism (12, 19, 23, 24). HRG moreover enhances the
ability of immune complexes to activate complement, allowing faster clearance of
necrotic material (18). HRG has also been implicated in the defense at the local site of
bacterial infection (25).

We now further validate and extend the concept that HRG is instrumental in the tumor
immune response. Using an \(hrg\) gene-inactivated mouse model, we show that lack of
HRG leads to i) increased tumor growth rate and metastatic dissemination, ii)
enhanced tumor vessel abnormalization, iii) critical changes in the activation state of
macrophages that lead to a tumor-promoting immune response, and iv) a constitutive
M2-like polarity of peritoneal macrophages. Furthermore, the immune stimulatory
transcript profile induced by HRG in peritoneal macrophages \(ex vivo\) is in accordance
with the inhibitory immune profile in the HRG-deficient tumors. Thus, through direct
and indirect effects, lack of HRG leads to alterations in several molecular and cellular
components within the tumor microenvironment.
MATERIAL AND METHODS

See Supplement for materials and methods used for lentivirus production, retinal vascularization, leukocyte count, chemotaxis, tumor studies, immunofluorescence and immunohistochemistry, hypoxia, necrosis assessment and tumor perfusion, flow sorting analysis, immunoblotting, microarray expression analysis, and for primer sequences.

Tissue Culture
Murine T241 fibrosarcoma cells (American Type Culture Collection; ATCC) were maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA), 10% FBS (Sigma-Aldrich, St. Louis, MO). The murine pancreatic Panc02 tumor cell line (provided by Dr. B. Wiedenmann, Charité, Berlin), were cultured in RPMI (Invitrogen), 10% FBS. Where indicated, T241 and Panc02 cells were starved overnight in medium with 0.5% FBS before being treated with HRG (1 μg/ml) for 6 or 24 hours.

Human embryonic kidney HEK-293 cells (ATCC) were cultured in DMEM, 10% FCS and given 0.25 mg/ml G418 (Calbiochem) to ensure selection of EBNA-1 expression. The 293-EBNA were used to produce recombinant HRG as described previously (15).

A subclone of the human monoblastic cell line U-937, U-937-1 (26), a kind gift from Dr. Kenneth Nilsson, Uppsala University, was maintained in RPMI 1640 supplemented with 10% FBS. Cells were treated with 25α-Dihydroxicholecalciferol (Vitamin D3) for 4 days before stimulation with recombinant hHRG (1 μg/ml) alone or in combination with lipopolysaccharide (LPS, 100ng/ml) for 24 hours.

Cell lines were not authenticated after purchase (T241, 293-EBNA) or after transfer from other laboratories (Panc02, U-937-1), but were routinely tested negative for mycoplasma using the Mycoplasma Detection Kit (Lonza).

Animals
All animal work was performed according to the guidelines for animal experimentation and welfare provided by Uppsala University and approved by a regional ethics committee. Mice deficient for HRG (hrg−/−) have been described previously (27). C57BL/6 wild type mice (8-10 weeks) were obtained from Taconic M&B (Bomholt, Denmark).
Isolation of naïve peritoneal macrophages
Peritoneal macrophages were isolated from naïve hrg−/− and age-matched C57BL/6 wild type mice. Mice were anaesthetized with ketamine/xylazin and injected intraperitoneally with 5 ml RPMI (Invitrogen) + 5% FBS. Cells were extracted by peritoneal lavage, counted, plated overnight and stimulated with HRG (1 μg/ml) for 24 hours.

Statistical analysis
Data was analysed using GraphPad Prism 5.0 (GraphPad Software Inc., La Jolla, CA). Bars represent mean values ± SEM. For comparison of two groups, Student’s unpaired t-test was used. Two-way analysis of variance (ANOVA) followed by Bonferroni post-tests were performed to assess for significant differences in tumor volume. p-value < 0.05 was considered statistically significant.

