Cancer Therapy: Preclinical

Anti-HDGF Targets Cancer and Cancer Stromal Stem Cells Resistant to Chemotherapy

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Abstract

Purpose: Approximately one third of the patients with advanced non–small cell lung carcinoma (NSCLC) will initially respond to platinum-based chemotherapy, but virtually all tumors will progress (acquired resistance). The remainder will progress during initial treatment (primary resistance). In this study, we test whether the treatment can be improved by inhibiting hepatoma-derived growth factor (HDGF).

Experimental Design: Thirteen primary NSCLC heterotransplant models were used to test four treatment regimens, including platinum-based chemotherapy with and without bevacizumab (VEGF-neutralizing antibody) or HDGF-H3 (HDGF-neutralizing antibody) and chemotherapy with bevacizumab and HDGF-H3. Expression of stem cell–related genes was measured using quantitative reverse transcription PCR (qRT-PCR) and immunohistochemistry.

Results: Among 13 primary NSCLC heterotransplant models, three (23%) responded to chemotherapy but all relapsed within 20 days. The residual tumors after response to the chemotherapy exhibited an increased expression in 51 (61%) of 84 genes related with stem cell proliferation and maintenance, particularly those in Notch and Wnt pathways, suggesting enrichment for stem cell populations in the residual tumors. Interestingly, tumors from two of three models treated with HDGF-H3, bevacizumab, and chemotherapy combination did not relapse during 6 months of posttreatment observation. Importantly, this treatment combination substantially downregulated expression levels in 57 (68%) of 84 stem cell–related genes, including 34 (67%) of 51 genes upregulated after the chemotherapy.

Conclusion: These data support the hypothesis that cancer stem cells (CSC) are a mechanism for chemotherapy resistance and suggest HDGF may be a target for repressing CSCs to prevent relapse of NSCLC sensitive to chemotherapy. Clin Cancer Res; 19(13); 1–10. ©2013 AACR.

Introduction

Lung cancer is the leading cause of cancer mortality in the industrialized world (1). Of note, 80% to 90% of lung cancer is the non–small cell lung carcinoma (NSCLC) type. The median survival of patients with advanced NSCLC in randomized trials is approximately 12 months with very few survivors beyond 3 years (2, 3).

Hepatoma-derived growth factor (HDGF) is a heparin-binding growth factor and has been implicated in angiogenesis (4–6). Although the mechanisms explain how HDGF functions at the molecular level, its binding to a candidate cell surface receptor as well as its reentering cells are thought to be critical for its cellular functions (7–9). We have shown that HDGF is overexpressed in NSCLC and higher expression is strongly associated with poorer clinical outcomes (10). We further showed that HDGF promotes tumorigenicity of NSCLC cells and targeting HDGF inhibits growth of these cancer cells both in vitro and in vivo (11). To test HDGF as a therapeutic target, we developed monoclonal antibodies (mAb) against HDGF and evaluated them in NSCLC xenograft models and selected HDGF-H3 for further evaluation (12).

In this study, we used a panel of NSCLC heterotransplant models developed directly from primary NSCLC to test a potential impact of inhibiting HDGF in treating NSCLC using a treatment design similar to randomized phase II clinical trials.

Materials and Methods

NSCLC heterotransplant tumor models

Primary NSCLC tissues from patients who underwent surgical resection were obtained from Departments of Pathology, the University of Texas MD Anderson Cancer
Center (Houston, TX) and the University of Maryland School of Medicine (Baltimore, MD) according to protocols approved by the Institutional Review Boards. Fresh tumor tissues were cut into pieces of 1 to 2 mm³ in sterile culture medium. Three to 4 pieces of the tissues were inoculated into the lower back and anterior chest of 6- to 8-week-old female Nu/Nu mice according to the protocols approved by Institutional Animal Care and Use Committees. Two to 3 mice were used for each tumor. Tumors grown and reached at least 10 mm in diameter were considered established. Each heterotransplant tumor model established was confirmed by pathology examination. Among the 38 primary NSCLC tissues, 17 heterotransplant tumor models were established (45% take rate). Tumors can be reestablished in nude mice in a reasonable time frame to allow drug testing in 13 of 17 models. The third or fourth generations of the 13 models were selected for this study (Table 1). The tumor generation is defined as the number of passages in animals starting from the implant of the primary tumors directly obtained from patients.

