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HGF Airway Over-expression Leads to Enhanced Pulmonary Vascularization without Induction of VEGF

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Abstract

The hepatocyte growth factor (HGF)/c-Met signaling pathway mediates angiogenesis. We have previously reported that airway expression of a human HGF transgene (HGF TG) produced mice that were more susceptible to lung tumorigenesis induced by 4-(methylnitrosoamino)-1-(3-pyridyl)-1-butanone (NNK). Here we show untreated HGF TG mice display enhanced vascularization (40 wks) and enhanced lymph vessel formation (20 wks) in the lungs compared to wild-type (WT) littermates, as ascertained by microvessel density. We profiled mRNA expression from HGF TG and WT mice for genes involved in angiogenesis. We consistently found significant decreases in expression of the VEGF family of angiogenic genes, including *Vegfa*, *Vegfb*, *Vegfc*, and *Vegfd* / *Figf*. Decreases were confirmed in whole lung protein extracts by immunoblot. Similar patterns of down-regulation were observed at 10, 20, and 40 wks of age. Vandetanib, an inhibitor of VEGFR2 and VEGFR3, did not prevent the increase in microvessel density observed in HGF TG mice. Reduction in VEGF pathway genes was also detected in lung tumors derived from NNK-treated HGF TG mice. HGF TG lung tumors also showed increased expression of five *Cxcl* family genes including *Cxcl1* and *Cxcl2* (murine forms of IL8). These results suggest increased vascularization produced by airway over-expression of HGF occurs through direct activation of c-Met on endothelial cells, rather than induction of VEGF pathways. Elevated HGF may also increase expression of inflammatory mediators that contribute to lung tumor progression.

Keywords

Angiogenesis; vascularization; HGF; non-small cell lung cancer; VEGF; c-Met

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AUTHOR DISCLOSURE

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SUPPLEMENTARY MATERIAL

Supplementary material is available on the publishers Web site along with the published article.

INTRODUCTION

Lung cancer is the leading cause of cancer death in the United States and worldwide [1]. The growth of non-small cell lung cancer (NSCLC), which accounts for more than 80% of all lung cancers, is usually dependent on angiogenesis [2, 3]. Blockade of angiogenesis is expected to prevent both the growth of tumor cells and the emergence of tumor progression, thereby improving the prognosis of patients with NSCLC [4]. The vascular endothelial growth factor (VEGF) pathway is considered one of the major regulators in angiogenesis in normal and malignant tissues [3]. However, recent evidence suggests that other pathways such as hepatocyte growth factor (HGF)/c-Met signaling are sufficient to promote angiogenesis. Although induction of VEGF has been reported in response to HGF, HGF has also been shown to induce neovascularization independently of VEGF [5]. For example, HGF-dependent signaling through c-Met expressed on lymphatic endothelial cells is known to directly promote blood vessel formation during tissue repair, and does not require VEGFs [6]. HGF can activate integrins on endothelial cells to trigger development of the lymph vascular system with endothelial cell proliferation, survival and migration [7]. HGF also activates macrophages, which express the c-Met protein; macrophages can trigger angiogenic processes through release of cytokines such as IL-8 [8].

C-Met, the receptor for HGF, is a tyrosine kinase receptor whose activation can lead to angiogenesis and tumorigenicity [9, 10]. In normal cells, c-Met activation is a transient event, whereas in tumor cells c-Met activity may be up-regulated by both receptor and ligand over-expression [11, 12]. Overexpression of HGF and c-Met has been reported in approximately 50% of lung adenocarcinomas [12–14]. Expression of HGF and c-Met have been found to be strong independent prognostic factors in various cancers including lung, breast, biliary tract, gastric, and nasopharyngeal carcinoma [15–17]. A retrospective analysis in patients with surgically resected NSCLC (stages I to IIIA) revealed that high levels of HGF in the primary tumor were associated with a significantly worse overall survival compared to patients with low HGF [18, 19]. The strong correlation between HGF/c-Met expression and patient survival supports the hypothesis that the HGF/c-Met pathway plays an important role in the pathogenesis of human cancers and is a potential target for therapeutic intervention. Activation of the c-Met pathway is also associated with resistance to other targeted therapies, such as epidermal growth factor receptors inhibitors [20].

To better understand the effects of HGF/c-Met signaling pathway in lung cancer, we previously generated transgenic (HGF TG) mice with elevated HGF expression in the airway epithelium under the control of the Clara cell secretory protein promoter (CCSP) [21]. We previously documented that when exposed to the tobacco carcinogen, 4-(methylnitrosoamino)-1-(3-pyridyl)-1-butanone (NNK), the HGF TG mice exhibited a significant increase in tumor multiplicity compared to wild-type (WT) littermates [21]. In that study, we observed high pulmonary blood vessel production in HGF TG mice after NNK exposure. Here we further characterize the vessel formation in HGF TG versus WT animals. Furthermore to analyze which genes contribute to the differences in blood vessel formation, we profiled the expression of genes representative of pathways involved in angiogenesis and tumorigenesis using commercial microarrays from individual untreated

lungs as well as from lung tumors that arose in NNK-treated HGF TG and WT mice. Our results indicate that HGF over-expression in the small airways induces vascularization without VEGF induction. Reinforcing the idea of inter-tumoral angiogenic heterogeneity, VEGF may be less important for vessel formation in tumors with high HGF/c-Met pathway activation.