GEO accession number
Microarray results were submitted to the Gene Expression Omnibus (28) and given the accession number (GSE34164).
RESULTS

Hrg<sup>−/−</sup> mice show normal vascular and hematopoietic development

Hrg gene targeted C57BL/6 mice (hrg<sup>−/−</sup>; (27)), were examined to determine if HRG has a general role in vascular development. Hrg<sup>−/−</sup> mice showed the same area and radius of the vascular plexus in the developing retina as wild type mice (Supplemental Fig. S1). Given the role of HRG on skewing of TAMs (11), we investigated whether HRG-deficiency affects the blood count. Assessment of different peripheral blood populations showed no statistically significant difference between hrg<sup>−/−</sup> and wt mice in any of the blood cell subtypes analyzed (Supplemental table 1). Thus, both vascular and hematological development appeared undisturbed by hrg gene inactivation.

HRG-deficiency leads to increased tumor growth and metastasis

We have previously shown that growth of T241 fibrosarcoma is attenuated in mice treated with human HRG (hHRG) or express hHRG as a result of lentivirus-mediated transduction (11, 15). Importantly, expression of mouse HRG (mHRG) in T241 fibrosarcoma suppressed tumor growth (Fig. 1a and b) to the same extent as reported earlier for hHRG (11).

To test the effects of endogenous HRG on tumor growth, wt and hrg<sup>−/−</sup> mice were inoculated with T241 fibrosarcoma or Panc02 pancreatic cancer cells. These C57-syngeneic tumor models do not express endogenous HRG, but are known to respond to HRG treatment (11). Moreover, T241 fibrosarcoma grows subcutaneously and in a non-metastatic manner under the conditions used here, whereas Panc02 grows orthotopically in the pancreas with local metastatic spread. We confirmed the presence of host-derived HRG in tumors from wt, but not knockout mice (Supplemental Fig. S2a), and lack of expression in the tumor cell lines in vitro (Supplemental Fig. S2b). As shown in Fig. 1c, d and e, HRG-deficiency was associated with increased tumor growth and end-point tumor weight in both tumor models. Furthermore, hrg<sup>−/−</sup> mice carrying Panc02 tumors presented a significant increase in the number of metastatic lymph nodes as compared to wt mice (Fig. 1f).

HRG-deficiency is accompanied by dysfunctional tumor angiogenesis

Analysis of the properties of T241 neovascularization in hrg<sup>−/−</sup> mice by immunostaining for the endothelial cell marker vascular endothelial (VE)-cadherin, displayed significantly increased area in tumors from hrg<sup>−/−</sup> mice vs wt mice (Fig. 2a). T241 tumor vessels from hrg<sup>−/−</sup> mice showed increased length and surface area (Supplemental Fig. S2c) and decreased coverage by αSMA-positive cells in the hrg<sup>−/−</sup> mice (Fig. 2b).
Vessel perfusion with FITC-lectin to determine the functionality of the T241 vasculature showed markedly reduced perfusion in tumors from hrg−/− mice (Fig. 2c). In Panc02 tumors, the vessel area was comparable in tumors from wt and hrg−/− mice (Fig. 2d). On the other hand, PancO2 tumor vessels from hrg−/− mice showed decreased αSMA-positive pericyte coverage (Fig. 2e), indicating certain shared properties with T241 tumor vessels (Fig. 2b), in the absence of HRG. The extent of vessel perfusion was comparable in Panc02 tumors from wt and hrg−/− mice (Fig. 2f).

We next evaluated parameters within the tumor microenvironment that might influence tumor angiogenesis and tumor growth. Tumor hypoxia was assessed by immunohistochemical detection of pimonidazole (PIMO) adducts. Hypoxia was increased in T241 fibrosarcoma from hrg−/− mice vs wt mice (Fig. 3a). T241 fibrosarcomas from hrg−/− mice also contained larger hemorrhagic (Fig. 3b) and necrotic areas (Supplemental Fig. S3a). Moreover, tumors from hrg−/− mice were more apoptotic and proliferated more than those from wt mice (Fig. 3c and 3d), as judged by detection of phospho-Histone H3, identifying mitotic cells, and cleaved caspase-3, a marker of apoptotic cells.

In the Panc02 tumors, hypoxia was similar in wt and hrg−/− mice (Fig. 3e); still, hemorrhaging (Fig. 3f) and necrosis (Supplemental Fig. S3b) were significantly increased as a consequence of HRG-ablation. Neither apoptosis nor proliferation, were significantly changed by HRG-deficiency in the Panc02 model (Fig. 3g-h).