### Translational Relevance

Patients with advanced non–small cell lung carcinoma (NSCLC) are not curable by current treatment strategies. A major obstacle to this poor outcome is due to treatment resistance. In this study, we used NSCLC heterotransplant models that resemble human primary tumors to test a novel target, hepatoma-derived growth factor (HDGF). We tested platinum-based chemotherapeutic regimens with and without bevacizumab or HDGF-H3 (HDGF-neutralizing antibody) and chemotherapy with bevacizumab and HDGF-H3. The control regimens are part of standard treatment for patients with advanced NSCLC and the testing design is similar to a randomized phase II trial. The findings that the anti-HDGF–based regimen may prevent tumor recurrence and that such antitumor effort may be due to inhibition of cancer stem cell (CSC) features have potentially significant clinical implications in preventing relapse of NSCLC that is initially sensitive to chemotherapy and developing novel strategies to target cancer-related stem cells.

### Treatment and assessment of treatment responses

For each tumor model, 5 mice were inoculated with tumor pieces (3–4 pieces per mouse) subcutaneously under the lower back. Tumors were allowed to grow to at least 10 mm in diameter before being randomly selected for 1 of 4 treatment arms: arm A, cisplatin (CDDP) + gemcitabine + bevacizumab + control mAb (M31); arm B, CDDP + gemcitabine + bevacizumab + Anti-HDGF mAb (HDGF-H3); arm C, CDDP + gemcitabine + M31; and arm D, CDDP + gemcitabine + HDGF-H3 (Fig. 1A). All the drugs were given intraperitoneally. CDDP was given weekly at 1 mg/kg based on previous report (13) and the dosing toxicity test; gemcitabine, weekly at 125 mg/kg; bevacizumab, twice weekly at 5 mg/kg; HDGF-H3, twice weekly at 10 mg/kg; and M31, twice weekly at 10 mg/kg.

The tumors in the fifth mouse from each model were used as treatment-naive controls in molecular analyses. Four cycles of treatment were given to each animal with each cycle lasting for 1 week. Tumor sizes were measured twice a week and the tumor volumes were calculated using the following formula: length (mm) × width (mm) × height (mm) = mm³. Response Evaluation Criteria in Solid Tumors (RECIST) criteria (14) were used to evaluate tumor responses after 4 cycles of treatment. Animals were weighed twice a week during treatment. The weight loss during chemotherapy was not detectable in some tumor models but observed in other models. The loss was

### Table 1. Clinical and pathologic characteristics of the heterotransplant models

<table>
<thead>
<tr>
<th>Model</th>
<th>Age, y</th>
<th>Sex</th>
<th>Histology</th>
<th>Differentiation</th>
<th>Stage</th>
<th>Prior treatment</th>
<th>Response</th>
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<tbody>
<tr>
<td>MDA2131-1</td>
<td>76</td>
<td>M</td>
<td>AD</td>
<td>Moderate</td>
<td>IA</td>
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</tr>
<tr>
<td>MDA2131-4</td>
<td>65</td>
<td>F</td>
<td>SQ</td>
<td>Moderate</td>
<td>IIA</td>
<td>3 cycles: cisplatin/docetaxel</td>
<td>PR</td>
</tr>
<tr>
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<td>78</td>
<td>F</td>
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<td>Poor</td>
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</tr>
<tr>
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<td>AD</td>
<td>Poor</td>
<td>IIA</td>
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<td>NA</td>
</tr>
<tr>
<td>MDA2131-8</td>
<td>84</td>
<td>M</td>
<td>AD</td>
<td>Poor</td>
<td>IIA</td>
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<td>NA</td>
</tr>
<tr>
<td>MDA2131-11</td>
<td>65</td>
<td>F</td>
<td>SQ</td>
<td>Poor</td>
<td>IIIA</td>
<td>3 cycles: docetaxel/carboplatin</td>
<td>SD</td>
</tr>
<tr>
<td>MDA2131-15</td>
<td>64</td>
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<td>NA</td>
</tr>
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</tr>
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<td>SQ</td>
<td>Poor</td>
<td>IB</td>
<td>1 cycle: cisplatin/docetaxel</td>
<td>SD</td>
</tr>
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<td>Poor</td>
<td>IV (brain metastasis)</td>
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<td>NA</td>
</tr>
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<td>MDA132</td>
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<td>M</td>
<td>AD</td>
<td>Moderate</td>
<td>IIIA</td>
<td>2 cycles: cisplatin/docetaxel</td>
<td>SD</td>
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<td>NA</td>
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</tbody>
</table>

