Outgrowth of Drug-Resistant Carcinomas Expressing Markers of Tumor Aggression after Long-term TβRI/II Kinase Inhibition with LY2109761

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Abstract
TGF-β is produced excessively by many solid tumors and can drive malignant progression through multiple effects on the tumor cell and microenvironment. TGF-β signaling pathway inhibitors have shown efficacy in preclinical models of metastatic cancer. Here, we investigated the effect of systemic LY2109761, a TGF-β type I/II receptor (TβRI/TβRII) kinase inhibitor, in both a tumor allograft model and the mouse skin model of de novo chemically induced carcinogenesis in vivo. Systemic LY2109761 administration disrupted tumor vascular architecture and reduced myofibroblast differentiation of E4 skin carcinoma cells in a tumor allograft. In the 7,12-dimethyl-benzanthracene plus phorbol myristate acetate–induced skin chemical carcinogenesis model, acute dosing of established naive primary carcinomas with LY2109761 (100 mg/kg) every 8 hours for 10 days (100 mg/kg) diminished phospho-Smad2 (P-Smad2) levels and marginally decreased the expression of inflammatory and invasive markers. Sustained exposure to LY2109761 (100 mg/kg/d) throughout the tumor outgrowth phase had no effect on carcinoma latency or incidence. However, molecular analysis of resultant carcinomas by microarray gene expression, Western blotting, and immunohistochemistry suggests that long-term LY2109761 exposure leads to the outgrowth of carcinomas with elevated P-Smad2 levels that do not respond to drug. This is the first description of acquired resistance to a small-molecule inhibitor of the TβRI/TβRII kinase. Resultant carcinomas were more aggressive and inflammatory in nature, with delocalized E-cadherin and elevated expression of IL23A, laminin V, and matrix metalloproteinases. Therefore, TGF-β inhibitors might be clinically useful for applications requiring acute administration, but long-term patient exposure to such drugs should be undertaken with caution. Cancer Res; 71(6); 2339–49. ©2011 AACR.

Introduction
TGF-β is a potent growth inhibitor of normal epithelial cells but is produced in excessive quantities by many advanced tumor types (1). This excess TGF-β is thought to drive malignant progression through multiple effects both on the tumor cell and on the tumor microenvironment (1–3). In accordance with this hypothesis, local or systemically elevated levels of TGF-β signaling pathway inhibitors have become the focus for the design of specific inhibitors for cancer therapy (4, 5). TGF-β1 signals via a heteromeric complex of type I and type II TGF-β receptors (TβRI/TβRII) that activates the canonical Smad pathway by TβRI-mediated phosphorylation of Smad2 and Smad3. Nuclear shuttling of the resultant hexameric complex, composed of receptor-associated phospho-Smads (P-Smad) bound to Smad4, culminates in TGF-β–driven transcrip-
from an epithelial toward a fibroblastoid tumor cell phenotype (11). In the chemically induced mouse skin model of tumorogenesis, resultant fibroblastoid spindle cell tumors are the most aggressive cutaneous lesions, although distant metastases are frequently squamous in character.

The goal of TGF-β inhibition has been to target its tumor-promoting properties, both cell autonomous and microenvironmental, while avoiding inhibition of its tumor suppression arm (5). There have been several preclinical reports on the use of both large- and small-molecule inhibitors of the TGF-β signaling pathway for various oncology applications. These inhibitors have been particularly efficacious in metastatic carcinoma models (12–15), utilizing multiple mechanisms to elicit effects. Clinical trials have commenced, which, like their preclinical counterparts, show promise for the treatment of metastatic melanoma, renal cell carcinoma, and glioblastoma (16, 17).

In mice, negative consequences of long-term exposure to a large-molecule inhibitor of TGF-β, Fc:TβRII, were few (12), yet this drug was efficacious in reducing metastasis of injected melanoma cells (13). More important, this soluble receptor antagonist inhibited spontaneous metastasis of primary mammary tumors that arise in MMTV-Neu transgenics (12). Taken together with preliminary reports on clinical trials with an anti–TGF-β antibody (17), these data suggest that anti–TGF-β drugs are well tolerated in mice and humans.