We conclude that HRG-deficiency had a similar yet distinct (see Discussion) impact on the overall condition of the two tumor models with enhanced tumor growth and metastatic dissemination, unstable pericyte-deficient vasculature and increased hemorrhaging and necrosis as shown in Figs. 1-3 and Supplemental Fig. S3a-b.

**HRG-deficiency is associated with increased pro-tumorigenic immune cell infiltration**

The contribution of tumor inflammation to the vascular changes and tumor growth in HRG-deficient mice was assessed by immunostaining for the macrophage marker F4/80, which showed significantly more F4/80-positive cells in tumors from HRG-deficient mice as compared to wt (Fig. 4a). Moreover, there was increased infiltration of CD45-positive cells expressing the mannose receptor MCR1, an M2-associated marker, in tumors from HRG-deficient mice (Fig. 4b). Furthermore, infiltration of CD11c-dendritic cells (DCs) into T241 tumors decreased with HRG ablation (Fig. 4c). Most of
the CD11c cells were positive for the macrophage marker CD68 and may therefore represent a subset of proinflammatory TAMs able to promote antitumor immune responses (29). Moreover, the CD11c population expressed lower levels of the costimulatory molecule CD86, suggestive of decreased capacity to activate T cells (30) (Supplemental Fig. S4a). In agreement, the number of infiltrating cytotoxic CD8-positive T lymphocytes decreased in HRG-deficient fibrosarcomas (Fig. 4d).

Importantly, compared to HRG-deficient T241 tumors, mHRG-overexpressing T241 tumors showed the reversed pattern with a clear trend towards decreased infiltration of MCR1-positive macrophages and significantly increased numbers of CD8 T cells (Supplemental Fig. S4b and S4c).

Also, Panc02 tumors showed increased infiltration of F4/80-positive cells in the absence of HRG (Fig. 4e). However, Panc02 TAMs from hrg<sup>-/-</sup> and wt mice expressed similar levels of MCR1 (Fig. 4f). On the other hand, infiltration of CD11c cells was decreased in the Panc02 tumors from hrg<sup>-/-</sup> mice (Fig. 4g), in agreement with the pattern in the T241 tumors. Around 25-30% of the CD11c cells co-expressed F4/80 in both genotypes (data not shown). Moreover, Panc02 tumors from hrg<sup>-/-</sup> mice showed reduced numbers of CD8 T cells (Fig. 4h).

Overall, these data indicate that HRG-deficiency was accompanied by altered infiltration of several important immune cell types, potentially contributing to the increased tumor burden and metastatic dissemination observed in HRG-deficient mice.

**TAMs and peritoneal macrophages (pMOs) from hrg<sup>-/-</sup> mice display a proangiogenic, immunoinhibitory phenotype**

Transcript profiling was used to explore the mechanisms underlying the increased tumor angiogenesis and altered immune cell profile in HRG-deficient mice. As shown in Fig. 5a, tumors from hrg<sup>-/-</sup> mice expressed increased levels of the proangiogenic growth factors PlGF and VEGF, while the angiostatic members of the CXC chemokine family CXCL10 and CXCL11 (31) were downregulated. CXCL10 and CXCL11 are T cell chemotactic, and their reduced expression is in keeping with the reduced number of tumor infiltrating CD8 T cells in HRG-deficient mice.

Profiling of TAMs isolated from T241 fibrosarcoma growing in hrg<sup>-/-</sup> mice compared to TAMs from wt mice showed that HRG-deficiency was accompanied by increased expression of the M2-associated gene Arginase-1 (Arg-1); of Angiopoietin-2 (Ang-2), a Tie-2 ligand that promotes tumor angiogenesis and infiltration of Tie-2 expressing
TAMs (32), and of CCL12, a potent chemoattractant for peripheral blood monocytes (33). The metalloproteinase MMP-8, which has been linked to inflammation and cancer progression (34) was also upregulated. TAMs from hrgr−/− mice furthermore contained reduced levels of CXCL10 and CXCL11 and of the costimulatory molecule CD80, important for T cell priming during DC activation (Fig. 5b). Taken together, these results suggest that HRG-deficiency leads to important alterations in the activation state of TAMs.