Abbreviations: AD, adenocarcinoma; F, female; M, male; NA, not available; SD, stable disease; SQ, squamous cell carcinoma.
generally less than 20% and no significant differences were observed among the 4 treatment arms. For the tumor models responding to chemotherapy and other combinations, the experiments were repeated with 2 animals for each treatment arm to verify earlier observations and to obtain sufficient tumor tissues for molecular analyses. One of the animals in each arm was scarified at the end of treatment (28 days) to obtain tumor tissues for molecular analyses, whereas other animals were further observed to verify early observations. For molecular analyses, resected tumors were divided into 2 parts, one fresh-frozen and the other formalin-fixed. These tissues were subsequently used for hematoxylin and eosin (H&E) staining, immunohistochemistry staining, and DNA/RNA extraction.

Immunohistochemistry staining

Part of the resected tumor tissues were fixed in formalin for 24 hours and then embedded in paraffin. For immunohistochemistry staining, the formalin-fixed and paraffin-embedded tissues were sectioned at 5 μm thickness sections and placed on glass pathology slides. The slides were deparaffinized and rehydrated in graded concentrations of alcohol using standard techniques. The slides were incubated with 1:50 anti-cleaved Notch1 (Val1744; D3B8) rabbit mAb (Cell Signaling Technology) for 24 hours. The slides were
then incubated with biotinylated secondary antibody (sheep anti-rabbit) in PBS with 0.05% Tween-20 for 30 minutes at room temperature followed by sequentially in ABC-Peroxidase Solution for 30 minutes, substrate-chromagen solution (DAKO Liquid DABþ Substrate Chromogen System) for 5 minutes. Cells with nuclear staining were considered positive of Notch intracellular domain (NICD) expression. For HDGF, the slides were blocked with 5% normal horse serum and Mouse-on-Mouse Blocking Reagent (M.O.M; Vector Laboratories) for 30 minutes followed by incubating with anti-HDGF H3 mouse mAb (1:500) at 4°C overnight. After 3 washes with PBS containing 0.05% Tween-20, the slides were incubated with biotinylated horse anti-mouse immunoglobulin G (IgG; 1:200; Vector Laboratories) for 30 minutes. The expression was detected using Vectastain Elie ABC Reagent (Vector Laboratories) and 3,3′-diaminobenzidine (DAB) reagent (Vector Laboratories) according to manufacturers instruction.

**Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assay**

Slides were deparaffinized and rehydrated in graded concentrations of alcohol and then incubated with proteinase K for 15 minutes. Each slide was incubated with 200 µL reaction mixture (terminal deoxynucleotidyl transferase, TdT, and biotin dUTP) for 1 hour at room temperature, followed by TB buffer for 15 minutes and then washed with double-distilled water (ddH2O). The slides were then incubated with 2% bovine serum albumin for 10 minutes at room temperature followed by incubation in ABC-Peroxidase Solution for 30 minutes, substrate-chromagen solution (DAKO Liquid DABþ + Substrate Chromogen System) for 5 minutes, methyl green staining for 2 minutes, and dehydration. Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL)–positive cells were counted in the tissue sections.