This study explores the therapeutic potential of LY2109761, a small-molecule inhibitor of TβRI/TβRII receptor kinase that targets activation of P-Smad2 and P-Smad3 and inhibits the related kinase receptors Acvr1b (activin activated) and Acvr1c (nodal activated). We show in a mouse skin model of chemokine receptor Acvr1b (activin activated) and Acvr1c that LY2109761 is well tolerated in mice and humans. LY2109761 is a small-molecule inhibitor of TβRI/TβRII kinase and was efficacious in reducing metastasis of injected melanoma cells (13). More important, this soluble receptor antagonist inhibited spontaneous metastasis of primary mammary tumors that arise in MMTV-Neu transgenics (12). Taken together with preliminary reports on clinical trials with an anti–TGF-β antibody (17), these data suggest that anti–TGF-β drugs are well tolerated in mice and humans.

This study explores the therapeutic potential of LY2109761, a small-molecule inhibitor of TβRI/TβRII receptor kinase that targets activation of P-Smad2 and P-Smad3 and inhibits the related kinase receptors Acvr1b (activin activated) and Acvr1c (nodal activated). We show in a mouse skin model of chemically induced carcinogenesis that despite the favorable outcomes of LY2109761 in inhibiting EMT in vitro and its short-term effects on carcinomas in vivo, sustained pharmacologic inhibition of TGF-β signaling may have a protumor effect with expansion of a more aggressive inflammatory drug-resistant carcinoma phenotype expressing markers of invasion.

Materials and Methods

Cell lines

Murine cell lines used in this study were isolated from chemically induced cutaneous carcinomas and have been very well characterized with respect to genetic mutations and gene expression profiles. These were provided by Allan Balmain at the University of California San Francisco (UCSF). E4 and H11 cells were both single-cell clones from SN161, derived from a lymph node metastasis of an F1.I.129 × NIH mouse (18). D3 cells were isolated from a primary squamous cell carcinoma (SCC) from an F1 M. musculus NIH × M. spreus animal (19). Where indicated, cells were treated with 5 ng/mL rhTGF-β1 (R&D Systems) and/or the TβRI/TβRII kinase inhibitor LY2109761 (20). Media containing TGF-β, LY2109761, and/or dimethyl sulfoxide vehicle were refreshed every other day.

Animals

All animal work was done in accordance with a UCSF Institutional Animal Care and Use Committee protocol. Nude mouse tumor allografts were generated by subcutaneous injection of E4 cells (1 × 10⁷ cells/mL; 100 ul per site/mouse). Tumors were harvested 10 days later.

Pharmacokinetic analysis

Plasma samples were harvested from blood collected at 0.5, 2, 4, and 8 hours after oral administration of LY2109761 (100 mg/kg of body weight). Plasma LY2109761 levels were determined by high-performance liquid chromatography (HPLC) at the UCSF Drug Study Unit, Analytical Division.

Chemical carcinogenesis

Mice (8-week-old female inbred NIH/OlaHsd) underwent standard dimethylbenzanthracene (DMBA)/phorbol myristate acetate (PMA) tumor induction as previously described (11, 21). Tumors were initiated with DMBA, 25 µg/200 µL acetone. The tumor promoter was PMA, 2 µg/200 µL acetone. The experiment was terminated once carcinoma reached 1.5 cm, became ulcerated, or if the mouse showed signs of morbidity. Carcinomas were diagnosed clinically, which was confirmed by histologic analysis. LY2109761 (7.5 mg/mL) or vehicle alone was administered by oral gavage in a polytronized suspension in CMC/SLS/PVP/antifoam. Two dosing regimens were used: the sustained dosing regimen was a daily single 100 mg/kg dose of LY2109761 every day, 7 days per week, from week 6 post–DMBA administration throughout tumor outgrowth, as specified. The short-term dosing regimen was administered to animals that had already established DMBA/PMA-induced carcinoma, that is, 8 to 9-month-old female mice roughly 25 weeks after DMBA treatment. This regimen was 100 mg/kg LY2109761 given every 8 hours for 10 days.

Immunohistochemistry

Immunohistochemistry (IHC) was carried out on 5-µm 4% PFA-fixed, paraffin-embedded tissue sections. Antibodies included anti–α-smooth muscle actin (αSMA; Sigma), CD45 (Caltag Laboratories), E-cadherin (BD Biosciences), F4/80 (Caltag Laboratories), P-Smad2 (Cell Signaling), P-Smad2/3 (Santa Cruz), and matrix metalloproteinase 13 (MMP13; Santa Cruz), and vimentin (Cell Signaling). CD31 staining was carried out using a Biocare Medical kit. TUNEL staining was carried out using the DeadEnd Colorimetric TUNEL system (Promega). Quantification employed 10 fields of view per sample. NIH ImageJ was used to determine pixel density (P-Smad staining). Tumor stroma area (vimentin staining), and TUNEL (terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling)-positive cell counts.