M2 marker expression was further examined in pMOs isolated from naïve hrgr−/− and wt mice. pMOs from HRG-deficient mice expressed about 10-fold higher levels of IL-10, Arg-1 and CCL22 in comparison to wt pMOs, indicating that under conditions of constitutive HRG-deficiency, macrophages are strongly skewed towards a proangiogenic and immunoinhibitory M2 phenotype (Fig. 5c). In agreement, immunoblotting showed a 3.5-fold increase in phosphoSTAT6 levels in pMOs from hrgr−/− mice compared to wt (Supplemental Fig. S5); STAT6 is a known regulator of several M2 genes such as Arg1 and MRC1 (35). On the other hand, activation of the stress kinase p38MAPK was markedly downregulated in the hrgr−/− pMOs compared to wt (Supplemental Fig. S5).

HRG-induced gene regulation
We detected decreased expression of the anti-tumor chemokines CXCL10 and CXCL11 in tumors from hrgr−/− mice (Fig. 5a), as well as in freshly isolated TAMs (Fig. 5b) and tumor cells (Supplemental Fig. S6a) derived from T241 tumors grown in hrgr−/− mice. Further analyses showed that treatment of pMOs or T241 tumor cells in vitro with HRG did not alter expression of CXCL10 (Supplemental Fig. S6b) whereas CXCL11 was undetectable, overall suggesting that their regulation is not directly mediated by HRG. Furthermore, we excluded potential direct effects of HRG on Panc02 tumor cells, as treatment of tumor cells with HRG did not affect the migration of these cells in vitro (Supplemental Fig. S7a). Loss or gain of HRG also did not significantly affect the expression of different genes involved in epithelial-mesenchymal transition (EMT), a process known to confer an invasive and metastatic phenotype in tumor cells (Supplemental Fig. S7b) (36).

To determine global HRG-induced gene regulation in relation to macrophage activation and function, we isolated pMOs from wt mice, treated them ex vivo with HRG and assessed for differences in gene expression by oligonucleotide microarrays. Interestingly, treatment of pMOs with HRG resulted in significant upregulation of 96 genes and downregulation of 55 (defined as fold change ± 1.8; p<0.05), which
clustered in different functional categories. Overall, validation analyses by qRT-PCR showed results consistent with the data obtained in microarray experiments (Supplemental Fig. S8). As shown in Table 1, the most significant changes occurred for genes involved in regulation of the extracellular matrix and immune responsiveness. The latter category includes humoral immune response genes such as components of the complement system and Fcγ receptors, several chemokines regulating chemotaxis and activation of monocytes and T cells, and genes implicated in antigen presentation and priming of T cells. The HRG-induced upregulation of genes related to DC maturation and T cell priming (CD11c, CD83 and MHC class II; see Table 1) is compatible with an immunocompetent anti-tumor microenvironment in the presence of HRG, and, conversely, with the immunosuppressed pattern described here for tumors growing in HRG-deficient mice.

To predict which transcriptional factors are engaged downstream of HRG to regulate the listed target genes in peritoneal macrophages, we used the tool TFactS. This approach resulted in a list of factors including POU2F1 (Oct-1), POU2F2 (Oct-2), SPI1 (PU.1), NF-E2-related factor 2 (Nrf2), JUN, Forkhead box protein 01 (FoxO1) and GLI1, which thus may be at least in part, responsible for the gene expression profile in the HRG-treated pMOs (Table 1).

To address the mechanisms of HRGs effects on macrophages, we exposed different cell lines to HRG in vitro. Mature macrophage cell lines such as Monomac (37) or RAW 264.7 (38) were unresponsive to HRG and failed to show gene regulatory effects such as those observed in pMOs (Supplemental Fig. S8). We further analyzed the monoblastic cell line U-937 (39), which differentiates towards the monocytic/macrophage lineage when treated with vitamin D3. As shown in Fig. 6a, combined treatment with vitamin D3 and HRG significantly increased expression of the monocyte-macrophage antigen CD14. Importantly, treatment with HRG in the presence of vitamin D3 led to significantly increased expression of FcγRI and CD11c (Fig. 6b), responses that were further strengthened in the presence of LPS. These genes were upregulated by HRG also in pMOs (Table 1). These data show that HRG acts on monocytes/macrophages in a specific differentiation window, where it exerts its effects at least in part by inducing certain markers of proinflammatory macrophages.
Discussion

