Nonamplified FGFR1 Is a Growth Driver in Malignant Pleural Mesothelioma

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Abstract
Malignant pleural mesothelioma (MPM) is associated with asbestos exposure and is a cancer that has not been significantly affected by small molecule-based targeted therapeutics. Previously, we demonstrated the existence of functional subsets of lung cancer and head and neck squamous cell carcinoma (HNSCC) cell lines in which fibroblast growth factor receptor (FGFR) autocrine signaling functions as a nonmutated growth pathway. In a panel of pleural mesothelioma cell lines, FGFR1 and FGFR2 were coexpressed in three of seven cell lines and were significantly associated with sensitivity to the FGFR-active tyrosine kinase inhibitor (TKI), ponatinib, both in vitro and in vivo using orthotopically propagated xenografts. Furthermore, RNAi-mediated silencing confirmed the requirement for FGFR1 in specific mesothelioma cells and sensitivity to the FGF ligand trap, FP-1039, validated the requirement for autocrine FGFs. None of the FGFR1-dependent mesothelioma cells exhibited increased FGFR1 gene copy number, based on a FISH assay, indicating that increased FGFR1 transcript and protein expression were not mediated by gene amplification. Elevated FGFR1 mRNA was detected in a subset of primary MPM clinical specimens and like MPM cells; none harbored increased FGFR1 gene copy number. These results indicate that autocrine signaling through FGFR1 represents a targetable therapeutic pathway in MPM and that biomarkers distinct from increased FGFR1 gene copy number such as FGFR1 mRNA would be required to identify patients with MPM bearing tumors driven by FGFR1 activity.

Implications: FGFR1 is a viable therapeutic target in a subset of MPMs, but FGFR TKI-responsive tumors will need to be selected by a biomarker distinct from increased FGFR1 gene copy number, possibly FGFR1 mRNA or protein levels. Mol Cancer Res; 12(10); 1460–9. ©2014 AACR.

Introduction
Malignant pleural mesothelioma (MPM) arises from the mesothelial cells lining the pleural cavity surrounding the lungs, and less frequently from the peritoneum (1). The incidence of MPM increased over the second half of the 20th century, coinciding with an increased industrial use of asbestos (1) and is associated with an extremely long latency of approximately 50 years (2). Although the predicted incidence of MPM (2,000 to 3,000 cases per year) may have peaked in the United States, incidence is expected to continue to increase worldwide due to variable asbestos regulation (1, 3). Three histologic subtypes of MPM have been defined: epithelioid, sarcomatoid, and biphasic (mixed). Median survival ranges from 6 to 13 months with epithelioid tumors exhibiting the longest survival time (11–13 months) and sarcomatoid tumors the shortest (6–7.5 months; refs. 4, 5). Combined cisplatin and pemetrexed is currently the only approved therapy with a median survival of 12.1 months (6).

Self-sufficiency in growth signaling, frequently through deregulation and activation of receptor tyrosine kinase (RTK) pathways, is a hallmark of cancer (7). On the basis of success in identifying and targeting specific mutated oncogene drivers in lung adenocarcinomas (8–10), similar investigations have proceeded in MPM. Although gain-of-function mutations in EGFR are exceedingly rare in MPM (11, 12), epidermal growth factor receptor (EGFR) is highly expressed in as many as 97% of tumors. However, no clinical benefit was observed in patients with MPM treated with EGFR-specific TKIs, gefitinib and erlotinib (13, 14). Besides EGFR, evidence...
supports activity of other RTKs in MPM, including MET (15, 16), platelet-derived growth factor receptors (PDGFR; refs. 17, 18), vascular-endothelial growth factor receptors (VEGFR; ref. 19), AXL (20, 21), and insulin-like growth factor receptor (IGFIR; refs. 22, 23). In fact, a growth network comprising multiple RTKs has been proposed such that combined inhibition of multiple pathways yields greater efficacy (15, 24).