**DNA/RNA extraction**

Frozen tissues were lysed and homogenized in a highly denaturing guanidine isothiocyanate–containing buffer. The lysate was then passed through an AllPrep DNA spin column. This column, in combination with the high-salt buffer, allows selective and efficient binding of genomic DNA. The column was washed and pure, ready-to-use DNA was then eluted with water. Ethanol was then added to the flow-through from the AllPrep DNA spin column to provide appropriate binding conditions for RNA, and the sample was then applied to an RNasey spin column, where total RNA binds to the membrane and contaminants were efficiently washed away. High-quality RNA was then eluted in 30 µL water.

**Expression analysis of stem cell–related genes**

Approximately, 120 ng total RNA was used for cDNA synthesis using the RT² First Strand kit (SA Biosciences) following the manufacturer’s instructions. Gene expression was quantitatively analyzed using Human Stem Cell RT² Profiler PCR Array and RT² SYBR Green Master Mix (SA Biosciences) as per the manufacturer’s instructions. PCR was conducted on ABI 7900HT Fast Real-Time PCR system (Applied Biosystems). For data analysis, ΔΔCt values were calculated online at the manufacturer’s website (http://www.sabiosciences.com/pcr/arrayanalysis.php).

Fold-changes were calculated as the differences in gene expression levels before and after treatment in each model and treatment arm. A positive value indicates gene upregulation and a negative value indicates gene downregulation. Genes that showed more than 2-fold differences in the expression levels were highlighted in the data presentation.

**Primary culture of tumor-derived fibroblasts**

Tissues for primary culture of fibroblasts were obtained from a lung cancer heterotransplant model. These specimens were dissected with scissors into small pieces (after washing with PBS) and transferred into flasks containing collagenase IV (200 U/mL) in Dulbecco’s modified Eagle medium (DMEM) with 10% FBS for 6 hours followed by exhaustively rinsed (8 times) with 40 mL of PBS under vigorous agitation. After disaggregation, the suspension of cells was washed by settling and then centrifuged and the cells were then put into a flask with DMEM for 15 minutes and the cell suspension was aspirated. The fibroblasts were cultured in DMEM and used for experiments.

**Microdissection of individual cells**

Ten micrometer tissue sections were mounted on glass slides. Tumor nests and stromal parts were microdissected using Arcturus laser capture microdissection (LCM), respectively.

The isolated cells were put into 1.5-ml plastic tubes for DNA extraction.

**DNA amplification and PCR product visualization**

DNA from microdissected tissues was amplified by PCR using the primer set: 5′-gaggtggaaaagataagcc-3′ (forward) and 5′-tgctgcttcgattctgctgat-3′ (reverse) to amplify a fragment in HDGF gene. Human DNA would result in a 153-bp PCR fragment, whereas mouse DNA would result in a 144-bp fragment. For tumor-derived fibroblasts and mouse tissues, DNA was amplified by PCR using either human-specific primers (forward: 5′-tcggggtggctgctgtgctc-3′ and reverse: 5′-aggaacctctcctctctctca-3′) to amplify a 122-bp fragment of a region located in 7p15-p12 or mouse-specific primers (forward: 5′-ttgcttcatcactctccc-3′ and reverse: 5′-cagacaggtcctccctcc-3′) to amplify a 181-bp fragment of a region located in B2m gene. Amplified PCR products were separated in 2% agarose gels and visualized using ethidium bromide.