We report that lack of HRG promotes increased tumor growth and dissemination accompanied by an altered gene expression profile of infiltrating TAMs, leading to a tumor-promoting microenvironment and dysfunctional tumor vasculature. Our data extend a recent report on enhanced angiogenesis in HRG-deficient Rip1-Tag2 mice (40), which was sensitive to treatment with a platelet-depleting antibody. The potential contribution of platelets to the immunomodulatory role of HRG remains to be addressed.

The T241 fibrosarcomas growing in hrg-/- mice presented several features of impaired vessel function. The vessel area was increased, probably through increased production of proangiogenic VEGF and PlGF and reduced production of the angiostatic chemokines CXCL10 and CXCL11. Vessels were also more immature, as indicated by the reduced pericyte coverage, and poorly perfused, resulting in increased hypoxia, bleeding and necrosis in the tumor tissue. The role of hypoxia in promoting tumor angiogenesis and inflammation is generally accepted. Moreover, hypoxia is a well-known instigator of tumor aggressiveness and promoter of EMT, which is associated with increased local invasiveness (36). There were no significant changes in expression of EMT-related genes either in tumor cells treated with HRG in vitro or in tumors from wt and hrg-/- mice.

Oxygen-deficiency alters expression of a wide range of genes implicated in inflammation. In agreement, we detected elevated expression of hypoxia-sensitive genes such as Arg-1, Ang-2, MMP8 and CCL12 in TAMs isolated from hrg-/- mice. In accordance with the established function of these genes, HRG-deficiency was accompanied by a tumor-promoting immune response, characterized by increased infiltration of MCR1-expressing TAMs and decreased infiltration of CD11c cells and cytolytic CD8 T cells. Notably, isolated pMOs that had never been in contact with HRG showed an activated phenotype reminiscent of M2 TAMs. Thus, HRG-deficiency appears to prime macrophages to a constitutive M2 polarity favoring tumor growth and immunosuppression. It must be stressed that the definition of M1 and M2 genotypes needs to take the context into consideration, in particular as tumors were growing in a constitutively HRG-deficient environment. Phenotypically, the M2 classification is highly relevant, since we noted M2-typical changes in infiltration of several immune cells (decreased DCs and T cells), which may account for the increased growth and dissemination of the tumor. M2 TAMS are also known to be pro-angiogenic (5, 9), promoting formation of dysfunctional and excess tumor vasculature, which agrees with
the altered vessel morphology and impaired function that we detected in the two tumor models in hrГіГ— mice, studied here.

T241 and Panc02 tumors from hrГіГ— mice contained reduced numbers of CD11c-positive DCs, which expressed lower levels of CD86, a costimulatory molecule from the B7 family important for antigen presentation and T cell priming (30). Tumor progression is as a rule accompanied by reduced DC recruitment and maturation, and DC depletion and dysfunction are critical in the escape from immune surveillance (41). Immunosuppressive factors such as IL-6, VEGF, TGF-β and IL-10, have been shown to inhibit DC recruitment and/or functions (42). The decreased DC recruitment and maturation in the HRG-deficient condition presents a mirror image of our previous report on more active DCs in HRG-expressing T241 fibrosarcoma in wt mice (11). HrgГ— mouse T241 and Panc02 tumors furthermore showed reduced infiltration of CD8 T cells. Decreased expression of the antiangiogenic chemokines CXCL10 and CXCL11 both by tumor cells and by TAMs, combined with decreased numbers and function of DCs in the HRG-deficient condition, could account for the reduced T cell infiltration.