MATERIALS AND METHODS

Mouse Models

Mice for all experiments were WT (murine HGF) or human HGF transgenic (FVB/N strain). Construction of the human HGF transgene, as well as generation and identification of HGF TG mice, was previously described [21]. For microarray analysis, whole lungs from untreated WT and HGF TG animals were dissected after sacrifice at 10, 20, or 40 wks of age. To induce lung tumors, mice were given a total of four intraperitoneal injections of 3mg NNK (15mg/ml) over 2 weeks. NNK induced lung tumors were dissected from the animals at 20 or 40 wks of age. For the *in vivo* inhibitor experiment, WT and HGF TG mice (5wks of age) were treated with gefitinib (75mg/kg, ChemieTek, Indianapolis, IN), vandetanib (25mg/kg, ChemieTek), or vehicle control (1% Tween-80/0.9% saline). Treatment was administered by oral gavage twice a week for five weeks. At the end of treatment, animals were sacrificed and lungs were processed as described previously [22]. Animal care was in strict compliance with the institutional guidelines.

Immunohistochemistry

Lungs were fixed in 10% buffered formalin, embedded in paraffin, and sectioned. Slides were deparaffinized, rehydrated, and steamed in EDTA (pH 8.0) followed by 3% hydrogen peroxide at room temperature for antigen retrieval. To identify vessel expression of the c-Met receptor, slides were stained with anti-c-Met (C-28, 1:75, 1 hr, Santa Cruz Biotechnology, Santa Cruz, CA). Antibody against PECAM-1 (M20, 1:100, 1.5 hrs, Santa Cruz Biotechnology) was used as a pan-endothelial marker. Staining of lymphatic vessels was accomplished with anti-LYVE-1 (1:100, 1 hr, Abcam, Cambridge, MA). H&E staining was also performed on serial sections from the same lung specimens. Microvessels, as detected by PECAM, were counted in five high-magnification areas per lung from five HGF TG and five WT mice per time point. Lymphatic vessels, as detected by LYVE-1, were counted in ten high-magnification areas per lung from six HGF TG and six WT mice at 20 wks of age. Results are presented as the mean number of blood vessels per area $\pm$ standard error.

Immunofluorescence

Dual immunofluorescence was utilized to distinguish the maturity of blood vessels. Deparaffinized, rehydrated paraffin sections (processed as described above) were subjected to antigen retrieval via steaming in citrate buffer (pH 6.0) for ten minutes. After blocking in 10% normal serum, slides were incubated overnight at 4°C with mixture of anti-PECAM (M20, 1:100), and anti-smooth muscle actin (SMA) (1:50, R&D, Minneapolis, MN). Vessels as detected by PECAM or SMA were counted in seven high-magnification areas per lung from six HGF TG and six WT mice at 20 wks of age. Results are presented as the mean

number of mature vessels per area (as stained by SMA), total blood vessels per area (as stained by PECAM), and percentage of SMA-positive vessels (SMA stained vessels/total vessels stained by PECAM) $\pm$ standard error.

RNA Isolation and Microarray Analysis

Total RNA was extracted from whole lung or isolated tumors from HGF TG or WT mice and subjected to qPCR analysis (Angiogenesis PCR Array [PAMM-024], SABio-sciences) or hybridization to Oligo GEArray Mouse Angiogenesis or Cancer Pathway Finder Microarrays (SuperArray OMM-44 and OMM-33, respectively). Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) and the Array Grade Total RNA Isolation Kit (SABiosciences, Frederick, MD) according to manufacturer's instructions. cDNA, reverse-transcribed from total RNA, was subjected to qPCR analysis (Angiogenesis PCR Array [PAMM-024], SABiosciences), or biotin-16-labeling. Overnight hybridization of biotin labeled cDNA to Oligo GEArray Mouse Angiogenesis or Cancer Pathway Finder Microarrays (SuperArray OMM-44 and OMM-33, respectively) was followed by exposure to x-ray film. These arrays contained 162 unique genes and 32 overlapping genes (see Supplemental Table 1). Data analysis was performed using GEArray Expression Analysis Suite, or RT2 Profiler PCR Array Data Analysis (www.sabiosciences.com). Data are presented as the relative induction of each gene normalized to housekeeping genes, and are representative of two to four independent experiments. These data have been deposited in National Center for Biotechnology Information's Gene Expression Omnibus (GEO) and are accessible through GEO Series accession number GSE20468. (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE20468>)

Protein Isolation and Western Analysis

Protein was extracted from tissue samples and quantified as described [23]. Western analyses for AKT (C-20, 1:000, Santa Cruz Biotechnology), VEGFD (C-18, 1:000, Santa Cruz Biotechnology), and VEGFA (A-20, 1:500, Santa Cruz Biotechnology) were performed as previously described [23]. Blots were stripped and reprobed for β -actin (1:5000, Chemicon/Millipore, Billerica, MA). Immunoreactive bands were quantitated by densitometry and ImageQuant analysis.

Statistical Analysis

All values were obtained using Student's t-test, except for the *in vivo* growth factor inhibitor experiment, for which a one-way ANOVA with Turkey post-test was utilized. All tests were two-sided with the threshold for significance defined as $P < 0.05$. For array analysis, adjusted p-values were calculated using the Benjamini Hochberg method. Comparisons that remained significant using this correction method are indicated with an asterisk within the tables.