RNA isolation, labeling, and microarray hybridization

Total RNA was extracted from snap-frozen carcinomas by TRizol (Invitrogen), using Ambion’s DNA-free kit, and RNAs were purified using RNeasy (Qiagen). Quantified RNAs were amplified using the Illumina TotalPrep RNA amplification kit (Ambion). The effects of the sustained drug dosing (LY2109761, n = 3; vehicle, n = 3) and short-term drug dosing (LY2109761, n = 4; vehicle, n = 5) regimens on gene expression were analyzed using the Illumina Mouse-WG-6 v2 platform. Slides were scanned on an Illumina Beadstation, and data...
were extracted using Illumina BeadStudio software and normalized by quantile normalization. Of 46,644 probes on the microarray, 32% (14,818) had a present P value of 0.05 or less in at least 80% of samples as assigned by BeadStudio, and these probes were used for further analysis. Gene expression in both treatment conditions was compared with that of matched vehicle-treated control mice, using the Significance Analysis of Microarrays algorithm (22), with a false discovery rate (FDR) cutoff of 10%.

Western blotting
Protein extraction and Western blotting was carried out as described previously (21). Antibodies included anti-P-Smad2 (Eli Lilly or Cell Signaling), total Smad2/3 (BD Biosciences), E-cadherin (BD Biosciences), RBPlk (Santa Cruz), Lgr6 (Santa Cruz), glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Cell Signaling), or β-actin (Sigma-Aldrich).

Results
LY2109761 inhibits and reverses TGF-β1–induced EMT in vitro
Murine carcinoma cell lines, isolated from different stages of chemically induced mouse skin tumorigenesis, were evaluated for their response to the LY2109761 inhibitor. Squamous E4 cells, isolated from a lymph node metastasis of a squamous cell carcinoma, convert to a fibroblastoid spindle phenotype on addition of TGF-β in vitro or, if grown, as a subcutaneous tumor in vivo. This EMT is driven cell-autonomously by TGF-β signaling (23). A dose of 2 μmol/L LY2109761 was sufficient to prevent Smad2 phosphorylation of both squamous and spindle E4 cells (Supplementary Fig. S1A and B). Prolonged LY2109761 exposure elevated E-cadherin protein levels higher than in the absence of exogenous TGF-β (Supplementary Fig. S1A) and prevented and reversed (Supplementary Fig. S1B and C) TGF-β1–induced EMT.

The D3 and H11 carcinoma lines are both innately spindle, independently of exogenous TGF-β (18, 19), and both have higher basal TGF-β signaling levels than E4 cells (ref. 24; Supplementary Fig. S1D and E). Short- or long-term treatment with LY2109761 reduced innate P-Smad2 levels in both cell lines (Supplementary Fig. S1D and E), supporting the view that in these cell lines, TβRI, Acvrlb, and/or Acvrlc actively signal to P-Smad2 via a positive autocrine loop (24). In addition, the D3 cells showed an LY2109761 dose-dependent increase in E-cadherin protein expression (Supplementary Fig. S1D), illustrating the partial contribution of Smad2 signaling to the maintenance of mesenchymal properties and the ability of LY2109761 to steer the D3 cells toward a more epithelial phenotype.

Pharmacokinetics and pharmacodynamics of LY2109761 in vivo
To determine LY2109761 effects on TGF-β signaling in vivo, 3 doses of the drug were administered to mice at 50, 75, or 100 mg/kg body weight and tissue P-Smad2 levels were assessed 2 hours postoral gavage. In both lung and skin, P-Smad2 levels were suppressed dose dependently, with partial inhibition at 50 and 75 mg/kg and almost complete inhibition at 100 mg/kg (Fig. 1A). LY2109761 pharmacokinetics were determined by HPLC analysis of murine plasma after a single oral dose of the drug (100 mg/kg). Mouse strains analyzed were 129, 129Sv/SvHsd; C57BL6; C57BL6Ntac; NIH, NIH/OlaHsd; 129/NH, F1 between 129Sv/SvHsd and NIH/OlaHsd; Nude, NCrNu/juTac. C, inhibition of P-Smad2 levels by LY2109761 was determined by Western blot analysis of lung tissue from mice after a single oral dose of 100 mg/kg LY2109761. D, tumor-bearing mice that had been subjected to thrice daily oral dosing with LY2109761 at 100 mg/mL for 10 days (short term) were administered an additional oral bolus of 100 mg/kg LY2109761 and protein lysates from papilloma, carcinoma, and lung were isolated 2 hours later. Western blot analysis was carried out to detect P-Smad2, total Smad2, and β-actin levels.
LY2109761 treatment of E4 tumor allografts in vivo reduces carcinoma myofibroblasts and disrupts vascular integrity