Deregulation of the FGFR signaling pathways is observed in various tumor types through multiple mechanisms (25). Activating mutations occur in FGFR2 and FGFR3 leading to constitutive dimerization in bladder, endometrial, and squamous cell lung cancers (26–28). Chromosomal translocations in FGFR genes have also been observed in various malignancies (29, 30). Amplification of FGFR2 is commonly seen in gastric cancers and FGFR1 in breast cancer, squamous cell lung cancers, and HNSCC (FGFR1; refs. 31–34). The increase in FGFR1 gene copy number is especially relevant to lung cancer, and serves as the key biomarker for patient recruitment to two open trials of FGFR-specific TKIs in solid tumors (NCT01004224 and NCT00979134). In contrast, ligand-dependent autocrine and paracrine signaling provides a mechanism that is independent of somatic mutation and has been demonstrated by our group in both non–small-cell lung cancer (NSCLC) and HNSCC (35, 36). Herein, we demonstrate that FGFR1 is coexpressed with FGF2 in a subset of MPM cell lines and is strongly associated with constitutive dimerization in bladder, endometrial, and squamous cell lung cancers (26–28). Chromosomal translocations in FGFR genes have also been observed in various malignancies (29, 30).

Materials and Methods
Cell lines
H28, H513, H2052, H2452, MSTO211H, and Met5A cells were obtained from ATCC and passaged for less than 6 months after receipt for completion of the studies. H226 and H290 cells were obtained from and authenticated by (OriGene; #TA301021) or FGFR2 (sc-122), FGFR3 (sc-13121), and NaK-ATPase α-subunit (sc-21712; Santa Cruz Biotechnology). For immunoblot analysis of FGFR1, FGFR2, FGFR3, and the α-subunit of the NaK-ATPase, cells were collected in PBS, centrifuged, and suspended in MKLB. Aliquots of the cell lysates containing 150 μg of protein were submitted to SDS-PAGE and immunoblotted for FGFR1 (OriGene; #TA301021) or FGFR2 (sc-122), FGFR3 (sc-13121), and NaK-ATPase α-subunit (sc-21712; Santa Cruz Biotechnology).

Measurement of FGFR1 gene copy number in cell lines by fluorescence in situ hybridization
Genomic FGFR1 status of all cell lines was analyzed using a fluorescence in situ hybridization (FISH) assay. Cells (60%–80% confluent when harvested) were incubated with 10 μL of 0.075 mol/L KCl at 37°C, and then fixative solution (acetic acid/methanol) was added. Slides were pretreated with a 2× SSC at 37°C for 60 minutes and digested with Digest-All III (dilution 1:25) at 37°C for 6 minutes. FGFR1 target probe and a commercially available centromeric reference probe of chromosome 8 (CEP8; MetaSystems) were denatured at 73°C for 5 minutes and immediately placed on the slides. Codenaturation of the cells and the FGFR1 probes (commercial green reference probe; MetaSystems; BAC Strand cDNA Synthesis Kit (Fermentas). Aliquots (5 μL) of a 1:25 dilution of the reverse transcription reactions were submitted to PCR in 25-μL reactions with SYBR Green JumpStart Taq ReadyMix (Sigma) with FGFR1 and FGF2 primers previously described (35, 36) using a MyiQ Real-Time PCR Detection System (Bio-Rad). GAPDH mRNA levels were measured as a housekeeper gene for normalization and data are presented as "relative expression."

Immunoblot analysis
For analysis of the phosphorylation status of signaling proteins in response to drug treatment, cells were seeded in 6-well dishes at 400,000 cells per well. After 24 hours, cells were placed in HITES medium for 2 hours and subsequently treated with ponatinib (0–300 nmol/L) for 2 hours. Cells were rinsed in phosphate-buffered saline (PBS), lysed in 500 μL of MAP Kinase Lysis Buffer (MKLB; 1× Protease Inhibitor Cocktail #8340 (Sigma), 0.5% Triton X-100, 50 mmol/L β-glycerophosphate (pH 7.2), 0.1 mmol/L Na3VO4, 2 mmol/L MgCl2, 1 mmol/L EGTA, 1 mmol/L DTT, 0.3 mol/L NaCl, and 4 mg/mL aprotinin), and centrifuged (5 minutes at 13,000 rpm). The supernatants were mixed with the SDS sample buffer and submitted to SDS-PAGE. Following electrophoretic transfer onto nitrocellulose filters, the filters were blocked in 3% bovine serum albumin (Cohn Fraction V; ICN Biomedicals, Inc.) in Tris-buffered saline with 0.1% Tween 20 (TTBS) and then incubated with antibodies to phospho-ERK-T202/Y204 (Cell Signaling Technology) for 16 hours at 4°C. The filters were washed thoroughly in TTBS, then incubated with alkaline phosphatase–coupled goat anti-rabbit or mouse antibodies and developed with Lumi-Phos reagent (Pierce) according to the manufacturer’s instructions. The filters were subsequently stripped and reprobed for total ERK1 (sc-93) and ERK2 (sc-154; Santa Cruz Biotechnology, Inc.).
RP11-148D21 was used as a target probe spanning the FGFR1 gene locus 8p11.23 to 8p11.22 (Invitrogen) was performed at 85°C for 4 minutes and then hybridization proceeded at 37°C overnight. The slides were extensively washed with 0.5× SSC and then signals were visualized with a fluorescence microscope.