RESULTS

HGF Over-expression Leads to Enhanced Blood and Lymph Vessel Formation

HGF/c-Met signaling has been linked to the promotion of angiogenesis and neovascularization [5]. To determine if HGF over-expression led to significantly increased

blood vessel formation in the lungs, we used H&E staining and PECAM immunohistochemistry to quantify blood vessel density. We consistently observed increased blood vessel production throughout the lungs of HGF TG mice compared to WT littermates. A considerable excess of blood vessels was seen by both H&E staining (Figs. 1A and 1B) and PECAM immunohistochemistry (Figs. 1C and 1D) in the lungs of HGF TG (Figs. 1B and 1D) animals compared to WT littermates (Figs. 1A and 1C). Blood vessel counts showed a significant increase in blood vessel density in HGF TG lungs at all time points (10 – 40 wk). The ratio of TG:WT blood vessel density also increased over time. The effect was maximal at 40 wk with more than a 4-fold increase in blood vessel density in HGF TG mice (24.6 ± 3.9 microvessels/area) compared to WT lungs (5.8 ± 1.6 microvessels/area, $P=0.0022$, Fig. 1E).

Endothelial cells in pulmonary vessels are known to express c-Met receptors in WT mice. c-Met immunohistochemistry confirmed c-Met is also expressed by pulmonary vessel endothelial cells in transgenic mice, as expected, allowing them to respond directly to HGF signaling (see Supplemental Fig. 1). No difference in the percentage of c-Met positive endothelial cells was observed between HGF TG and WT lungs, however c-Met staining in blood vessels in the HGF TG animals appeared more intense compared to the staining in WT vessels. HGF has been reported to up-regulate its own receptor [10].

HGF has also been reported to be the major inducer of lymphangiogenesis [24]. To determine if the HGF-induced blood vessel formation included increased lymphatic vessel formation in the lungs, we stained lung tissues for lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1). LYVE-1, a cell-surface receptor specifically expressed on lymphatic endothelial cells [25], has been utilized as a marker for lymphangiogenesis in cancer [26, 27]. LYVE-1 immunohistochemistry (Fig. 2A and B) was used to compare vessel counts in lungs of HGF TG and WT mice. LYVE-1 positive vessels were observed at a greater density in HGF TG compared to WT mice. At 20 wks, lymphatic vessel density was 9.05 ± 0.2 vessels/ mm^2 , compared to 6.32 ± 0.3 vessels/ mm^2 in WT littermates, $P=0.0001$, (Fig. 2C). The ratio of HGF TG:WT lymphatic vessel density at 20 wks was 1.43, compared to a ratio of 3.33 for all vessels (Fig. 1E). Approximately one-third of the total increase in vessel density was accounted for by vessels arising from the lymphatics.

HGF-stimulated Vessels Show a Lesser Degree of Maturation

The PECAM immunohistochemistry results in Fig. (1C and 1D) show that HGF over-expression continuously stimulated microvessel formation as mice aged. Dual immunofluorescence staining of PECAM and SMA (pericyte marker) was utilized to determine the degree of maturation of excess vessels. Since nascent endothelial tubes form prior to pericyte coverage [28], the acquisition of pericytes indicates vessel stabilization and maturation [28]. PECAM, as a pan-endothelial marker, does not distinguish between vessel maturity; however, only mature, pericyte containing vessels stain positive for SMA [29, 30]. Whole lungs from WT (n=6) and HGF TG mice (n=6) were dual stained with SMA and PECAM to determine the average number of mature vessels (WT= 4.05 ± 1.43 , TG= 6.33 ± 1.54) and total vessels (WT= 5.02 ± 1.72 , TG= 9.42 ± 1.86). Representative images are shown in (Fig. 3A). Staining was performed at an intermediate time point (20 wks) when

enhanced vessel growth was not complete. A significantly smaller fraction of vessels stained positive for SMA in HGF TG mice ($67 \pm 1.8\%$ SMA positive vessels) compared to WT littermates ($80 \pm 2.9\%$ SMA positive vessels, $P=0.0032$, Fig. 3B). These data further demonstrate new blood vessel formation in the lungs is continually promoted by HGF over-expression over time.

A Majority of Angiogenic-related Genes are Down-regulated in Untreated Transgenic Mice Compared to Wild-type Littermates

To identify if changes in gene expression might be associated with the enhanced vascularization observed in HGF TG mice, we profiled the expression of 113 genes using the Oligo GEArray Cancer Pathway Finder microarray using intact whole lungs from HGF TG ($n=4$) and WT ($n=4$) non-NNK treated mice (40 wks, Table 1 and Supplemental Table 2). The significance threshold was set at a 1.5- or 2- fold increase or decrease relative to the wild-type. Genes are listed in order from most to least changed (TG:WT ratio) with the gene symbol, functional grouping, average expression as normalized to *Gapdh*, standard deviation, and P value. Of the six cancer-related pathways represented on the arrays, mRNA expression was preferentially altered in genes that are involved in angiogenesis (16 of 32 genes were down-regulated). All members of the VEGF family of angiogenic ligands were significantly reduced (*Vegfd* $P=0.0031$, *Vegfb* $P=0.0043$, *Vegfa* $P=0.01$, *Vegfc* $P=0.0361$, Table 1). Ratios of TG:WT expression for the four VEGF genes were as follows: *Vegfd* 0.34, *Vegfb* 0.48, *Vegfa* 0.53, *Vegfc* 0.56 (Table 1). Most genes on the array (91 of 113) involved in cell cycle, cell senescence, signal transduction, invasion, and adhesion were not significantly different in HGF TG murine lungs compared to WT lungs and no genes were up-regulated above the 2-fold boundary. We also observed that the murine HGF gene was decreased in expression (2-fold), although not significantly, in HGF TG lungs compared to WT littermates (see Supplemental Table 2). Since these mice overexpress human HGF, not murine HGF, we did not expect to observe an increase in the murine HGF gene. A slight decrease in the murine HGF gene may reflect feedback inhibition due to over-expression of the human HGF transgene. We previously documented Clara cell-specific over-expression of human HGF in airways of TG mice, as well as elevated levels of human HGF in bronchoalveolar lavage fluid from HGF TG mice [21]. The mRNA expression of the HGF receptor, c-Met, was not significantly different in lungs of HGF TG mice (see Supplemental Table 2), even though by immunohistochemistry, c-Met protein in the endothelial cells in HGF TG lungs appears somewhat higher. This localized change may not be reflected in RNA isolated from whole lungs.