Subcutaneously injected E4 cells grow as aggressive, highly vascularized spindle tumors. LY2109761 administered in drinking water (2 mg/mL) ad libitum for 10 days significantly reduced nuclear P-Smad2 expression (Fig. 2A and B) but had no effect on tumor outgrowth (data not shown), apoptosis, or proliferation.

Figure 2. LY2109761 reduces P-Smad2 expression, myofibroblast phenotype, and vascular integrity without effects on proliferation or apoptosis in E4 carcinoma allografts in vivo. Fourteen nude mice were inoculated bilaterally and subcutaneously with E4 carcinoma cells. For 10 days from the time of inoculation, 2 mg/mL LY2109761 was provided ad libitum in drinking water to the experimental group (n = 7) whereas the control group was fed normal water. A, tumor sections from vehicle- and drug-treated mice were stained for P-Smad2. LY2109761 treatment led to a decrease in nuclear P-Smad2 staining. Nuclei were scored as strong, weak, or negative for nuclear P-Smad2. Five sections each of 5 independent allografts were quantified from both the LY2109761 and vehicle-treated mice. The decrease in P-Smad2 nuclear staining was significant (P < 0.001 by 2-way ANOVA). B, ImageJ was used to determine the average pixel intensity of the nuclear P-Smad2 staining. Nuclei were scored as strong, weak, or negative for nuclear P-Smad2. Five sections each of 5 independent allografts were quantified from both the LY2109761 and vehicle-treated mice. The decrease in P-Smad2 nuclear staining was significant (P < 0.001 by 2-way ANOVA). C, tumor sections were stained by TUNEL, and apoptosis was estimated as the average of TUNEL-positive cells per representative field (5 allografts/treatment). D, mice were injected with bromodeoxyuridine (BrdU) 2 hours before harvest of the tumor. LY2109761- and vehicle-treated mice were examined for BrdU incorporation by anti-BrdU staining (5 allografts/treatment). Proliferation was estimated as the average of the BrdU-positive cells per representative field. There was no significant difference between the drug and vehicle treatments in the percentage of either apoptotic or proliferating cells. E and F, tumor sections from vehicle- and drug-treated mice, as indicated, were stained for (E) CD31/PECAM-1 or (F) αSMA. Vehicle-treated tumors possessed a fine reticular capillary network of pericyte-invested, PECAM-1+ vessels, whereas the tumor vasculature of LY2109761-treated mice was poorly invested with αSMA+ cells. Note that αSMA staining of vascular smooth muscle cells was more intense than that of the myofibroblasts and that vessels within uninvolved skin were unaffected by LY2109761 (E, top). Vessels within drug-treated tumor stained weakly for PECAM-1, were dilated, and filled with pooling red blood cells, with hemorrhaging into tumor tissue. Scale bar, 50 μm.
proliferation (Fig. 2C and D). However, LY2109761 treatment did alter tumor histology. Whereas control tumors showed a fine reticular network of CD31+ vessels invested with pericytes and features of active angiogenesis such as noninvested CD31+ cells (Fig. 2E), LY2109761-treated tumors displayed disrupted vascular architecture. Vessels present in drug-treated tumors were dilated, with weak or no CD31+ staining, and pooling of red blood cells suggestive of indolent blood flow and hemorrhage (Fig. 2E).

It has been reported that increased carcinoma levels of αSMA in tumors may be predictive of an aggressive phenotype and the most invasive tumor types take on a myofibroblast phenotype (24, 25). E4 spindle tumor cells from vehicle-treated mice showed high levels of αSMA staining, and treatment with LY2109761 decreased this expression (Fig. 2F). However, unlike genetic inhibition of TGF-β signaling in this model (23), there was not a complete reversion from spindle to squamous morphology.