**Results**

**Rodent-based randomized phase II treatment trial**

To recapitulate treatment strategies used in treating patients with advanced NSCLC in animal models, we selected the commonly used regimen of cisplatin plus gemcitabine in treating patients with advanced NSCLC. We also tested this regimen with bevacizumab based on data.
showing prolonged progression-free survival (3). We included HDGF-H3 in each of the regimens to determine a role of targeting HDGF in treating NSCLC (Fig. 1A). Thirteen NSCLC heterotransplant models (15), which are derived directly from primary NSCLC without in vitro passage (Table 1), were evaluated. For each model, 5 animals were implanted with third- or fourth-generation heterotransplant tumors with an average tumor size of 448 mm² at the time treatment started. The large size of the tumors mimics the actual presentation of patients with advanced NSCLC. Four of 5 animals were then randomly selected to be treated with of 4 treatment arms (Fig. 1A) with 13 animals in each arm. Together with repeated experiments in 2 of the models, a total of 62 animals were used in the animal trial. The identical genetic background of all the animals minimizes the impact of host factors. In addition, each treatment arm used the same tumor panel, which minimizes the impact of tumor heterogeneity. Together, this experimental strategy recapitulates an initial biomarker-integrated randomized phase II human therapeutic trial.

NSCLC heterotransplants contain human-origin stroma

Tumor stroma is increasingly recognized as an important component of tumor biology, contributing to the malignant phenotypes and treatment resistance (16, 17). In the NSCLC heterotransplant tumors, which reflect properties of their clinical counterparts (18, 19), stromal cells of human origin were observed in the third- and fourth-generation tumors using assays that are sensitive and specific to differentiate the genetic origins of these cells (Fig. 1B and C).

Responses to treatment regimens

Tumor response was assessed using the response criteria in solid tumors (RECIST) after 4 treatment cycles with each cycle lasting for one week. The chemotherapy only arm (arm C) resulted in 3 partial responses (PR), 6 stable diseases, and 3 progressive diseases (Table 2) for a 23% response rate (RR). This degree of response is comparable with previous reports using similar models and clinical trials (18, 20). We analyzed HDGF expression levels in the tumor models and found no direct relationship between the expression levels and tumor responses (Table 2). The 3 models with PR also showed PR or complete response (CR) in other treatment arms (Table 2). Consistent responses were also observed in other tumor models with stable diseases and progressive diseases with limited variation (Table 2).

Impact of chemotherapy on expression of stem cell-related genes

Expression patterns of 84 genes representing putative stem cell–specific markers, stem cell differentiation markers, and stem cell maintenance signaling pathways were quantitatively analyzed in 6 tumor models, 3 (MDA2131-1, MDA889, and UMB410) with primary chemotherapy resistance, and 3 (MDA2131-5, MDA2131-8, and UMB710) with acquired resistance, before and after chemotherapy. There was minimal change in the expression patterns of these 84 genes in the tumor models with primary resistance (Fig. 2A). Only one of the genes was upregulated more than 2-fold (TERT; 2.2-fold) and one downregulated (FGFR1; -2.6-fold). In contrast, 51 (61%) of 84 genes were upregulated more than 2-fold in the tumor models with acquired resistance but only 4 (5%) of the genes downregulated (Fig. 2B). As shown in Fig. 2C, the upregulated genes include 7 of 12 critical for Notch pathway, 4 of 10 for Wnt pathway as well as stem cell–specific molecules including 11 of 12 cytokines/growth factors, 4 of 25 metabolic markers, 3 of 6 self-renewal markers, 9 of 17 cell-cycle regulators, 2 of 6 chromosome/chromatin modulator, 3 of 5 symmetric regulators, 4 of 6 cell–cell communication regulators, and 5 of 10 cell-adhesion regulators. Upregulated genes also included 5 of 7 mesenchymal lineage markers, 7 of 9 embryonic lineage markers, 4 of 5 hematopoietic lineage markers, and 2 of 5 neural lineage markers (Fig. 2C).

