Molecular Imaging of TGFβ-Induced Smad2/3 Phosphorylation Reveals a Role for Receptor Tyrosine Kinases in Modulating TGFβ Signaling

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Abstract

Purpose: The dual modality of TGFβ, both as a potent tumor suppressor and a stimulator of tumor progression, invasion, and metastasis, make it a critical target for therapeutic intervention in human cancers. The ability to carry out real-time, noninvasive imaging of TGFβ-activated Smad signaling in live cells and animal models would significantly improve our understanding of the regulation of this unique signaling cascade. To advance these efforts, we developed a highly sensitive molecular imaging tool that repetitively, noninvasively, and dynamically reports on TGFBR1 kinase activity.

Experimental Design: The bioluminescent TGFBR1 reporter construct was developed using a split firefly luciferase gene containing a functional sensor of Smad2 phosphorylation, wherein inhibition of TGFβ receptor1 kinase activity leads to an increase in reporter signaling. The reporter was stably transfected into mammalian cells and used to image in vivo and in vitro bioluminescent activity as a surrogate for monitoring TGFBR1 kinase activity.

Results: The reporter was successfully used to monitor direct and indirect inhibition of TGFβ-induced Smad2 and SMAD3 phosphorylation in live cells and tumor xenografts and adapted for high-throughput screening, to identify a role for receptor tyrosine kinase inhibitors as modulators of TGFβ signaling.

Conclusion: The reporter is a dynamic, noninvasive imaging modality for monitoring TGFβ-induced Smad2 signaling in live cells and tumor xenografts. It has immense potential for identifying novel effectors of R-Smad phosphorylation, for validating drug–target interaction, and for studying TGFβ signaling in different metastasis models. Clin Cancer Res; 17(23); 7424–39. © 2011 AACR.

Introduction

TGFβ signaling pathway regulates growth inhibition and tumor suppression through control of cell proliferation, differentiation, apoptosis, migration, and adhesion (1). Consequently, when the TGFβ signaling pathway is disrupted, either through genetic mutations of key signaling components or due to loss of its growth inhibition capabilities, tumorigenesis occurs. It has been shown that TGFβ plays a dual role in cancer development (2, 3). During early stages of tumorigenesis, when the tumor cells are premalignant, TGFβ suppresses cellular outgrowth whereas at later stages, following alteration of TGFβ responsiveness and increased ligand expression, it promotes tumor progression, invasion, and metastasis. Tumor cells that have lost TGFβ-mediated tumor suppression but maintain intact components of its core signaling pathway are especially aggressive. These cells exploit TGFβ for pro-oncogenic activities: epithelial-to-mesenchymal transition (EMT), tumor invasion, mitogen production, metastatic dissemination, and immune evasion (2, 3).

TGFβ cellular activity is mediated through a receptor serine–threonine kinase (RSK) complex. TGFβ initiates the interaction of TGFβ type-1 and type-2 receptors (TGFBR1 and TGFBR2, respectively), which phosphorylate the C-terminal SXS motif of receptor-regulated Smads (R-Smads), Smad2, and Smad3. The phosphorylated R-Smads complex with Co-Smad4 translocate into the nucleus and, along with cell-specific transcriptional cofactors, interact with Smad-binding elements (SBE) in the DNA to regulate transcription of target genes. The C-terminal phosphorylation of R-Smads is a critical event in TGFβ signaling and transcriptional regulation. It is necessary for Smad complex assembly and disassembly, nuclear translocation, and transcriptional activity and stability required for Smad-mediated cellular responses (4).

Despite the importance of TGFβ-mediated Smad signaling in the progression of human cancers, methods for monitoring in vivo and in vitro Smad phosphorylation are
Translational Relevance

The response of cells to TGFβ stimulation is highly contextual in cancer. Loss or mutational inactivation of genes that mediate the TGFβ pathway early in carcinogenesis is indicative of its role as a tumor suppressor. Yet, paradoxically, TGFβ also modulates processes such as cell invasion and modifications of the tumor microenvironment that are critical for metastasis and tumor establishment at distant sites. Although therapeutic agents have been developed targeting this pathway, their rational use will require a better understanding of the regulation of its signaling. In this effort, we in this article describe the development of a reporter molecule wherein TGFβ receptor I kinase activity can be imaged dynamically and noninvasively in living subjects. We also provide preliminary results that TGFβ signaling activity is impacted by seemingly unrelated signaling cascades, providing potential explanation for the contextual differences in TGFβ downstream signaling events in cancer.