To further elucidate genes that might be responsible for the enhanced vascularization, we profiled the expression of 113 genes involved in angiogenesis using commercial angiogenic arrays (Oligo GEArray and Angiogenesis PCR Array) (Figs. 4A and 4B). Microarray analysis on individual whole lungs from HGF TG ($n=4$) and WT ($n=3$) non-NNK treated mice (10 wks) showed a statistically significant 2-fold decrease in the expression of 25 genes including VEGF receptor 2 (*Kdr*), neuropilin 1 (*Nrp1*), and transforming growth factor beta 2 (*Tgfb2*) (Table 1). A significant 2-fold increase was seen in two genes: chemokine (C-X-C motif) ligand 5 (*Cxcl5*) and *Il1b* (Table 1). Analysis of individual whole lungs from HGF TG ($n=4$) and WT ($n=4$) non-NNK treated mice (40 wks) showed a

statistically significant 2-fold decrease in the expression of eight of the same genes observed at 10 wks, such as *Mmp2*, *Mmp9*, and neuropilin 2 (*Nrp2*) (Table 1). In addition, expression of 21 other genes associated with angiogenesis including angiopoietin-like 4 (*Angptl4*), *Cxcl9*, *Tgfb3*, *Figf* (*Vegfd*), *Vegfa*, *Vegfb*, and *Vegfc* were also reduced (Table 1). No genes were found to be significantly up-regulated 2-fold or greater at 40 wks of age. Lung tissue analyzed from mice sacrificed at 20 wks of age showed similar trends in decreased gene expression to that of the 10 and 40 wk old mice (data not shown).

A two-dimensional clustergram of the individual lung tissue expression profiles (at 40 wks) from the angiogenesis array was generated using the normalized expression and an average join type. The expression profiles effectively clustered individual HGF TG samples from the WT lungs (see Supplemental Fig. 2). Clustergram and statistical tests identified the same genes in the VEGF signaling pathway as being reduced in HGF TG lungs compared to WT as scatterplot results: *Vegfa*, *Vegfb*, *Vegfc*, *Vegfd/Figf*, *Nrp2*, *Csf3*, *Pi3k*, and *Grb2*. The majority of genes on the angiogenesis array showed no significant difference between WT and HGF TG lungs. Genes with an expression ratio of at least 0.80 compared to WT at both 10 and 40 wks included epidermal growth factor (*Egf*), angiopoietin 1 (*Angpt1*), fibroblast growth factor 2 (*Fgf2*), integrin alpha 5 (*ItgaV*), matrix metalloproteinase 19 (*Mmp19*), and serpin peptidase inhibitor clade F member 1 (*Serpinf1*) (see Supplemental Table 2).

Western blot analysis was used to verify decreases in gene expression identified by microarrays. Protein was isolated from the lung tissue from the same mice that were used for the RNA microarray analysis. Decreases observed in the gene expression of VEGFA, VEGFD, and AKT1 from the 10 wks (Fig. 4A) and 40 wks (Fig. 4B) microarray analyses were also seen in protein levels in the 10 wks (Fig. 4C) and 40 wks (Fig. 4D) whole lung blots. After normalization to β -actin, the percent decrease in protein expression in HGF TG compared to WT was determined as follows: VEGFA (40.2% 10 wks, 62.4% 40 wks), VEGFD (73.3% 10 wks, 94.4% 40 wks), and AKT1 (60.2% 10 wks, 49.7% 40 wks). Relative densitometry values are shown beneath each band.

VEGFR Inhibition does not Block Increased Vascularization in Transgenic Mice

VEGF mRNA and protein levels were significantly reduced in HGF TG mice at 40 wks and also found to be down-regulated or at similar levels of expression in mice at 10 wks. These data suggest the increased vascularization of transgenic animals is largely VEGF-independent. To further confirm VEGF is not required in enhanced blood vessel formation, WT and HGF TG mice were treated with vandetanib, a tyrosine kinase inhibitor that inhibits VEGFR2, VEGFR3 (the major receptor responsible for endothelial cell proliferation), and to a lesser extent, epidermal growth factor receptor (EGFR) [31]. Thus, we also treated mice with gefitinib (a selective EGFR inhibitor, [31]) in order to determine whether any differences seen in vascularization after vandetanib treatment could be attributed to VEGF inhibition alone. We treated HGF TG (n=3) and WT mice (n=3) with vehicle control (1% Tween-80/0.9% saline), gefitinib (75mg/kg), or vandetanib (25mg/kg) for five wks, beginning at 5 wks of age, collecting whole lungs at 10 wks. We observed a significant increase in blood vessel density in transgenic animals compared to WT littermates regardless of treatment as determined by immunohistochemistry of PECAM (see Supplemental Table 3).