**Anchorage-independent growth and cell proliferation assays**

For measurement of anchorage-independent cell growth in soft agar, 15,000 cells (H2052, MSTO211H, H513, H226, Mer5A, and H2452) were suspended in 1.5 mL RPMI-1640 containing 10% fetal bovine serum and 0.35% agar noble and overlaid on base layers containing 1.5 mL RPMI-1640 containing 10% fetal bovine serum and 0.5% agar noble in 6-well plates. The wells were overlaid with 2 mL growth media containing ponatinib or FP-1039 and replaced once per week. The plates were incubated for 14 days and viable colonies were stained for 24 hours with 200 µL of 1 mg/mL nitroblue tetrazolium. The wells were photographed and submitted to quantification using the MetaMorph (Molecular Devices) imaging software program. Data are presented as percentage of the DMSO-treated control. For measurement of proliferation, 10,000 cells (H28) or 15,000 cells (H290) were plated in 2 mL RPMI-1640 containing 10% fetal bovine serum in 6-well plates. The cells were treated with ponatinib for 7 days and following trypsinization, viable cells were counted with a Cellometer (Nexcelom) and compared with the DMSO-treated control. For measurement of proliferation, 10,000 cells (H28) or 15,000 cells (H290) were plated in 2 mL RPMI-1640 containing 10% fetal bovine serum and 0.5% agar noble in 6-well plates. The cells were treated with ponatinib for 7 days and following trypsinization, viable cells were counted with a Cellometer (Nexcelom) and compared with the DMSO-treated control for each cell line and presented as percentage control.

**FGFR1 silencing with shRNAs**

Distinct FGFR1-targeting shRNAs (clone IDs TRCN0000121185 and TRCN0000121186) in the pLKO.1 lentiviral vector were obtained from University of Colorado Denver Anschutz Medical Campus. One pas- sage before use, puromycin was removed from the growth medium for luciferase-expressing H226 (H226-luc) cells. H226-luc cells were collected by trypsinization, washed three times with sterile PBS, and resuspended in sterile PBS at a concentration of 1.0 × 10⁵ cells/100 µL. Female mice were anesthetized with isoflurane by inhalation, the left chest wall was cleaned with betadine followed by rinsing with 70% ethanol. A small skin incision in the left chest wall (approximately 5 mm in length) was made along the left lateral axillary line at the level of the xyphoid process. Subskin fat was dissected away until the lung was visualized through the intercostal muscles and pleura. A 1-mL insulin syringe with a 30-gauge needle was used to inject 100 µL of the cell suspension (1 × 10⁶ cells) into the pleural space and the skin incision was closed using Nexaband Liquid Topical Tissue Adhesive (Abbott Laboratories). Following 6 weeks to allow the tumors to establish, the animals were injected with luciferin (300 mg/kg in 150 µL PBS). Approximately 15 minutes later, the mice were anesthetized and submitted to bioluminescence imaging (BLI) using a Xenogen IVIS Imaging System 50 Series (PerkinElmer). Tumor-bearing mice were randomized into groups (5 per group) and treated daily by oral gavage with ponatinib (20 mg/kg) or diluent alone as a control. Tumor burden was monitored every 2 weeks for the duration of the study.