Sustained suppression of TGF-β signaling by LY2109761 increases chemically induced papilloma incidence

To determine the long-term effects of LY2109761 treatment on DMBA/PMA-induced tumorigenesis, we dosed mice once daily with LY2109761 during the papilloma and carcinoma outgrowth phases of skin carcinogenesis. Three experiments were conducted (Fig. 3A and Supplementary Fig. S2). A pilot
experiment (75 mg/kg dosing) showed a significant drug-induced increase in papilloma numbers (Supplementary Fig. S2A), but a larger replication study failed to show a difference (Supplementary Fig. S2B). In a third experiment, the drug dose was elevated to 100 mg/kg, which significantly increased papilloma numbers in drug-treated mice compared with vehicle-treated mice (Fig. 3A). These findings are concordant with our previous studies, showing that elevation of TGF-β1 expression via transgenesis reduced benign papilloma number, but genetic reduction of TGF-β1 levels led to increased papilloma numbers (11, 21).

The LY2109761 increase in papilloma number was not due to a decrease in apoptosis (data not shown). Histologic examination of papillomas revealed that compared with vehicle-treated mice, sustained LY2109761 treatment increased the number of inflammatory (CD45+) cells within the papilloma stroma (Supplementary Fig. S3), suggesting that inflammation might contribute to increased papilloma incidence (26). By morphology, most of the infiltrate seemed to be neutrophilic, with a minority of macrophages. Interestingly, there was no significant change in carcinoma number, malignant conversion frequency, or carcinoma latency in the LY2109761-treated mice compared with vehicle-treated mice (Supplementary Table S1).

**Failure to downregulate P-Smad2 levels in tumors after sustained LY2109761 treatment**

P-Smad2 Western blot analysis of carcinoma cell lysates (Fig. 3B) and P-Smad2 and P-Smad2/3 IHC analysis of tumors (Fig. 3C) were undertaken on carcinoma that developed following sustained LY2109761 treatment and that were collected 2 hours following the last drug dose. Surprisingly, this analysis revealed that P-Smad2 was not significantly downregulated by LY2109761 (Fig. 3C and Supplementary Fig. S4A) and, in several carcinomas, P-Smad2 was in fact upregulated in drug-treated mice compared with vehicle-treated mice (Fig. 3B and C and Supplementary Fig. S4C). In contrast, nonmalignant fibroblast cells of the carcinoma stroma showed decreased P-Smad2 staining in response to LY2109761 treatment even after sustained drug exposure (Fig. 3C and Supplementary Fig. S4B).

We hypothesized that drug-refractile P-Smad2 signaling in the tumor parenchyma was a malignant adaptation to sustained TGF-β signaling inhibition, either by outgrowth of a drug-resistant carcinoma population or by activation of P-Smad2 by alternative intersecting pathways (27). To address this issue, we investigated the effect of a shorter but more frequent drug-dosing regimen (short term) on established DMBA/PMA-induced primary carcinoma. Mice bearing full-blown DMBA/PMA-induced carcinoma 25 weeks postinitiation were orally dosed with LY2109761 (100 mg/kg) every 8 hours for 10 days (n = 7 drug treated; n = 7 vehicle treated). This treatment had no overt effect on carcinoma morphology, but IHC showed variable degrees of P-Smad2 downregulation (Supplementary Fig. S4A). We also evaluated lung tissue from these carcinoma-bearing mice to determine the response of nonmalignant tissue to LY2109761 treatment. Indeed, Western blot analysis (not shown) and IHC of lung tissue from mice on both the short-term or sustained treatment regimens showed reduced nuclear P-Smad2 levels in response to LY2109761 treatment (Fig. 3D). Our findings of high levels of drug-insensitive nuclear P-Smad2 after sustained LY2109761 treatment were validated by IHC analysis of P-Smad2 levels in carcinoma from an independent study, in which mice received LY2109761 on a sustained dosing regimen from week 6 until week 17 post–DMBA administration (Sustained Exp 2; Fig. 3C and Supplementary Fig. S4C). Together, these data suggest that acquired drug resistance was limited to malignant tissue and occurred predominantly after sustained drug dosing.

We investigated whether the high levels of P-Smad2 seen after sustained treatment might be due to a rebound effect on TGF-β signaling leading to hyperactivation of P-Smad2 between drug doses. Mice were treated once daily for 7 or more days with the LY2109761 inhibitor (100 mg/kg), and lung tissue was collected at various times after the last drug dose. Western blot analysis showed that P-Smad2 levels were suppressed at 2 hours, returning to near baseline by 16 hours posttreatment, but even after 24 hours, when the next drug dose would normally be administered, there was no increase in P-Smad2 levels above those observed in the control arm (Supplementary Fig. S5). Increased P-Smad2 levels in carcinomas following sustained LY2109761 treatment were therefore unlikely due to a rebound effect.