Materials and Methods

Construction of the bioluminescent TGFβR reporter

To image TGFβRI kinase activity, we constructed a recombinant chimeric reporter (Fig. 1A and B) consisting of a 15 amino acid target peptide sequence derived from the C-terminus of Smad2 containing the SXS motif, aa 453–467, a TGFβRI substrate (13). The bioluminescent TGFβR reporter (BTR) reporter was constructed through fusion of the Smad2 peptide sequence and the FHA2 phospho-serine-threonine binding domain of Rad53, which were flanked by the amino-(N-Luc) and carboxyl-(C-Luc) terminal domains of the firefly luciferase reporter molecule (14). The cloning primers were designed with a linker sequence (GlyGlyAlaGlyGly) on both ends and XhoI/Xmal precut sites. The BTR-WT was constructed using the appropriate primers (CTA GAG GAG GAA GTG GAG GGT TAA CTC AGATGG GAT CCC CIT CAG TGG GTT GCT CTT CGA GGA TTCG ACT GAA GGG GAT CCC ATG GTA GTT AAC CCT CCA CTT CTT CTT). The mutant reporter BTR-MUT was generated by substituting both serines of the SXS motif with alanine using a single-primer mutagenesis protocol, with minor modifications as described earlier (15), using the following primer: CCT TCA GTG CTT GTC ATG CCC ACT GGA GGA GCT GTA ATC AAC CCT CCA CTT CTT CTT. All the clones were sequence verified.

Cell culture and transfection

The human lung carcinoma cell line A549 [American Type Culture collection (ATCC)] was maintained in RPMI-1620 media supplemented with 10% heat-inactivated FBS, 1% glutamine, and 0.1% penicillin/streptomycin/gentamycin ( Gibco-Invitrogen). Cell cultures were grown in a humidified incubator at 37°C and 5% CO2. Breast cancer cell line 1833 derived from MDA-MB-431 (16) was kindly provided by Dr. Joan Massague and maintained in Dulbecco’s modified Eagle’s medium in the same conditions mentioned above. Cell cultures were grown in a humidified incubator at 37°C and 5% CO2. ATCC cell line was tested routinely for Mycoplasma and purity. All ATCC lines were expanded immediately upon receipt, and multiple vials of low passage cells were maintained in liquid N2. No vial of cells was cultured for more than 1 to 2 months. Stable cell lines were developed by transfecting the BTR reporter plasmids into A549 and 1833 cells using Fugene (Roche Diagnostics), and resulting clones were selected using 500 μg/mL (A549) or 250 μg/mL (1833) G418 (Invitrogen) containing media. Twenty-four stable clones were selected for both wild-type and mutant cell lines. The BTR-WT expressing clones were analyzed by bioluminescent imaging following treatment with 10 μmol/L SB431542 (Cayman Chemical). Single-cell subclones were generated and maintained in 250 μg/mL G418 containing media.

Antibodies and reagents

Rabbit polyclonal antibodies to phospho(S465/467) Smad2, Smad2, p(S423/425) Smad3, Smad6, TGFβRI/ALK5, TGFβRII, C-Myc, C-Jun, p(Y845)EGFR, epidermal growth factor receptor (EGFR), p(Y1248)HER2, p(T180/Y182)P38MAPK, P38MAPK, p(F85-Y155/F55-Y199)P38-K, P38-K, p(T183/Y185)JNK, c-jun-NH2-kinase (JNK), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were obtained from Cell Signaling Technology; anti–I-cadherin and N-cadherin from BD Biosciences; anti–p65, Smad3, and SMAD7 from Invitrogen; Smad4 from Santa Cruz Biotechnology.
SMAD2 453-467
Bioluminescence
N-Luc
C-Luc

FHA2 domain

TGFβ

SMAD2 453-467 (phospho-Ser465/67)

LTQMGSPSVRCS A

LTQMGSPSVRCS

A

B

C

D

E

F

pSMAD2

Luciferase

GAPDH

SB431542 + TGFβ

SB431542 10 μmol/L + TGFβ

SB431542

Mock

0.1 μmol/L

1 μmol/L

5 μmol/L

10 μmol/L

25 μmol/L

SB431542 concentration

R² = 0.9703

R² = 0.773

SB431542 (μmol/L)