Vandetanib and gefitinib treatments had no effect on normal microvessel density in WT mice (5.52 ± 0.33 microvessels/area, 6.52 ± 0.36 microvessels/area respectively) as compared to WT littermates (4.63 ± 0.28 microvessels/area (n.s., vandetanib or gefitinib vs. control). Microvessel density was still significantly increased in HGF TG mice at 10 wks (8.00 ± 0.48 microvessels/area, $P < 0.001$) compared to WT littermates as expected. Vandetanib treatment did not alter HGF TG microvessel density (8.79 ± 0.59 , $P > 0.05$ compared to control HGF TG). The microvessel density of HGF TG lungs from vandetanib-treated mice remained significantly different from WT mice either with or without vandetanib treatment ($P < 0.001$ for both comparisons). Gefitinib treatment of HGF TG mice slightly increased blood vessel density (10 ± 0.42 , $P = 0.049$), suggesting that inhibiting EGFR in the presence of HGF overexpression may somewhat promote new blood vessel formation. Gefitinib has been reported to inhibit recruitment of pericytes to blood vessels [32]. This might sustain new blood vessels in an immature state, and allow for continued endothelial cell growth and migration in the presence of elevated HGF. These data further suggest that VEGF pathways do not play a major role in microvessel formation in the lungs when HGF/c-Met activation is high.

Lung Tumors from Transgenic Mice Demonstrate Similar Gene Expression Profiles as Seen in Whole Lungs, with Increased Expression of Inflammatory Related Genes

To determine if similar differential expression of VEGF genes was found within lung tumors induced by NNK in HGF TG mice compared to WT littermates, microarray analysis of four individual lung tumors dissected from lungs of HGF TG mice were compared to four individual lung tumors dissected from lungs of WT mice. Representative arrays from HGF TG and WT are shown in Fig. (5A). The tumor profiles showed a statistically significant 2-fold or greater decrease in the expression of many of the same angiogenic genes observed in normal lung in HGF TG mice. The down-regulated genes included *Mmp2*, *Akt1*, *Timp3*, and the *Vegfa*, *Vegfb*, *Vegfc* and *Vegfd/Fgf* (Fig. 5B). Many genes were shown to have comparable expression on both arrays when comparing HGF TG individual tumors to WT individual tumors. The three genes with a relatively high ratio of HGF TG whole lung expression compared to WT, *Angpt1*, *Fgf2* and *ItgaV*, also showed a ratio of 1.0 or higher in HGF TG tumors. A number of other genes not in the VEGF pathway also had comparable expression, or were slightly increased (over 1.0-fold) in HGF TG and WT lung tumors (see Supplemental Table 4). Five genes in the chemokine (C-X-C motif) ligand family were also found to be significantly up-regulated in HGF TG tumors: *Cxcl1* and *Cxcl2* (murine homologs of IL8), *Cxcl10*, *Cxcl11*, and *Cxcl9* (Table 2).

DISCUSSION

The angiogenesis process in lung cancer has been considered to be largely dependent on the VEGF pathway [2, 3]. As a result, the use of angiogenesis inhibitors in NSCLC and other cancers has been non-selective and has not taken into consideration the possibility of inter-tumoral angiogenic heterogeneity [4, 33]. Heterogeneity in angiogenic pathways has been described in different head and neck squamous cell carcinomas (HNSCC) [34]. Molecular profiling of primary human HNSCC specimens by these investigators revealed two major groups of tumors. HGF pathway genes predominated in one type, while VEGF pathway

genes predominated in the other. Previous reports support the concept that HGF can induce angiogenesis independently of VEGF. VEGF levels were not correlated with survival in patients with resected stage I NSCLC [33], HGF remains highly angiogenic when VEGF signaling is blocked [5], and analysis of lymphatic endothelial cells revealed HGF induction of proliferation was partially mediated via integrin α -9 and did not require VEGFR3 activation [6].

Our results support the hypothesis that different signaling pathways can drive vessel formation. In lungs containing high HGF expression, robust and continuously increasing vascularization was induced, and vessels were significantly less mature than in WT animals. Induction of lymphatic vessels, which is known to be stimulated by HGF alone [24], accounted for only about one-third of the increased vessel density, suggesting that both blood and lymphatic vessels are induced. These microvessels uniformly express c-Met protein, and thus are able to respond to the increased HGF ligand. The lungs of HGF over-expressing mice also displayed mRNA expression profiles in which a major driver of angiogenesis, VEGF, and genes associated with the VEGF pathway such as *Nrp2* were significantly down-regulated. Furthermore, treatment of HGF TG mice with the VEGFR inhibitor vandetanib failed to reduce the increase in microvessel density. These results highly suggest HGF acts independently of the VEGF pathway to stimulate vascularization.

It is possible that HGF-induced vascularization occurs through transcription of genes not represented on the gene arrays utilized, thus accounting for the lack of up-regulated gene expression seen in this study. Alternatively, much of the increase in blood vessels could be through direct effects of the transgenic HGF protein on endothelial cells that do not require increased synthesis of the mRNAs examined. For example, HGF, via the c-Met receptor, could directly activate integrins expressed on endothelial cells to promote their movement and proliferation [7]. Release of pre-synthesized angiogenic proteins from infiltrating macrophages and neutrophils, such as Glu-Leu-Arg (ELR+) CXC chemokines, also would not require new mRNA synthesis of angiogenic factors and could act directly on endothelial cell receptor [35]. It is also possible that since the blood vessels were not microdissected for RNA extraction, we were only able to observe gene expression changes occurring in the whole lung, and could not measure local changes in mRNAs needed for increased vascularization in response to HGF.