There were clear similarities in the overall effects of HRG-deficiency on T241 and Panc02 tumor properties. The T241 tumors from hrГіГ— mice displayed a more deteriorated condition probably due to the larger size of these tumors (see Fig. 1d vs e), which grew in a relatively avascular anatomical location (intradermally) as compared with the orthotopic Panc02 tumors. There may be additional aspects of the microenvironments of the two cancer models due e.g. to differences in lymphatic drainage, different interstitial composition etc, which may have influenced the tumor host interactions. That HRG deficiency led to increased metastatic dissemination in the Panc02 orthotopic model is important in relation to our previous finding of decreased metastatic spread from HRG-expressing Panc02 tumors (11). However, the blood vessel area, vascular perfusion and hypoxia were similar in Panc02 tumors from wt and hrГіГ— mice. On the other hand, tumor vessels from HRG-deficient mice were only partially covered by Г–SMA+ cells and the tumor tissues were more hemorrhagic and necrotic. Clearly therefore, vessel parameters were affected also in the Panc02 model, which in turn might contribute to the increased metastatic spread. The changed immune profile of Panc02 tumors from hrГіГ— mice with increased infiltration of TAMs and decreased numbers of CD11c cells and CD8 T cells may also have contributed to tumor aggressiveness. Of note, the metastatic index (nodules/gram tumor weight) did not differ between wt and hrГіГ— genotypes (data not shown), indicating that increased dissemination could be due to the combined effects on pro-tumorigenic immunity and
dysfunctional blood vessels and therefore enhanced tumor growth in the absence of HRG.

The biological consequence of HRG and HRG-deficiency, prompts the question of the identity of a potential HRG receptor. FcγRs are important candidates and well-known HRG binders. However, it has still not been possible to tie HRG-induced gene regulation to FcγR expression in vivo (data not shown), perhaps due to the redundancy within this family of immunoglobulin receptors (43). Constitutive HRG-deficiency affects signalling, as we noted clear differences in the activity states of STAT6 and p38 MAPK when comparing wt and hrg−/− pMOs. To better understand HRGs effects, we studied gene regulation in pMOs. The range of genes regulated in pMOs in response to HRG (humoral immune response, interferon inducible proteins, T cell/monocyte chemotaxis and activation, antigen presentation and regulation of extracellular matrix), agree with the consequence of loss of HRG in the tumor microenvironment. The HRG-induced upregulation of genes related to DC maturation and T cell priming (CD11c, CD83 and MHC class II) is of particular interest, since they would favor a more efficient, anti-tumoral immunostimulatory tumor microenvironment. HRG-induced upregulation of M1 markers was also reproduced in vitro in monoblastic U-937 cells. It is essential to further identify HRG’s mechanism of action to successfully apply HRG to therapeutic steering of tumor macrophage activity in cancer forms characterized by proangiogenic and pro-tumor inflammation.

Acknowledgements
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References

Table 1. Functional categories of genes regulated by HRG in pMOs.

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I. REGULATION OF THE IMMUNE RESPONSE

I. 1. HUMORAL IMMUNE RESPONSE

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I. 2. INTERFERON INDUCIBLE PROTEINS

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<td>10402347</td>
<td>interferon, alpha-inducible protein 27 like 2A</td>
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I. 3. T CELL/MONOCYTE CHEMOTAXIS AND ACTIVATION

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<tr>
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<tr>
<td>10389222</td>
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<td>10590635</td>
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<td>10357472</td>
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<td>Cxcr4</td>
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I. 4. ANTIGEN PRESENTATION/ T CELL PRIMING

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<td>CD83 antigen</td>
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<td>10450154</td>
<td>histocompatibility 2, class II antigen A, alpha</td>
<td>H2-Aa</td>
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<td>10456005</td>
<td>CD74 antigen (invariant polypeptide of MHC class II antigen-associated)</td>
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I. 5. OTHERS

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<td>Trem4</td>
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<td>10469786</td>
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II. REGULATION OF THE EXTRACELLULAR MATRIX

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<td>secreted phosphoprotein 1</td>
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<td>Timp3</td>
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III. OXIDATIVE STRESS/METABOLIC PROCESS

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<td>10487011</td>
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<td>Gatm</td>
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<td>monooamine oxidase A</td>
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<td>glutathione S-transferase, alpha 4</td>
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IV. OTHERS

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<td>neuropilin 1</td>
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* log fold change HRG-treated relative to untreated cells.
Figure legends