**Surgical mesothelioma cohort and preparation of a tissue microarray**

We screened the archives of the Institute for Pathology, Heidelberg University, Germany, for available paraffin-embedded tissue of surgically obtained mesotheliomas treated between 2002 and 2010 in the Thoraxklinik at Heidelberg University, Germany. Usage of the human tissue was approved by the local ethics committee (No. 206/2005). All diagnoses were made by at least two experienced pulmonary pathologists according to the current (2004) WHO classification. Finally, a collection of 168 pleural mesotheliomas was used for tissue microarray (TMA) construction. Before TMA construction, a hematoxylin and eosin (H&E)-stained slide of each block was analyzed to select tumor-containing regions. A TMA machine (AlphaMetrix Biotech) was used to extract tandem 1.0-mm cylindrical core sample from each tissue donor block.

**Chromogenic-Silver in situ Hybridization assay of formalin-fixed, paraffin-embedded tissues for FGFR1 gene copy number**

A rapid fully automated Chromogenic-Silver in situ Hybridization (CS-ISH) dual-color FGFR1 target assay was performed using the Ventana automated platform (Ventana Medical Systems). Four to 5-µm thick sections were cut and mounted on Superfrost Slides, which were then deparaffinized, pretreated with sodium citrate (90°C for 36 minutes, sodium citrate buffer pH 6; Ventana Medical System, Inc.) and digested (8 minutes, ISH-Protease2; Ventana Medical Systems, Inc.). The mixture of the FGFR1 DNP probe (Ventana Medical Systems, Inc.) and the chromosome 8 DIG probe (Ventana Medical Systems, Inc.) was denatured...
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Expression of FGF2–FGFR1 pathway components in mesothelioma cell lines

To validate these gene expression findings, a panel of seven mesothelioma cell lines was used for cell and molecular studies in which five (H28, H226, H290, H513, and H2052) represent the epithelioid histology of MPM and two (H2452, MSTO211H) represent the biphasic variant. In addition, immortalized M5A mesothelial cells were included. Immunoblot analysis of FGFR1-3 was performed using three lung cancer cell lines as positive controls for each of the FGFR proteins (Fig. 1). Whereas FGFR2 expression was limited to H28 cells and no mesothelioma cell line expressed detectable FGFR3, FGFR1 protein was expressed at levels equal to Colo699 and SW1573 lung cancer cells in H226 and MSTO211H cells with lower expression in H2452 and M5A cells. Because FGFR1 gene copy-number gain has been identified as a mechanism for increased FGFR1 expression in lung squamous cell carcinomas and HNSCC (31, 32, 34), the FGFR1 gene amplification status was measured in the mesothelioma cell lines using a FISH assay (see Materials and Methods). The FGFR1 FISH assay result for H226 cells is shown in Supplementary Fig. S1 and demonstrates a normal diploid FGFR1 gene status in these cells (FGFR1:CEP8 ratio = 1). H1703 lung cancer cells are shown as a positive control for focal FGFR1 gene amplification (FGFR1:CEP8 ratio = 3.5. The FGFR1 gene copy-number status was assessed for five additional mesothelioma cell lines and none exhibited FGFR1:CEP8 ratios > 2 (Table 1). To validate the FISH results, the relative FGFR1 gene copy number was assessed in CCLE SNP array datasets and presented in Table 1. Again, no mesothelioma cell line exhibited evidence for increased FGFR1 gene copy number. Thus, the data indicate that increased expression of FGFR1 in H226, MSTO211H, and H2452 cells is not associated with FGFR1 gene amplification.
Measurement of FGF2 mRNA and protein by quantitative real-time PCR (RT-PCR) and ELISA, respectively, revealed generally high expression levels in the mesothelioma cell lines except H513 cells (Table 1), a finding consistent with the CCLE gene expression data in mesothelioma cell lines (Supplementary Table S1). Measurement of FGF9 mRNA by quantitative RT-PCR revealed levels in the mesothelioma cell lines that were less than 1% of FGF9 mRNA levels in H520 cells (data not shown), again consistent with the findings in Supplementary Table S1. Thus, these results indicate coexpression of FGFR1 or FGFR2 with FGF2 in five of eight mesothelioma cell lines tested.