IL-8 and IL-1 β have been identified as potential mediators for HGF-induced angiogenesis in HNSCC [34, 36] and nasopharyngeal cancer (NPC) [37]. Co-expression of HGF and IL-8 in NPC has been shown to be associated with increased microvessel density and poor prognosis [37]. In this published study, exogenous HGF promoted induction of IL-8 in NPC cells. In NSCLC, IL-1 β , a pro-inflammatory macrophage secreted cytokine, has been shown to induce expression of proangiogenic ELR+ CXC chemokines [36]. HGF has also been reported to induce IL-8 expression in lung adenocarcinoma cells [38]. We observed no significant change in the mRNA expression for whole lung samples of the functional mouse homologues of IL-8, MIP-2 (*Cxcl2*) and KC (*Cxcl1*) or IL-1 β at 40 wks. However, the expression of *Cxcl5* (another ELR+ CXC chemokine) and IL-1 β was found to be significantly up-regulated in HGF TG mice at 10 wks, suggesting expression of inflammatory associated chemokines play a role in HGF-mediated vascularization.

Similarly, IL-1 β , along with MIP-2 and KC, were elevated in HGF TG tumors, indicating these chemokines also play a role in HGF-mediated tumorigenesis in response to NNK. Angiostatic ELR- CXC chemokines (*Cxcl9*, *Cxcl10*, *Cxcl11*) were also slightly up-regulated in these tumors (though not in non-treated animals), possibly in attempt to balance the rise in proangiogenic factors.

There was no significant down-regulation of genes such as caspase-9 or *Bax* that would imply that decreased apoptosis is occurring in untreated HGF TG lungs. Additionally, no other genes related to proliferation or oncogenesis contained on these microarrays were found to be elevated, including many genes involved in oncogenic pathways such as *Src* and *Bcl2*. These observations suggest that in the absence of injury, such as a carcinogenic insult, the angiogenic effects of moderate HGF over-expression in the lungs of these heterozygous HGF TG mice predominate over directly oncogenic signals. This is consistent with the role of HGF in wound healing. We observed that heterozygous HGF TG mice do not develop lung tumors spontaneously, although they do have a higher level of spontaneous airway preneoplasia compared to WT mice [21]. Mild Clara cell hyperplasia was also detected in the small airways of HGF transgenic mice [21]. However, lung adenoma and adenocarcinoma formation remain dependent on carcinogen exposure in these mice.

Our results suggest that the main spontaneous effect of HGF over-expression in these mice is continually enhanced vascularization, which was accompanied by a decrease in gene expression for many VEGF pathway genes in lung tissue, so is likely VEGF-independent. Decreases in VEGF pathway genes may be a feedback mechanism in response to the heightened blood vessel formation initiated by HGF. According to our data, HGF-dominant angiogenesis and lymphangiogenesis might be accompanied by decreased VEGF pathway utilization. Down-regulation of VEGF through high HGF expression could explain resistance to anti-VEGF therapies currently being tested in several types of cancer including NSCLC [4, 39]. Tumor growth inhibition by a VEGF inhibitor in mice was not as effective in HNSCC cells that showed relative VEGF down-regulation [34]. It has been reported that several proangiogenic cytokines including HGF were elevated before progression in colorectal cancer patients being treated with bevacizumab, a VEGF inhibitor [40]. Recently, it has been documented that HGF/c-Met activation plays a role in resistance to sunitinib treatment, a VEGFR and PDGFR inhibitor [39]. Resistant tumors were found to contain higher HGF concentrations and c-Met expression compared to sensitive tumors. In this study, dual treatment with sunitinib and a c-Met inhibitor significantly inhibited tumor growth compared to sunitinib alone and largely targeted the vasculature in resistant tumors [39].

Our HGF TG animal model could be used to examine agents that selectively inhibit angiogenesis via VEGF-independent pathways. This approach could potentially lead to new insights into the pathogenesis of NSCLC, and examination of new anti-angiogenic approaches, either through HGF/c-Met inhibition or combinations with VEGF inhibitors. The redundancy of multiple angiogenic signals, and ability of tumors to adapt to inhibitory challenges, might limit the efficacy of single agent angiogenic treatment. The complete inhibition of angiogenesis in lung cancer may require novel compounds that target angiogenesis through multiple pathways such as VEGF and HGF pathways.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. (1). Continually enhanced blood vessel formation in HGF TG mice compared to WT littermates.

Representative pictures from 20 wk old WT (A,C) and HGF TG (B,D) mouse lungs. A,B. H&E staining. C,D. PECAM immunohistochemistry. Arrows indicate areas of increased blood vessel formation. E. Average number of blood vessels $\pm$ standard error from 5 high-magnification areas per lung of 5 HGF TG and 5 WT mice per time point. Asterisk denotes level of significance of HGF TG to similarly aged WT mice (* P < 0.05, ** P < 0.005, *** P < 0.001).