SB431542 concentration

SB431542 + TGFβ

SB431542 10 μmol/L + TGFβ

Mock

TGFβ

0.25h

1 h

2 h

4 h

6 h

psmad2 level

reporter fold change

reporter fold induction

P = 9.04 E-6

P = 1.25 E-6

P = 0.0003

P = 0.01

r = -0.985

r = 0.88

r² = 0.773

P = 0.0003

P = 0.01

P = 4.037 E-6

P = 1.25 E-6

P = 9.04 E-6

pSMAD2

tSMAD2

Luciferase

GAPDH

SB431542 (μmol/L)
Biotechnology); and anti-HER2 and anti-firefly luciferase antibodies from Millipore. The *Horseradish peroxidase* (HRP)-conjugated secondary antibodies were from Jackson Laboratories. TGFBR1/ALK5 inhibitor SB431542, AG1296, Typhostin AG1478, and SP600125 were obtained from Cayman Chemical; pan-RTK (receptor tyrosine kinase) inhibitor PP2 from Calbiochem-EMD chemicals; and SB203580 (Invivogen). Lapatinib in tablet form (Tykerb) was purchased from the University of Michigan pharmacy. Erlotinib was a kind gift from Genentech. α-Luciferin was purchased from Promega Corp. An 84-compound kinase-specific library was obtained from Timtek Drug Discovery. SBE4-Luc reporter plasmid (17) was a kind gift of Dr Bert Vogelstein (Addgene plasmid #16495).

**Bioluminescence reporter assay and live-cell imaging**

For the reporter assay, A549 and 1833 BTR cell lines were seeded in 96-well (5 × 10^4 cells per well), clear bottom, white-walled plates (Corning, Inc.) 48 hours before assay- ing. Cells were treated in serum-free media with varied concentrations of test compounds for indicated time periods. Luminescence was read with an Envision 2104 multi-label plate reader (PerkinElmer) for 5 repeats after addition of α-luciferin (100 μg/ml final concentration) to the cell medium; each experiment was done in triplicate. For the high-throughput screen, 10 μL of an intermediate dimethyl sulfoxide (DMSO) stock of each compound in the kinase inhibitor library was added to the cell medium using a Beckman Biomek NX i Laboratory Automation Workstation (Beckman Coulter Inc.), yielding a final assay concentration of 10 μmol/L for each compound; all appropriate controls were included and cells incubated for 4 hours before α-luciferin was added and bioluminescence measured.

Live-cell luciferance imaging was achieved by adding α-luciferin (100 μg/ml final concentration) to the growth medium on cells seeded in black-walled, clear-bottom, 96-well plates. Photon counts for each condition were acquired 5 minutes after incubation with α-luciferin using an IVIS 200 imaging system (Xenogen).

**siRNA transfection analysis**

TGFBR1 and TGFBR2 siRNA, an siGENOME Smart Pool, and nonsilencing siRNA (NSS) were synthesized by Dharmacon Research. Knockdown experiments were done by transfecting A549 and 1833 BTR-WT reporter cells with 100 nmol/L siRNA for TGFBR1 and TGFBR2, as well as for nontargeted siRNA (NSS) as a negative control using Dharmafect1 (Dharmacon Research). The transfected cells were incubated for 60 hours, serum starved overnight, and 10 ng/mL TGFβ added 1 hour before evaluating reporter activity with bioluminescent imaging and Western blot analysis. Fold change in reporter activity was calculated over change in activity in NSS-treated cells.

**Western blot analysis and immunoprecipitation**

Western blot analysis was carried out using standard protocols. A549 and 1833 BTR cell lines were grown in culture dishes, treated with select compounds for designated time periods, and cell lysates resolved on SDS-PAGE gels and transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were probed against specific primary antibodies followed by HRP-conjugated secondary antibodies, then visualized using the Enhanced Chemiluminescence (ECL) Western Blotting System (GE Healthcare).

Immunoprecipitation of the BTR reporter molecule was carried out by incubating cell lysate (400 μg protein) with 5 μg luciferase-specific antibody for 1 hour. The immune complex was captured using 20 μl slurry of protein A/G-coupled Sepharose beads (GE Healthcare) for 1 hour, washed 3 times with radioimmunoprecipitation assay buffer. The resulting pellet was resolved by SDS-PAGE and transferred to PVDF membrane for Western analysis.