Expression of FGF2–FGFR1 pathway components in primary malignant mesothelioma specimens

We determined the expression of FGFR1 mRNA in a collection of 168 primary mesothelioma specimens with a commercially available ISH assay suitable for formalin-fixed, paraffin-embedded tissues as described in Materials and Methods. The findings in Supplementary Table S2 revealed that 21 (12.5%) of the 168 evaluable tumors exhibited FGFR1 expression of ≥2+. FGFR1 mRNA positivity was distributed among epithelioid (n = 9), biphasic (n = 5), sarcomatoid (n = 3), and mixed/not otherwise specified (n = 4) histologies. Although recent evidence indicates that the FGFR1 gene undergoes amplification or copy-number gain in lung squamous cell carcinomas and HNSCC (31, 32, 34), none of the 168 tumors, regardless of FGFR1 mRNA expression levels, exhibited significant gains or amplification of the FGFR1 gene (Supplementary Table S2).

To validate the findings with the MPM TMA, we interrogated published MPM mRNA expression and gene copy-number data (37) deposited in GEO DataSets (GSE29354 and GSE29902). As shown in Supplementary Table S3, 22 of 53 (42%) primary MPM tumors were positive for FGFR1 mRNA as defined by mRNA expression ≥ mean expression of the 53 tumors. These tumors included both epithelioid and biphasic histologies, but no sarcomatoid tumors. When the overlap of positivity for FGFR1 and FGF2 was considered, eight tumors (15% of total) expressed both FGFR1 and FGF2. As with the malignant mesothelioma TMA (Supplementary Table S2), none of the tumors exhibited evidence of increased FGFR1 gene copy number (Supplementary Table S3). Combined, the primary malignant mesothelioma data indicate frequent expression of FGFR1 mRNA that is not associated with FGFR1 gene amplification or copy-number gain.

Table 1. Ponatinib sensitivity associates with FGF2 and FGFR1 expression in mesothelioma cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Cell Line morphology/histology</th>
<th>Ponatinib IC50 nmol/L</th>
<th>FGFR1 protein</th>
<th>FGFR1:CEP8 ratio (FISH)</th>
<th>FGFR1 GCN</th>
<th>FGFR1 mRNA (qPCR)</th>
<th>FGFR2 protein (pg/µg cell protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H226</td>
<td>Epithelial mesothelioma</td>
<td>6</td>
<td>5.4</td>
<td>1.0</td>
<td>1.1</td>
<td>0.24 ± 0.07</td>
<td>438 ± 15</td>
</tr>
<tr>
<td>MSTO211H</td>
<td>Biphasic mesothelioma</td>
<td>7</td>
<td>4.2</td>
<td>1.7</td>
<td>0.88</td>
<td>0.76 ± 0.22</td>
<td>888 ± 139</td>
</tr>
<tr>
<td>H2452</td>
<td>Biphasic mesothelioma</td>
<td>50</td>
<td>2.0</td>
<td>1.2</td>
<td>1.91</td>
<td>0.12 ± 0.02</td>
<td>2.49 ± 54</td>
</tr>
<tr>
<td>Met5A</td>
<td>Immortalized nonmalignant mesothelial cells</td>
<td>100</td>
<td>1.4</td>
<td>ND</td>
<td>ND</td>
<td>0.27 ± 0.05</td>
<td>191 ± 6</td>
</tr>
<tr>
<td>H290</td>
<td></td>
<td>200</td>
<td>1.3</td>
<td>0.9</td>
<td>ND</td>
<td>0.17 ± 0.06</td>
<td>94 ± 6</td>
</tr>
<tr>
<td>H28</td>
<td>Mesothelioma</td>
<td>500</td>
<td>0.1</td>
<td>ND</td>
<td>0.8</td>
<td>0.26 ± 0.07</td>
<td>135 ± 15</td>
</tr>
<tr>
<td>H513</td>
<td>Epithelial mesothelioma</td>
<td>500</td>
<td>0.1</td>
<td>1.2</td>
<td>0.88</td>
<td>0.00 ± 0.00</td>
<td>0 ± 4</td>
</tr>
<tr>
<td>H2052</td>
<td>Mesothelioma</td>
<td>500</td>
<td>0.4</td>
<td>0.8</td>
<td>0.7</td>
<td>0.97 ± 0.26</td>
<td>690 ± 54</td>
</tr>
<tr>
<td>H1703</td>
<td>NSCLC</td>
<td>—</td>
<td>—</td>
<td>3.5</td>
<td>3.0</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

NOTE: The ponatinib IC50 values were calculated from the data in Fig. 2 and FGFR1 protein levels were determined by immunoblotting and densitometry. The indicated cell lines were submitted to two-color FISH assays for FGFR1 and CEP8 and the ratios are tabulated. The CCLE SNP data were interrogated and the relative FGFR1 gene copy number (GCN) is presented. FGF2 mRNA and protein were measured by quantitative RT-PCR (Materials and Methods) and ELISA (R&D Systems), respectively, with mean and SEM presented. Abbreviation: ND, not determined.