<table>
<thead>
<tr>
<th>Model</th>
<th>Arm level</th>
<th>Arm</th>
<th>Arm</th>
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</tr>
</thead>
<tbody>
<tr>
<td>MDA2131-1</td>
<td>+++</td>
<td>PD</td>
<td>SD</td>
<td>SD</td>
<td>PD</td>
</tr>
<tr>
<td>MDA2131-4</td>
<td>+</td>
<td>SD</td>
<td>PR</td>
<td>SD</td>
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</tr>
<tr>
<td>MDA2131-5</td>
<td>+++</td>
<td>PR</td>
<td>PR</td>
<td>PR</td>
<td>PR</td>
</tr>
<tr>
<td>MDA2131-7</td>
<td>+++</td>
<td>SD</td>
<td>SD</td>
<td>SD</td>
<td>SD</td>
</tr>
<tr>
<td>MDA2131-8</td>
<td>++</td>
<td>CR</td>
<td>CR</td>
<td>PR</td>
<td>PR</td>
</tr>
<tr>
<td>MDA2131-11</td>
<td>+</td>
<td>SD</td>
<td>PD</td>
<td>PD</td>
<td>PD</td>
</tr>
<tr>
<td>MDA2131-15</td>
<td>++</td>
<td>PD</td>
<td>SD</td>
<td>SD</td>
<td>SD</td>
</tr>
<tr>
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<td>+</td>
<td>PD</td>
<td>SD</td>
<td>SD</td>
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<td>MDA513</td>
<td>+++</td>
<td>SD</td>
<td>SD</td>
<td>SD</td>
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</tr>
<tr>
<td>MDA889</td>
<td>+</td>
<td>PD</td>
<td>SD</td>
<td>PD</td>
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</tr>
<tr>
<td>MDA132</td>
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<td>+++</td>
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<tr>
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NOTE: +, weak expression; ++, moderate expression; ++++, strong expression.
Abbreviations: SD, stable disease; PD, progressive disease.

Table 2. Responses to treatment in the heterotransplant models

HDGF-H3 and NSCLC relapse

To test whether an anti-HDGF–based combination can disrupt tumor microenvironment and inhibit cancer-associated stromal stem cells to result in preventing or delaying NSCLC relapse, we extended the observation time of the 3 chemotherapy-sensitive tumor models. Ten to 20 days after treatment completion, all but 2 (MDA2131-5 and MDA2131-8) tumors in arm B (HDFD-H3, bevacizumab, and chemotherapy) relapsed (Fig. 3A–C). The experiment was repeated once for MDA2131-5 tumor model with similar results (Fig. 3A). In the MDA2131-5 case, the residual tumor in the treatment arm completely regressed 1 week after the completion of treatment (Fig. 3A). The mice remained relapse-free for more than 6 months (at which time they were sacrificed) showing a role for the combination in preventing or delaying relapse of the tumors.

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To determine whether this effect is due to inhibition of stem cells enriched by chemotherapy, we compared expression patterns of the 84–stem cell gene panel using real-time reverse transcription PCR (RT-PCR) after being treated with chemotherapy (arm C) in the tumor models not responding to the treatment. The genes are arranged on the basis of fold changes from the most downregulated to the most upregulated from left to right horizontally. B, fold changes of the 84 stem cell–related genes after treatment with chemotherapy (arm C) in the tumor models (MDA2131-5, MDA2131-8, and UMB710) that significantly responded to the treatment. The genes are arranged on the basis of fold changes from the most downregulated to the most upregulated from left to right horizontally. C, list of the 84 stem cell–related genes arranged by pathways or functionalities involved (some of the genes involved in multiple pathways or functionalities). The data are based on results exhibited in panel B. Gene names in red for those upregulated (>2-fold) and in green for those downregulated (fold changes are rounded to closest full digits except those <3-fold).

**Figure 2.** Responses of the NSCLC models to a combination of chemotherapy. A, fold changes of the 84 stem cell–related genes quantified using real-time reverse transcription PCR (RT-PCR) after being treated with chemotherapy (arm C) in the tumor models not responding to the treatment. The genes are arranged on the basis of fold changes from the most downregulated to the most upregulated from left to right horizontally. B, fold changes of the 84 stem cell–related genes after treatment with chemotherapy (arm C) in the tumor models (MDA2131-5, MDA2131-8, and UMB710) that significantly responded to the treatment. The genes are arranged on the basis of fold changes from the most downregulated to the most upregulated from left to right horizontally. C, list of the 84 stem cell–related genes arranged by pathways or functionalities involved (some of the genes involved in multiple pathways or functionalities). The data are based on results exhibited in panel B. Gene names in red for those upregulated (>2-fold) and in green for those downregulated (fold changes are rounded to closest full digits except those <3-fold).