To investigate the stage of onset of acquired LY2109761 resistance, benign papillomas harvested from the same carcinoma-bearing mice described earlier were assayed for nuclear P-Smad2 levels. There was a significant decrease in nuclear P-Smad2 levels in papillomas from the short-term dosing regimen compared with matched vehicle-treated controls, but no significant P-Smad2 response after sustained LY2109761 treatment compared with matched vehicle-treated papillomas (Supplementary Fig. S6). Therefore, drug resistance was observed even at the papilloma stage, albeit at experiment termination following many weeks of LY2109761 treatment. Papillomas at earlier stages were not collected.

**Sustained suppression of TGF-β signaling induces a proinvasive gene signature**

Because sustained LY2109761 administration seemed to paradoxically increase carcinoma P-Smad2 levels, we hypothesized that the resultant carcinomas may be more aggressive despite the fact that there was no drug-induced change in spindle to squamous character (Supplementary Table S2). To probe the molecular makeup of the carcinomas further, microarray gene expression analysis was conducted on carcinomas from both drug-dosing regimens and their matched vehicle controls. After statistical correction for genome-wide testing, there were no significant changes (≥1.5-fold) in gene expression between the 2 vehicle-treated arms of the 2 experiments or between matched drug- and vehicle-treated carcinoma on the sustained treatment regimen (FDR ≤ 10%). The short-term dosing regimen resulted in a significant reduction in expression of only one gene: short stature homeobox 2.
We speculated that using the stringent statistical analysis employed, inherent heterogeneity between different carcinomas masked any drug-induced changes in gene expression. In contrast, direct comparison between drug-treated carcinomas from both drug-dosing regimens revealed more than 800 significant differences in gene expression (FDR ≤ 10%) between the 2 drug regimens (FDR ≤ 10%), of which approximately 300 were 1.5-fold or more (Fig. 4 and Supplementary Table S3). These data indicate that subthreshold changes in gene expression had occurred in response to drug treatment under both regimens but that directionality of these changes was appositional such that they were only statistically detectable by direct comparison between the 2 drug arms (Fig. 4 and Supplementary Tables S3 and S4).

Gene ontology analysis indicated enrichment for the expression of genes encoding key extracellular matrix proteins and receptors, cytoskeletal proteins, and chemokines in the drug-treated carcinomas of the sustained versus short-term dosing regimens. All 3 components of laminin 332 (Lama3, Lamb3, and Lamc2) were elevated approximately 2-fold by sustained drug treatment. These same genes were downregulated approximately 30% by the short-term dosing regimen (Supplementary Table S3). Moreover, the Igfb1 gene, which encodes a component of a cognate laminin 322 receptor, exhibited increased expression following sustained LY2109761 treatment compared with the short-term dosing regimen (Supplementary Table S3). Both laminin 332 and Igfb1 have been shown to be centrally involved in SCC tumor invasion (29).

Increases in MMP expression and/or activity have also been linked to tumor cell invasion, and sustained LY2109761 treatment led to a 2.5- to 4-fold increase in Mmp3, Mmp10, Mmp9, and Mmp13 compared with the short-term dosing regimen (Supplementary Table S3). In the case of MMP13, this observation was validated by IHC (Fig. 5A). Many other genes involved in extracellular matrix remodeling showed similar increases in expression after sustained drug treatment but slight decreases after the short-term dosing regimen, including latent TGF-β binding protein 2 (Ltbp2), Bmp1, and Pcolce2 (Supplementary Table S3). In addition, markers of EMT, including Msx1 and Twisted2, were elevated by sustained drug treatment but downregulated by the short-term dosing regimen. Intriguingly, Lgr6, reportedly a multipotential...
keratinocyte stem cell marker (30), and Fermd4A, also reported to mark keratinocyte stem cells (31), were both found to be elevated in carcinoma after sustained drug treatment (Fig. 4C, Supplementary Table S4). Rbpjk, a component of the notch signaling pathway that is important in stem cell fate, angiogenesis, and EMT (32, 33), was also elevated in carcinomas after sustained drug treatment. These findings from microarray analysis were validated by Western blot estimation of protein levels for Lgr6 and Rbpjk (Fig. 5B).