**Figure 1.** HRG influences tumor growth and dissemination. a. Wt C57BL/6 mice challenged with subcutaneous T241 transduced with empty vector (EV; see (11)) or T241 expressing mHRG injected in the flank. Tumor size at indicated time points. n=10/group. b. Histogram shows tumor endpoint weights in grams (g) at day 21. c. Wt C57BL/6 and hrg/- mice challenged with T241 tumors as in a. n=15/group. d. Histogram shows tumor endpoint weights (g) at day 21; n=15/group. e. Panc02 endpoint weight, day 11 after implantation; n=20/group. f. Panc02 peritoneal lymph node metastasis; number of lymph nodes/mesentery; n=20/group. Data in panels a and c were analyzed using two-way ANOVA followed by Bonferroni post-tests. *p<0.05, **p<0.01 ***p<0.001; Data are mean values ± SEM.

**Figure 2.** Tumor properties in HRG-deficient mice. a. Immunostaining for VE-cadherin in T241 fibrosarcoma from wt and hrg/- mice. Quantification to the right shows % VE-cadherin-positive area/total tumor area; n=15/group. b. % αSMA-positive area/CD31+ vessel area in T241 tumors; quantification to the right; n=15/group. c. Vessel functionality in T241 tumors determined by perfusion with FITC-lectin. Quantification to the right, % FITC-lectin-perfused vessel area/CD31+ vessel area; n=15/group. d. Quantification shows % VE-cadherin-positive area/total tumor area in Panc02 tumors; n=15/group. e. Quantification shows % αSMA-positive area/total CD31+ area in Panc02 tumors; n=15/group. f. Quantification shows % FITC-lectin perfused vessel area/CD31+ area in Panc02 tumors; n=15. Data are mean values ± SEM. *p<0.05, ***p<0.001. Bars: 20 μm.

**Figure 3.** Hypoxia, hemorrhage, apoptosis and proliferation in T241 and Panc02 tumors. a. Increased hypoxia in T241 tumors in hrg/- mice; incorporation of pimonidazole followed by detection using hypoxyprobe. Quantification below shows % hypoxyprobe-positive area/total area; n=20/group. b. H&E staining of T241 tumor tissue shows increased hemorrhage in tumors from hrg/- mice. Scoring of degree of hemorrhaging according to clinical pathology routine below; n=20/group. c. Apoptosis in T241 fibrosarcoma estimated by immunostaining for cleaved caspase-3 (Casp-3); % cleaved caspase-3 area/total tumor area is shown below; n=20/group. d. Proliferation in T241 fibrosarcoma estimated by PHH3 immunostaining; % PHH3 area/total nuclear area is shown below; n=20/group. e. Quantification shows % hypoxyprobe-positive area/total tumor area in Panc02 tumors; n=15/group. f. Quantification shows degree of hemorrhage in Panc02 tumors; n=15/group; g. Quantification shows % cleaved caspase-3 area/total tumor area in Panc02 tumors; n=15/group. h. Quantification
shows PHH3 area in relation to total nuclear area in Panc02 tumors; n=15/group. *p<0.05, **p<0.01. Data are mean values ± SEM. Bars: 20 μm.

**Figure 4.** Tumor-infiltrating immune cells in T241 and Panc02 tumors. a. Immunostaining of T241 sections with anti-F4/80 (green); quantification below shows % F4/80+ macrophage area/total tumor area; n=20/group. b. Immunostaining of T241 sections with anti-MRC1 (green) and anti-CD45 (red); quantification below shows % MRC1 area/total CD45+ area; n=20/group. c. Immunostaining of T241 sections with anti-CD68 (green) and anti-CD11c (red) antibodies; quantification below shows % CD11c area/total tumor area; n=15/group. d. Immunostaining or T241 sections with anti-CD8 (red) and counterstaining with DAPI; quantification below shows % CD8 area/total tumor area; n=15/group. e. Quantification of % F4/80+ macrophage area/total tumor area in Panc02 tumors; n=15/group. f. Quantification of % MRC1 area/total CD45+ area in Panc02 tumors; n=15/group. g. Quantification of %CD11c area/total tumor area in Panc02 tumors; n=15/group. h. Quantification of % CD8 area/total tumor area in Panc02 tumors; n=15/group. *p<0.05, **p<0.01. Data are mean values ± SEM. Bars: 20 μm.