FGFR1 and FGF2 form an autocrine signaling pathway in mesothelioma cell lines

We tested the panel of mesothelioma cell lines for growth sensitivity to the multikinase TKI, ponatinib (38), which exhibits potent activity on FGFR1, 2 and 4 (IC50s, 2, 2, and 8 nmol/L, respectively) and weaker inhibition of FGFR3 (18 nmol/L; ref. 39). The findings in Fig. 2A demonstrate dose-dependent growth inhibition of H226, MSTO211H, and H2452 cells with IC50 values ≤ 50 nmol/L (Table 1). Although H28 cells express high levels of FGFR2 and FGF2, growth was insensitive to ponatinib treatment. Pearson correlation analysis of FGFR1 protein levels and ponatinib IC50 values (Fig. 2B) revealed a statistically significant association (r = −0.780; P = 0.023). In addition to growth inhibition, ponatinib induced a dose-dependent inhibition of basal phospho-ERK in H226, MSTO211H, and H2452 cells, but not H513 cells (Fig. 3). We also tested the effect of ponatinib on the phosphorylation status of FGFR1 by immunoblot analyses using commercially available antibodies to FGFR1-Y653/654 as well as by anti-phosphotyrosine immunoblotting following FGFR1 immune precipitation. In our hands, the
sensitivity of these assays was insufficient to provide clear evidence for decreased FGFR1 tyrosine phosphorylation following ponatinib treatment in these cell lines.

To define the requirement for autocrine FGF in the mesothelioma cell lines, we determined the effect of the ligand trap, FP-1039 (36, 40), on growth of a subset of the cell lines. As shown in Fig. 2C, FP-1039 potently inhibited anchorage-independent growth of H226 and H2452 cells and more modestly the growth of MSTO211H cells. In contrast, growth of ponatinib-insensitive and FGFR1-negative H513 and H2052 cells was not influenced by the ligand trap. Thus, the data support an autocrine activity of FGF2 in mesothelioma cell lines similar to that previously defined in lung cancer and HNSCC cell lines (35, 36, 40, 41).

Besides FGFR1, ponatinib potently inhibits VEGFR1-3, PDGFRA, PDGFRB, and RET (39). However, none of these RTKs are expressed at the mRNA level in H226, MSTO211H, and H2452 cells (Supplementary Table S1), suggesting that FGFR1 is a likely RTK driving growth of these cell lines and sensitivity to ponatinib. To directly test the requirement for FGFR1 in the ponatinib-sensitive cell lines, two independent FGFR1-targeting shRNAs in the pLKO.1 lentivirus vector were packaged and transduced into H226 and MSTO211H cells. Lentiviruses encoding a shRNA targeting GFP were used as a control for shRNA expression. The findings in Fig. 4 show that the FGFR1-specific shRNAs reduced FGFR1 mRNA levels and inhibited clonogenic growth of H226 and MSTO211H cells relative to the GFP control shRNA. Thus, growth of H226 and MSTO211H cells is inhibited by direct RNAi-mediated silencing as well as by an FGFR inhibitor ponatinib.