**HDGF-H3 and expression of stem cell–related genes**

To determine whether this effect is due to inhibition of stem cells enriched by chemotherapy, we compared expression patterns of the 84–stem cell gene panel using real-time PCR array system (http://www.sabiosciences.com/rt_pcr_product/HTML/PAHS-405Z.html) in tumors after each treatment regimen with the treatment-naïve tumors in the 2 models (MDA2131-5 and MDA2131-8). In arm B, 57 (68%) of the genes, including 34 (67%) of 51 upregulated in the chemotherapy arm (arm C), were downregulated more than 2-fold (Fig. 4A and B). There were 22 genes, including 14 upregulated by chemotherapy, upregulated...
Figure 3. A, tumor growth curves of model MDA2131-5 (third generation including results from the repeated experiment) treated in 4 arms. B, mice 1 month after treatment stopped. C, tumor growth curves of model MDA2131-8 (fourth generation) treated in 4 arms.

more than 2-fold (Fig. 4A and B). In contrast, only modest impacts were observed when either bevacizumab (arm A) or HDGF-H3 (arm D) was added to chemotherapy (Supplementary Fig. S1A and S1B).

To verify the inhibition of Notch in tumors from arm B, we measured NICD using immunohistochemistry and found that NICD was highly expressed in nucleus of tumor cells in treatment-naive tumors but undetectable in the residual tumor cells after treatment combinations containing HDGF-H3 (Supplementary Fig. S2A). We further evaluated Wnt signaling molecules because the pathway is important in embryonic stem cell self-renewal (21) and interaction between cancer stem cells (CSC) and stromal stem cells (22, 23). In the residual tumors, 9 of 10 Wnt pathway genes were significantly downregulated (Fig. 4B) with 8- and 51-fold reduction of Wnt1 and Frizzled (the key receptor of Wnt), supporting the impairment of Wnt signaling.

HDGF-H3 and apoptosis of tumor stromal cells

To determine whether the modulation of stem cell–related genes is in part due to the death of tumor-associated stromal cells, we used the TUNEL assay to measure apoptotic cells and observed substantially increased TUNEL–positive cells in the arm B–treated tumors, predominantly with morphology of stromal cells (Supplementary Fig. S2B).

Discussion

In this study, we used human NSCLC heterotransplant models in a therapeutic testing similar to a randomized phase II clinical trial. Because the tumors derived directly from primary tumors, the models avoid in vitro adaptation, and therefore better reflect in vivo tumor conditions. It has been well recognized that the morphologies of the tumors from heterotransplant models mimic their primary tumor counterparts. Here, we provide evidence indicating the presence of human stromal cells in these tumors. It is well known that stromal cells in cancer tissues play critical roles in supporting and promoting cancer initiation and progression (24, 25). Somatic mutations of tumor suppressor genes and epigenetic alterations commonly seen in cancer cells can be identified in the stroma of breast cancers (26, 27). Therefore, it is possible that some of the stroma cells in NSCLC are immortal and possess stem cell features. These cells may coproliferate together with cancer cells in these tumor models and become a critical tumor component for assessing treatments targeting cancer microenvironment. The identical genetic background of the host animals and the tumor source of each model helped the uniform treatment responses observed in the study (Table 2).