To study the effect of various inhibitors on Smad2/3 complex formations with Smad4, coimmunoprecipitation was carried out using a Smad3 antibody. A549 cells were serum starved and treated with 10 μmol/L inhibitors in the presence of 10 ng/mL TGFβ for 1 hour. Lysates were made in native lysis buffer (50 mmol/L Tris pH 7.4, 1% NP40, 0.25% deoxycholate sodium salt, 150 mmol/L NaCl, 10% glycerol, and 1 mmol/L EDTA) supplemented with 1× PhosStop (Roche), 1× protease inhibitor cocktail (Roche), sodium ortho vanadate, sodium fluoride, phenylmethylsulfonylfluoride, and β-glycerol phosphate. The samples were gently rocked for 2 hours at 4°C. Immune complexes were captured using 40 μl slurry of protein A/G-coupled Sepharose beads (GE Healthcare) for 1 hour. The resulting pellet was...
washed and resolved by SDS-PAGE and transferred to PVDF membrane and probed using a human anti-Smad4 antibody.

Transcriptional response assay
A549 cells were transiently transfected with SBE4-Luc reporter plasmid along with Gaussia luciferase plasmid (pEF-GLuc) as internal control. Transfected cells were incubated with 10 μmol/L inhibitors in the presence or absence of 10 ng/mL TGFβ in media containing 1% FBS. SBE4-Luc reporter activity was measured 22 hours posttreatment. Each condition was done in triplicate.

In vivo bioluminescence imaging of mouse tumor models
Tumor xenografts expressing BTR-WT were established by implanting 2.5 × 10⁶ stably transfected BTR-WT A549 cells on each flank of 4- to 6-week-old female athymic mice of the genotypic CD-1 nu/nu (Charles River Laboratories). When tumors reached a volume of approximately 40 to 60 mm³, in about 4 weeks, bioluminescence activity was monitored. The mice were anesthetized with 2% isoflurane/air mixture and given a single intraperitoneal injection of 150 mg/kg luciferin in normal saline. Image acquisition was initiated after injection of luciferin and serial background bioluminescence images acquired for 4 hours prior to drug administration. Mice were injected with a single intraperitoneal dose of 10 mg/kg body weight with SB431542, a dosing regimen previously reported to inhibit TGFβ signaling in vivo (18, 19), or vehicle control (DMSO) and bioluminescent images done before treatment as well as, at 1, 4, 8, and 24 hours.

In parallel, tumor xenografts were generated in mice using A549 BTR-WT and MUT reporter expressing cells. Mice were treated as described and bioluminescence was measured 4 hours after treatment. All mice experiments were approved by the University Committee on the Use and Care of Animals (UCUCA) of the University of Michigan.

Immunohistochemistry
Tumors from mice treated with SB431542 (10 mg/kg) or vehicle, for 4 hours, were excised and fixed in formalin. The tumors were processed at the UMCCC Tissue Core Facility and stained with anti-pSmad2 antibody (1:100); micrographs were taken using an Olympus microscope fitted with an Olympus DP-70 high resolution digital camera. Cell nuclei stained positive for pSmad2 were counted in 3 random fields for vehicle-treated (n = 3) and SB431542-treated (n = 3) tumors. A 2-sided student t test was done to assess statistical significance. Slides were adjusted for brightness and contrast with Adobe Photoshop CS2 (Adobe Inc.), but the micrographs underwent no other manipulations.

Data analyses
Western blot signal intensity was measured using the image processing and analysis program, ImageJ v1.45 (20). Pearson correlation coefficient (r) was estimated to confirm how well the SB431542 doses correlate to BTR reporter fold induction and pSmad2 levels. Analysis of statistical significance (student t test, P values) was done to estimate the significance of reporter fold induction with SB431542 doses. In addition, regression analysis (goodness of fit; R²) was carried out to confirm relationships between SB431542 dose, reporter fold induction, and pSmad2 levels. All the statistical analyses were done on Microsoft Excel 2010. GraphPad Prism v5 (GraphPad Software), nonlinear regression analyses, and sigmoidal dose response (variable slope) was used to generate EC₅₀ values.