Finally, inflammatory gene signatures showed divergent trends in gene expression between the 2 drug regimens. Il23a, which is known to promote inflammatory tumor progression in the DMBA/PMA model (26), was increased in carcinomas after sustained drug exposure. Several genes involved in leukocyte cell adhesion and cytoskeletal reorganization (Jam2, Icam, Nfat5, and Msn) showed the same reciprocal gene expression pattern between the 2 drug-dosing regimens. The potent macrophage chemotactants Ccl3 (MIP1-α) and Ccl4 (MIP1-β) were also elevated after sustained drug treatment but suppressed following short-term dosing. Similar observations were made for other inflammatory markers, including the tumor cell marker CD274 (increased 3.6-fold in the sustained drug treatment group) which is involved in suppression of antitumor CD8+ T cell. In conclusion, sustained drug treatment induced a gene expression signature indicative of a more aggressive and invasive carcinoma type with elevated inflammatory markers compared to vehicle-treated carcinoma, or to the short-term drug dosing regimen.

**Sustained LY2109761 exposure results in expansion of tumor stroma and delocalized E-cadherin within carcinoma cells in vivo**

Although histologic examination indicated no difference in spindle carcinoma incidence after sustained drug treatment (Supplementary Table S2), we further investigated the possibility of paradoxical induction of EMT by LY2109761 by using IHC for E-cadherin (epithelial) and vimentin (mesenchymal). There was consistent delocalization of E-cadherin in carcinoma cells after sustained LY2109761 treatment compared with the characteristic cell–cell junctional localization of E-cadherin seen in carcinomas either from vehicle-treated mice or after the LY2109761 short-term dosing regimen (Fig. 6A and Supplementary Table S5). There was no carcinoma cell vimentin expression in any of the treatments; however, vimentin staining revealed a significant expansion of the tumor stroma compartment after sustained LY2109761 treatment compared with vehicle-treated carcinoma or to drug-dosed carcinoma on the short-term dosing regimen (Fig. 6B and C). These data, showing delocalization of E-cadherin in carcinoma and an expanded vimentin-positive tumor stromal compartment, were replicated in an independent DMBA/PMA study in which mice underwent 11 weeks of sustained drug dosing during the tumor outgrowth phase (Fig. 6 and Supplementary Table S5).

**Discussion**

Here, we reveal that sustained suppression of TGF-β signaling in vivo results in the outgrowth of carcinomas that are apparently resistant to the small-molecule TβRI/TβRII inhibitor LY2109761. We show marked differences in the outcome of LY2109761 treatment of carcinoma cells in vitro, tumor allografts in vivo, and primary carcinomas in situ. Furthermore, the data illustrate that in the same in vivo model of carcinogen-induced primary tumor outgrowth and progression, the outcome of LY2109761 treatment can be either antitumorigenic or protumorigenic, depending on the precise drug-dosing regimen.

Short-term, high-dose LY2109716 treatment could not completely reverse TGF-β–induced carcinoma EMT in either the E4 allograft or primary chemically induced carcinoma models. These data were in contrast with our previous finding using genetic inhibition of autocrine TGF-β signaling in E4 cells in vitro (23). Nevertheless, short-term LY2109716 treatment did reduce the levels of αSMA and Shox2 in the allograft and DMBA/PMA models, respectively, which suggests a trend toward a less myofibroblast phenotype in mice treated continuously with drug for 10 days. Failure to definitively replicate inhibition of EMT after blockade of TGF-β signaling inhibition (23) could be due to additional effects of LY2109761 on the tumor stroma that potentiates tumorigenesis (34, 35) and/or inadequate drug delivery to the tumor parenchyma, especially in the light of vascular disruption in response to LY2109761 in the tumor allograft model.

In concordance with predictions from earlier studies using genetically manipulated TGF-β1 or TβRII (11, 21, 36), we show that long-term daily LY2109761 treatment during tumor outgrowth increased papilloma number, supporting a suppressive
effect of TGF-β in early tumorigenesis. Analysis of the papillomas suggests that these tumor-suppressive effects may be, at least in part, due to the immunosuppression of TGF-β signaling. Inflammation has been suggested to act either protectively via immune surveillance or via a protumorigenic manner, depending on the nature of the inflammatory cell infiltrate (37). In the mouse skin model of chemical carcinogenesis, inflammation has clearly been shown to be protumorigenic (26, 38). In agreement with these observations, we have shown that sustained LY2109761 treatment resulted in a significant increase in CD45+ neutrophilic infiltrate within the papilloma stroma.