CSC hypothesis implies that resistance to treatment occurs as a consequence of the presence of a small number of chemotherapy-resistant self-renewing tumor cells, which are primarily based on observation in cell-based models (28–31). Recently, the hypothesis has been validated in several animal models (32–34) but has not been confirmed in any human cancers. Here, the hypothesis was evaluated by examining expression patterns of 84 genes representing putative stem cell–specific markers, stem cell differentiation markers, and stem cell maintenance signaling pathways in tumors sensitive and resistant to chemotherapy. Compared with the treatment-naive tumors, the residual tumors from chemosensitive models showed a substantial upregulation of stem cell–related genes, suggesting an enrichment of stem cell populations, but showed virtually no expression change of these genes in the chemoresistant tumors. The data support the hypothesis that the emergence of a CSC population is associated with acquired chemotherapy resistance. However, it is also possible that these cells are resistant before chemotherapy stimulation and are simply enriched when the sensitive cells are eliminated during the treatment course. Notch signaling activation is critical in lung carcinogenesis and stem cell renewal (35–37). The
Figure 4. A, fold changes of the 84 stem cell–related genes after treatment with HDGF-H3 + bevacizumab + chemotherapy (arm B) in the tumor models MDA2131-5 and MDA2131-8. The genes are arranged on the basis of fold changes from the most downregulated to the most upregulated from left to right horizontally. B, list of the 84 stem cell–related genes arranged by pathways or functionalities involved in a format as presented in Fig. 1F. The fold changes are based on the data presented in panel A. Gene names with green highlight are those downregulated in the treatment arm, whereas those with pink highlight are those upregulated. Gene names in red are those upregulated in the tumor models sensitive in arm C.
upregulation of multiple Notch ligands (DLL1, DLL3, and JAG1). Notch itself, and transcriptional coactivators (EP300 and DTOX1) suggests that the Notch pathway is activated in the residual tumor tissues.

We noticed predominant mesenchymal and hematopoietic features in the residual tumor tissues after responses to chemotherapy, suggesting an enrichment of cancer-related mesenchymal and hematopoietic stem cells, a finding consistent with the increased tumor stroma observed in patients with NSCLC after neoadjuvant chemotherapy (38). Consistent with this notion, almost all the cytokines measured were upregulated in these tissues. Together with the evidence supporting the critical role of cancer-associated fibroblasts in tumorigenesis and progression (39, 40), these data suggest that the stromal stem cells may facilitate cancer relapse. The results are also consistent with a recent report showing the existence of distinct types of CSCs in a colon cancer model (41).

Thus, it is possible that the anti-HDGF–based treatment eliminated stem cells capable of regenerating, whereas the remaining "stem cells" had limited or no self-renewal capability. In addition, we observed significantly reduced expression levels of genes encoding cytokines, cell-adhesion molecules, and stem cell differentiation markers. The striking downregulation of COL1A1 (down >500-fold; Fig 4B), together with the downregulation of COL2A1 and COL9A1, may impact collagen structures and result in a loss of extracellular support for proliferation and differentiation of CSCs.

It should be noted that an increased rate of hemoptysis was observed in patients with lung squamous cell carcinoma (LSCC) treated with bevacizumab in combination with chemotherapeutic agents (2), which limits the use of bevacizumab only for patients with nonsquamous type NSCLC. In our earlier study (12), we showed that certain chemotherapeutic agent, such as gemcitabine, could substantially enlarge the vessel size in an experimental NSCLC model. Although bevacizumab could reduce the vessel number but not the vessel size in the model system. In contrast, the anti-HDGF antibody substantially reduced the vessel size but not the vessel number. When bevacizumab was used together with the anti-HDGF antibody, both the vessel size and numbers were substantially reduced. Therefore, combining anti-VEGF and anti-HDGF agents may reduce the possibility of hemoptysis seen in patients with LSCC treated with bevacizumab. More experimental and clinical studies will be needed to test this potential.

In summary, our data provide evidence supporting the CSC hypothesis and may have potential clinical implications. We show that chemotherapy-sensitive human lung cancer explants have enriched stem cell–like populations after chemotherapy. Importantly, a regimen combining chemotherapy/bevacizumab with an anti-HDGF antibody might have impaired the stem cell populations and prevented relapse. It is possible that this effect is due to a blockade of signaling and other factors required for the proliferation and/or maintenance of CSCs and cancer-related stem cells. Thus, our study suggests that simultaneously targeting CSCs and stromal stem cells in chemosensitive NSCLC may prevent relapse and prolong patient survival.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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