Results
Construction and mechanism of the bioluminescent TGFβR1 reporter
The key step in TGFβ signaling is the phosphorylation of receptor-regulated Smads by active TGFβR1 receptor. To study this essential step, we designed a bioluminescent TGFβR1 reporter using a split firefly luciferase construct (Fig. 1A), consisting of the critical TGFβ substrate, the C-terminal SXS motif of Smad2, linked to the phosphopeptide-binding domain FHA2, and flanked by the N-terminal (N-Luc) and C-terminal (C-Luc) domains of the firefly luciferase reporter molecule. The functional basis of the reporter is shown schematically in Fig. 1B. The reporter exhibits low level luminescence when the Smad2 substrate is phosphorylated allowing it to bind to the FHA2 domain, thereby sterically preventing reconstitution of the luciferase reporter molecule. A reduction in Smad2 phosphorylation through inhibition of TGFβR1 kinase activity, or other downstream modulators of Smad2 phosphorylation, releases the stearic inhibition allowing reconstitution of the luciferase reporter molecule, whose activity can be detected by bioluminescence imaging.

In addition to the wild-type reporter (BTR-WT), we constructed a BTR mutant (BTR-MUT) reporter based on reports that mutations of the SXS motif abolish Smad2 phosphorylation by TGFβR1 (13). The Smad2 C-terminal serine residues 465 and 467 were mutated to the neutral amino acid alanine (Fig. 1A).

Increased BTR reporter activity following treatment with TGFβR1 inhibitor SB431542
To evaluate whether the BTR reporter provides a sensitive and robust response to inhibition of TGFβ-mediated Smad2 phosphorylation, we imaged A549 and 1833 BTR-WT cells following treatment with varied concentrations of SB431542 (21–24) in the presence of 10 ng/mL TGFβ. Bioluminescence activity was measured sequentially for 6 hours revealing both concentration- and time-dependent increases in reporter activity, reaching peak luminescence within 60 minutes posttreatment (Fig. 1C; Supplementary Fig. S2A). The BTR reporter activity increased with greater concentrations of inhibitor,
reaching a maximum expression of more than 10-fold with 25 μmol/L SB431542 in A549 (Fig. 1C) and 8-fold in 1833 (Supplementary Fig. S2A) maintaining a steady, although lower, bioluminescence for approximately 4 hours followed by a gradual decline in signal. EC_{50} values from the same datasets (reporter fold induction at 1 hour) were estimated to be 2.09 and 17.67 μmol/L for A549 and 1833 cell lines, respectively. The increased BTR reporter activity correlates with a concomitant decrease in TGFβR1-dependent levels of phosphorylated Smad2 and with increasing concentrations of SB431542 (Fig. 1D; Supplementary Fig. S2B). EC_{50} values (pSmad2 levels at 1 hour) were calculated to be 1.6 and 0.36 μmol/L for A549 and 1833 cell lines, respectively. A very strong positive correlation (Pearson correlation coefficient \( r = 0.88 \) in Fig. 1C and \( r = 0.998 \) in Supplementary Fig. S2A) was found between reporter fold induction values and SB431542 concentration. In addition, we also estimated \( R^2 \) values (goodness of fit), which determines how closely our data fit linear regression. \( R^2 \) values were estimated to be 0.773 (Fig. 1C) and 0.996 (Supplementary Fig. S2A). An analysis of statistical significance between reporter fold induction and SB431542 concentration was done. \( P \) values were found to be statistically significant. Similarly, a strong negative correlation (\( r = -0.985 \) in Fig. 1E and \( r = -0.5308 \) in Supplementary Fig. S2B, bottom panel) between reporter fold induction and pSmad2 levels was found, which further confirms the robustness of the reporter. A549 BTR-MUT cells treated with SB431542 (10 μmol/L) in the presence of TGFβ show only a slight increase in reporter activity, which was not sustained throughout the duration of the experiment (Fig. 1F), confirming the specificity of the BTR-WT reporter activity to TGFβR1 kinase phosphorylation of the Smad2 substrate. These changes in BTR reporter activity were not the result of changes in levels of total Smad2 or reporter (Fig. 1D; Supplementary Fig. S2B and C).

**BTR reporter sensitivity to indirect inhibition of Smad2 phosphorylation**

Studies have shown that active src-kinase is a downstream mediator of TGFβ-induced cellular responses (25). To study the sensitivity of the BTR reporter assay to indirect effectors of Smad2 phosphorylation, we treated A549 BTR-WT cells with the src-kinase inhibitor, PP2 (26), in the presence of 10 ng/mL TGFβ. Inhibition of src-kinase activity results in both a concentration- and time-dependent increase in reporter response, with peak bioluminescence occurring approximately 2 hours post-treatment (Supplementary Fig. S1A). Western blot analysis confirms that increase in reporter activity corresponds to decrease in pSmad2 levels, 2 hours post-PP2 treatment (Supplementary Fig. S1B), as well as a concentration-dependent decrease in pSmad2 with increasing PP2 concentration (Supplementary Fig. S1B). Identical experiments using the BTR-MUT expressing A549 cell line show minimal effect of PP2 on reporter activity (Supplementary Fig. S1C). Similar to SB431542 treatment, these results were not due to any significant change in levels of total Smad2 (Supplementary Fig. S1B).