Importantly, we have shown that sustained treatment with LY2109761 during the tumor outgrowth phase resulted in resistance to drug-induced P-Smad2 downregulation, specifically in malignant tumor cells but not in tumor stromal fibroblasts or normal tissue. The development of acquired drug resistance to both conventional chemotherapeutics and targeted therapies is a common undesirable outcome in malignant disease. The mechanisms of acquired drug resistance are varied and, for targeted small-molecule therapies, might be somewhat complex (39, 40). However, understanding of these mechanisms of drug resistance will allow modification of therapeutic strategies that lead to more efficacious treatments. Mechanisms of acquired drug resistance include amplification of the target gene, as seen in acquired resistance to Met tyrosine kinase inhibitors (41), and activation of alternative signaling pathways (39, 42) and perturbations in the intermolecular cross-talk between interacting ligands, tyrosine kinases, or their kinase-inactive partners (39, 40, 43).

There are several possible explanations for our unexpected findings of outgrowth of tumor cells with high levels of LY2109761-resistant P-Smad2. The simplest would be inadequate drug delivery, possibly as a consequence of vascular disruption. However, this is unlikely, bearing in mind that LY2109761 can downmodulate P-Smad2 in the tumor stroma even after sustained drug treatment. Moreover, the sustained dosing regimen resulted in a molecular carcinoma phenotype distinct from that of either vehicle-treated or short-term drug-treated mice. Mutation of the ATP/drug-binding site of TβRI, Acvr1b, or Acvr1c may confer drug resistance (e.g., ref. 44), as would mutational hyperactivation of the kinase receptor (45). Genetic alterations that influence P-Smad2 levels downstream or parallel to TβRI (27) may also provide mechanisms to bypass LY2109761 effects. TGF-β signaling is known to be finely regulated by both negative and positive feedback.
mechanisms, and, in tumor cells, signaling may be regulated by trans receptor interactions that might be perturbed in the presence of LY2109761. This drug is known to inhibit type I receptors that signal for activins, GDF3, nodal, and myostatin. It is therefore conceivable that perturbation of one or more of these signaling pathways within the tumor cell or tumor microenvironment might lead to expansion of a more aggressive tumor type. In this study, it was noted that the LY2109761 target TβRI was upregulated more than 1.5-fold in the sustained LY2109761-treated carcinomas. This might be due to disruption of a negative feedback loop on TβRI levels and/or indirectly caused by expansion of a cellular compartment, characterized by high TβRI expression (46, 47).

Importantly, not only was there resistance to LY2109761-mediated attenuation of carcinoma P-Smad2 levels but also these carcinomas took on a more aggressive molecular profile, with upregulation of markers of EMT and inflammation, illustrated by delocalization of cell surface E-cadherin and expansion of a vimentin-positive tumor stroma, and elevated expression of proinflammatory markers. In contrast, carcinomas treated on the short-term LY2109761 dosing regimen showed the inverse trend, with downregulation of markers of EMT such as Mx1 and Shox2. It would seem that many of the protumorigenic effects of LY2109761 are likely driven by the action of this drug on the tumor stroma, leading to immune cell infiltration, stromal cell expansion, and subsequent feedback via growth factors such as hepatocyte growth factor, which drive the outgrowth of LY2109761-resistant carcinomas, as has been seen in genetic models of TGF-β signaling inhibition (34, 35). There is an increasing appreciation of the importance of the interaction between the carcinoma cell and its substratum in driving tumor progression, particularly with respect to matrix density and stiffness (48, 49). The expansion of the tumor stroma and the prevalence of ECM components, junctional adhesion molecules, and integrins in genes upregulated after sustained exposure to LY2109761 would all support the concept of a stroma-driven enhancement in aggressive molecular phenotype.

In conclusion, this study shows that although TGF-β inhibitors might be clinically useful for short-term patient exposure (50), long-term treatment with TGF-β inhibitors should be administered with caution. Dosing regimen is clearly critical, and recommended dosage might vary considerably on the basis of tumor type and TGF-β signaling status. Further investigation of mechanism of acquired drug resistance might provide more efficacious routes to therapy.

Disclosure of Potential Conflicts of Interest

J.M. Yingling, employee, Eli Lilly and Company. The other authors disclosed no potential conflicts of interest.

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