![Figure 2. Inhibition of in vitro growth of mesothelioma cell lines by the FGFR inhibitor, ponatinib. A, anchorage-independent growth (H2052, MSTO211H, H613, H226, Met5A, and H2452) or cell proliferation assays over 7 days (H28 and H290) were performed in the presence of the indicated concentrations of ponatinib as described in Materials and Methods. B, the IC50 values were calculated with Prism software (GraphPad) and plotted versus FGFR1 protein expression measured by densitometry of immunoblots. C, anchorage-independent growth of the indicated cell lines was measured in the presence of increasing concentrations of the FGF trap, FP-1039, and the data are presented as growth as a percent of control growth measured in the absence of FP-1039.](www.aacrjournals.org Mol Cancer Res; 12(10) October 2014 1465)
As an *in vivo* test of the ability of ponatinib to inhibit growth of FGFR1-dependent mesothelioma cells, we developed an orthotopic model of mesothelioma in which H226 cells transduced with firefly luciferase were implanted into the pleural space of immune-deficient mice. The tumor cells grow aggressively in the orthotopic site with local invasion into the lungs and heart (Supplementary Fig. S2). Following intrapleural injection and 6 weeks for tumor establishment, the mice were treated daily by oral gavage with diluent control or ponatinib at 20 mg/kg. Tumor size was monitored by BLI every 2 weeks. As shown in Fig. 5 (inset), ponatinib induced modest tumor shrinkage in 3 of the 5 mice after 6 weeks of treatment, yielding a statistically significant inhibition of tumor size compared with the control-treated mice. Importantly, growth of the ponatinib-treated tumors increased after 8 weeks of treatment and eventually grew at a rate equal to the control tumors. Thus, ponatinib inhibited the growth of H226 tumors in mice, although the response was transient and likely related to acquired resistance.

**Discussion**

In light of the poor control of malignant mesothelioma by approved cytotoxic chemotherapeutic regimens, RTKs have been actively explored in MPM as alternative therapeutic targets (15, 19, 42, 43). Although EGFR, VEGFRs, MET, and PDGFRs have been a focus in these studies, our findings support the novel role of FGFR1 as a growth driver in a subset of MPM cell lines and suggest that FGFR-active TKIs may serve as therapeutics in this cancer. RNAi-mediated silencing of FGFR1 inhibited clonogenic growth of two FGFR1-expressing MPM cell lines, H226 and MSTO211H (Fig. 4), and the TKI, ponatinib, selectively inhibited growth of MPM cell lines expressing FGFR1 (Fig. 2). We previously demonstrated with RNAi-mediated silencing the requirement of FGF2 for H226 cell growth (35), and we validate this finding by showing sensitivity of FGFR1-dependent mesothelioma cell lines to the ligand trap, FP-1039. Combined, the data support the activity of an FGF-FGFR1 autocrine pathway in a subset of mesothelioma cell lines similar to that described in NSCLC and HNSCC (35, 36, 41).

FGFR1 is under extensive evaluation as a therapeutic target in NSCLC, especially of the squamous cell histology,
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The data in Fig. 5 suggest rapid acquisition of ponatinib resistance in H226 cells propagated as orthotopic tumors. Chronic exposure of H226 cells to ponatinib cultured in vitro also results in outgrowth of resistant tumor cells, although no evidence for classic gatekeeper mutations or other secondary somatic mutations in FGFR1 was observed (data not shown). Thus, it is likely that FGFR TKIs will not provide cures when delivered as monotherapies to FGFR1-dependent MPM. The clinical experience with gefitinib and erlotinib in EGFR-mutant lung cancer patients and crizotinib in ALK-rearranged lung cancer patients provides precedent for the limitations associated with TKI monotherapy due to acquired resistance (44–46). In fact, as reviewed by Glickman and Sawyers (45), durable control of solid tumors will likely require combinations of targeted agents similar to the present strategy in which combinations of antiviral agents provide long-term control of HIV infections by preventing the outgrowth of drug-resistant virions. In addition to prevention of acquired resistance, combinations of TKIs may disrupt the function of RTK coactivation networks that are generally active in cancer cells (47) and MPM specifically (15). We are presently deploying functional genomics approaches to identify in an unbiased fashion additional signal pathways whose inhibition in combination with FGFR1 will achieve synergistic MPM growth inhibition. Thus, the goal is to build upon transient clinical efficacy with FGFR TKI monotherapy with eventual development of inhibitor cocktails to control malignant pleural mesothelioma.

Disclosure of Potential Conflicts of Interest
J.M. Gozgit has ownership interest (including patents) in ARIAD Pharmaceuticals. L.E. Heasley reports receiving a commercial research grant from ARIAD Pharmaceuticals. No potential conflicts of interest were disclosed by the other authors.

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References

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