Fig. (2). Enhanced lymph vessel formation in HGF TG mice compared to WT littermates. HGF TG mice displayed significantly higher numbers of LYVE-1 positive vessels than similarly aged WT littermates. **A,B.** Representative pictures from 20 wk old WT (**A**) and HGF TG (**B**) mouse lungs stained with the lymphatic vessel marker, LYVE-1. **C.** Average number of LYVE-1 positive vessels from 10 high-magnification areas (1mm²) per lung of 6 HGF TG and 6 WT mice. Asterisk denotes level of significance of HGF TG to WT mice (*** $P < 0.001$), Scale bar=100 μ m.

Fig. (3). Decreased blood vessel maturity in HGF TG mice compared to WT littermates. HGF TG mice had a significantly smaller fraction of SMA positively staining vessels compared to WT littermates, indicating they contained a larger percentage of immature vessels. **A.** Representative pictures of dual immunofluorescent staining of the blood vessel marker, PECAM (green), and pericyte marker, SMA (red), from 20 wk old WT and HGF TG mouse lungs. **B.** Vessels stained with PECAM or SMA were counted in 5–10 high-magnification areas per lung of 6 HGF TG and 6 WT mice. Graph depicts the % of SMA positive vessels as determined by dividing the average number of SMA stained vessels by PECAM stained vessels in HGF TG or WT animals (see text). Asterisk denotes level of significance of HGF TG to similarly aged WT mice (** $P < 0.005$).

Fig. (4). Whole Lung DNA Microarrays and Western Blot Analysis for genes related to angiogenesis.

Microarray analysis depicted decreased expression in genes associated with angiogenesis in HGF TG mice compared to WT littermates. **A,B.** Scatter plot analysis displaying the fold difference in the relative expression levels of genes in the angiogenesis array from 10 wk (**A**) and 40 wk (**B**) HGF TG mice vs. WT. A green (+) represents genes whose fold decrease is greater than the 2-fold boundary. Black asterisks depict unchanged genes. **C,D.** Protein expression of individual whole lungs from 10 wk (**C**) and 40 wk (**D**) old HGF TG and WT mice. Relative densitometry quantitation normalized to b-actin is shown beneath each lane. Percent decrease in protein concentration as compared to the wild type animals were determined by densitometry and are as follows: AKT1 (90.2% – 10 wk, 49.7% – 40 wk), VEGFD (93.3% – 10 wk, 94.4% – 40 wk), VEGFA (60.2% – 10wk, 92.4% – 40wk).

Fig. (5). Tumor DNA Microarray Analysis for genes associated with angiogenesis.

Similar patterns of down-regulated gene expression were seen in NNK induced tumors from HGF TG mice compared to WT littermates. **A.** Representative hybridization blots from HGF TG and WT mice. Colored boxes highlight specific significantly down-regulated genes. **B.** Scatter plot displaying the fold difference in the relative expression levels of genes of isolated NNK induced lung tumors from HGF TG and WT mice (40 wks old). A green (+) represents genes whose fold decrease is greater than the 2-fold boundary. Black (+) depicts unchanged genes. Red (+) represents genes whose fold increase is greater than the 2-fold boundary. Colored arrows point to corresponding genes as indicated on microarray blots.

Table 1.

Genes with Significantly Altered Expression (>1.5-fold)

			HGF WT		HGF TG			
Symbol	Functional Gene Grouping		Avg	STD	Avg	STD	Ratio (TG:WT)	p
10 wks								
Epas1	Transcription Factor		6.38	0.69	0.76	0.11	0.12	0.0038*
Cdh5	Adhesion Molecule		2.78	1.03	0.35	0.22	0.12	0.0018*
Kdr	Growth Factor Receptor		1.33	0.69	0.17	0.07	0.13	0.0017*
Tbx4	Transcription Factor		0.16	0.74	0.02	0.08	0.13	0.0018*
Eng	Adhesion Molecule		0.60	0.61	0.09	0.10	0.15	0.0039
Tgfb1	Growth Factor		0.28	0.68	0.04	0.10	0.16	0.0001*
Ephb4	Growth Factor Receptor		0.31	0.66	0.05	0.14	0.17	0.0006*
Lama5	Adhesion Molecule		0.18	0.70	0.04	0.12	0.21	0.0119
Mmp2	Invasion & Metastasis/Protease		0.88	0.55	0.22	0.14	0.25	0.0082*
Fgfr3	Growth Factor Receptor		0.58	0.72	0.15	0.13	0.26	0.0067*
Flt1	Growth Factor Receptor		0.40	0.68	0.10	0.18	0.26	0.0009*
Npr1	Growth Factor Receptor		0.12	0.71	0.03	0.14	0.29	0.0029*
Timp2	Invasion & Metastasis/ Angiogenic Inhibitor Protein		1.95	0.67	0.65	0.13	0.33	0.0103
Col4a3	Matrix Protein		0.12	0.97	0.04	0.35	0.35	0.0390
Mapk14	Growth Factor Receptor		0.19	0.65	0.07	0.10	0.38	0.0267
Stab1	Adhesion Molecule		0.10	0.64	0.04	0.20	0.40	0.0063*
Tgfb2	Growth Factor		0.07	0.48	0.03	0.17	0.40	0.0172
PECAM	Adhesion Molecule		2.85	0.64	1.25	0.15	0.44	0.0035*
Sphk1	Transcription Factor		0.02	0.67	0.01	0.20	0.46	0.0004*
Pdgfa	Growth Factor		0.17	0.68	0.08	0.28	0.46	0.0017*
Mmp9	Invasion & Metastasis/Protease		0.06	0.77	0.03	0.18	0.46	0.0391
Nrp2	Growth Factor Receptor		0.15	0.70	0.07	0.19	0.49	0.0333