**Figure 5.** Transcript expression in wt and hrg-/- T241 tumors. a. Expression of vegf, plgf, cxcl10 and cxcl11 in whole T241 tumors from wt or hrg-/- mice. b. Expression of indicated genes in TAMs isolated from T241 tumors. *p<0.05; Data are mean values ± SEM. c. Expression of il10, arg1 and ccl22 in pMOs isolated from wt and hrg-/- mice. Fold change in expression in hrg-/- pMOs vs wt. *p<0.05; **p<0.01. Data are mean values ± SEM.

**Figure 6.** Effects of HRG on human U-937-1 monoblasts a. Expression of CD14 in U-937-1 cells treated with Vitamin D3 (VitD3) and hHRG (1 μg/ml) for 4 days. b. Transcript expression of FcγRI and CD11c in VitD3 differentiated U-937-1 cells treated with HRG or LPS+HRG for 24 hours. *p<0.05; **p<0.01. Data are mean values ± SEM.
Fig. 1, Tugues et al.

(a) T241 tumor volume

(b) T241 tumor growth

(c) T241 tumor volume

(d) T241 tumor growth

(e) Panc02 tumor growth

(f) Panc02 lymph node metastasis
Fig. 2, Tugues et al.

(a) VE-cadherin staining in T241 and Panc02 cell lines. 
(b) αSMA and CD31 staining in T241 and Panc02 cell lines. 
(c) FITC lectin and CD31 staining in T241 and Panc02 cell lines.

Graphs showing:
- T241 vessel area
- Panc02 vessel area
- T241 vessel coverage
- Panc02 vessel coverage
- T241 vessel perfusion
- Panc02 vessel perfusion

Significance indicated: *** p < 0.001, * p < 0.05.
Fig. 3, Tugues et al.

a) PIMO staining in WT and hrg-/- cells under hypoxic conditions.
b) H&E staining and cleaved Casp-3 expression in WT and hrg-/- cells.
c) PPH3 staining in WT and hrg-/- cells.

d) Graphs showing the percentage of hypoxic area (T241 hypoxia), hemorrhagic score (T241 hemorrhage), percentage of cleaved Casp-3 area (T241 apoptosis), and percentage of pHH nuclei (T241 proliferation).

e) PIMO staining in Panc02 under hypoxic conditions.
f) H&E staining and cleaved Casp-3 expression in Panc02.
g) PPH3 staining in Panc02.

h) Graphs showing the percentage of hypoxic area (Panc02 hypoxia), hemorrhagic score (Panc02 hemorrhage), percentage of cleaved Casp-3 area (Panc02 apoptosis), and percentage of pHH nuclei (Panc02 proliferation).
Fig. 4, Tugues et al.

(a) T241 TAM infiltration

(b) T241 M2 infiltration

(c) T241 CD11c infiltration

(d) T241 CTL accumulation

(e) Panc02 TAM infiltration

(f) Panc02 M2 infiltration

(g) Panc02 CD11c infiltration

(h) Panc02 CTL accumulation
Fig. 5, Tugues et al.

(a) 

- VEGF T241
- PIGF T241
- CXCL10 T241
- CXCL11 T241

(b) 

- Arg-1 TAMs
- Ang-2 TAMs
- CCL12 TAMs
- MMP-8 TAMs
- CXCL10 TAMs
- CXCL11 TAMs
- CD60 TAMs

(c) 

- IL10 pMO
- Arg-1 pMO
- CCL22 pMO
Fig. 6, Tugues et al.

a

CD14 U937

Mean intensity fluorescence

Basal  H2O  VH3  VH3 + HMD

b

FCγRI U937

Fold change vs VEGF

VEGF  VEGF + HMD

FCγRI U937

Fold change vs VEGF + LPS

VEGF + LPS  VEGF + LPS + HMD

CD11c U937

Fold change vs VEGF

VEGF  VEGF + HMD

CD11c U937

Fold change vs VEGF + LPS

VEGF + LPS  VEGF + LPS + HMD
Genetic deficiency in plasma protein HRG enhances tumor growth and metastasis by exacerbating immune escape and vessel abnormalization.

Sónia Tugues, Satoshi Honjo, Christian König, et al.

Cancer Res Published OnlineFirst February 28, 2012.