Symbol	Functional Gene Grouping	HGF WT		HGF TG		Ratio (TG:WT)	p
		Avg	STD	Avg	STD		
Tnfrsf12	Adhesion Molecule	0.14	0.60	0.07	0.21	0.54	0.0017*
Plau	Invasion & Metastasis/Protease	0.02	0.71	0.01	0.20	0.63	0.0445
Pigs1	Signal Transduction Molecule	0.38	0.63	0.25	0.23	0.65	0.0161
Cxcl5	Cytokine	0.00	0.42	0.02	0.23	4.89	0.0252
Il1b	Cytokine	0.22	0.51	1.35	0.34	6.04	0.0343
40 wks							
Efna2	Signal Transduction Molecule	0.48	0.27	0.09	0.17	0.19	0.0286
Plk3cb	Signal Transduction Molecule	0.57	0.10	0.11	0.05	0.20	0.0285
Cxcl9	Cytokine	0.35	0.19	0.08	0.10	0.23	0.0304
Mmp9	Invasion & Metastasis/Protease	0.25	0.15	0.08	0.01	0.34	0.0250
Fgf(Vegfd)	Growth Factor	0.51	0.13	0.18	0.16	0.34	0.0031
Akt1	Signal Transduction Molecule	0.56	0.14	0.20	0.12	0.36	0.0013
Angptl4	Angiogenic Inhibitor Protein	0.63	0.21	0.25	0.18	0.40	0.0324
Syk	Signal Transduction Molecule	0.16	0.02	0.07	0.00	0.41	0.0172
Mmp2	Invasion & Metastasis/Protease	0.29	0.06	0.13	0.05	0.44	0.0004
Pofut1	Signal Transduction Molecule	0.22	0.07	0.10	0.03	0.44	0.0143
Tnfrsf12a	Adhesion Molecule	0.41	0.13	0.18	0.11	0.45	0.0376
Vegfb	Growth Factor	0.69	0.18	0.33	0.15	0.48	0.0043
Efna1	Signal Transduction Molecule	0.42	0.04	0.20	0.17	0.49	0.0459
TNF	Cytokine	0.17	0.04	0.08	0.02	0.49	0.0008
Pik3r1	Signal Transduction Molecule	0.13	0.02	0.06	0.00	0.49	0.0425
Nrp2	Growth Factor Receptor	0.19	0.04	0.10	0.02	0.52	0.0055
Mdk	Growth Factor	0.21	0.05	0.11	0.03	0.53	0.0107
Tgfb3	Growth Factor	0.72	0.15	0.38	0.19	0.53	0.0327
Vegfa	Growth Factor	0.83	0.11	0.45	0.28	0.53	0.0100
Mapk14	Growth Factor Receptor	0.89	0.10	0.48	0.35	0.54	0.0205
Flt1	Growth Factor Receptor	0.89	0.10	0.48	0.35	0.54	0.0205
Csf3	Cytokine	0.32	0.09	0.18	0.07	0.55	0.0422

Symbol	Functional Gene Grouping	HGF WT		HGF TG		Ratio (TG:WT)	p
		Avg	STD	Avg	STD		
Vegfc	Growth Factor	0.23	0.08	0.13	0.06	0.56	0.0361
Timp3	Invasion & Metastasis/ Angiogenic Inhibitor Protein	0.94	0.08	0.53	0.28	0.56	0.0065
Grb2	Signal Transduction Molecule	0.11	0.00	0.06	0.00	0.57	0.0046
Anpep	Protease	0.11	0.03	0.07	0.02	0.58	0.0344
Plau	Invasion & Metastasis/Protease	0.11	0.04	0.06	0.01	0.60	0.0457
Col18a1	Angiogenic Inhibitor Protein	0.13	0.03	0.08	0.03	0.62	0.0166
Pigs2	Signal Transduction Molecule	0.12	0.03	0.07	0.01	0.62	0.0328
Itga2	Adhesion Molecule	0.10	0.00	0.06	0.00	0.64	0.0069
Pdgfa	Growth Factor	0.93	0.08	0.60	0.28	0.65	0.0198
Timp1	Invasion & Metastasis/ Angiogenic Inhibitor Protein	0.11	0.03	0.07	0.01	0.65	0.0169
Eng	Adhesion Molecule	0.09	0.02	0.06	0.01	0.65	0.0357
FGF1	Growth Factor	0.09	0.03	0.06	0.01	0.66	0.0350
IGF1	Growth Factor	0.10	0.02	0.07	0.01	0.67	0.0019

* adjusted p-value <0.05 after application of Benjamini Hochberg correction

Table 2.

Expression of Genes in HGF TG Lung Tumors

Symbol	WT Tumor		TG Tumor		Ratio (TG:WT)	p-value
	Avg	STD	Avg	STD		
Cxcl1	0.16	0.01	0.41	0.01	2.55	0.002 *
Cxcl11	0.15	0.01	0.32	0.01	2.21	0.002 *
Cxcl10	0.25	0.02	0.45	0.01	1.79	0.006
Cxcl2	0.17	0.02	0.30	0.00	1.75	0.010
Cxcl9	0.43	0.04	0.73	0.00	1.71	0.008

* adjusted p-value <0.05 after application of Benjamini Hochberg correction