**In vitro A549 BTR-WT cellular responses to TGFβ stimulus**

To monitor the effect of the biological transducer on BTR reporter activity, we treated A549 BTR-WT cells with 10 ng/mL TGFβ, revealing a nearly 60% decrease in reporter activity at 1 hour posttreatment, whereas treatment of A549 cells expressing the mutant reporter show minimal effect on reporter response (Fig. 2A). Western blot analysis of similarly treated A549 BTR-WT cells indicate a corresponding increase in pSmad2 at 15 minutes that remained at maximal levels for 2 hours, followed by slightly lower levels at 4 and 6 hours (Fig. 2B).

Verifying that TGFβ induction phosphorylates the target peptide, the reporter molecule was immunoprecipitated from cell extracts with anti-luciferase antibody and immunoblotted with a phospho-serine–specific antibody. The BTR protein had a substantial increase in phosphoserine levels following TGFβ treatment as compared with controls (Fig. 2C; Supplementary Fig. S2D). Following treatment with SB431542, phosphoserine levels are significantly lower than control levels (Fig. 2C; Supplementary Fig. S2D) corresponding with increased BTR reporter activity, as determined directly by bioluminescence imaging.

To further confirm the link between reporter activity and TGFβ signaling, we suppressed the expression of the upstream kinases by transiently transfecting A549 BTR-WT cells with TGFβR1- and TGFβR2-specific siRNA resulting in approximately 6-fold and 3-fold increases in reporter activity, respectively (Fig. 2D). Cells transfected with nontarget siRNA (NSS) did not affect reporter activity (Fig. 2D). Similar observations were made in 1833 BTRWT cells which exhibited 2.5-fold increase in reporter activity with TGFβR1 siRNA (Supplementary Fig. S2E); although, no significant activation of the reporter was observed with siRNA to TGFβR2. Cell extracts prepared from parallel experiments and probed with antibodies against TGFβR1 and TGFβR2 confirm the siRNA-mediated suppression of these proteins. A substantial increase in pSmad2 was observed in NSS-transfected TGFβ-treated cells, whereas theTGFβR1 and TGFβR2 knockdown cells, with or without TGFβ stimulation, have minimal levels of pSmad2 (Fig. 2E; Supplementary Fig. S2F) which closely correlates with reporter bioluminescence activity (Fig. 2D; Supplementary Fig. S2E).

To show that A549 BTR WT cells undergo EMT transition and exhibit the characteristic mesenchymal phenotype with TGFβ treatment (3, 27, 28), we treated serum-starved cells with 10 ng/mL TGFβ for up to 96 hours and studied the changes in cell morphology every 24 hours posttreatment. TGFβ-treated cells lost cell-to-cell contact and exhibited characteristic elongated, spindle-shaped morphology (Fig. 2F). These samples were prepared for Western blot analysis and probed against epithelial marker E-cadherin and mesenchymal marker N-cadherin. An increase in levels of N-cadherin and corresponding decrease of E-cadherin...
Figure 2. BTR reporter activity is dependent on reporter substrate phosphorylation through TGFβ signaling. A, A549 cells stably expressing BTR-WT reporters and BTR-MUT treated with TGFβ (10 ng/mL) and bioluminescence measured serially for 6 hours. B, cell lysates prepared and analyzed by Western blot against anti–phospho-(S465–S467) Smad2, total Smad2, luciferase, C-jun, and GAPDH antibodies. C, BTR-WT reporter expressing A549 cells were treated with TGFβ (10 ng/mL) or TGFβ and SB431542 (10 μmol/L) for 1 hour; cell lysates were prepared and immunoprecipitated with luciferase-specific antibody. The level of substrate phosphorylation was determined by Western blot analysis with phospho-Ser antibody. The immunoblot was probed with luciferase-specific antibody as a control and the input lysate was probed against phospho-(S465–S467) Smad2, total Smad2, luciferase, and GAPDH. D, A549 cells stably expressing the BTR-WT reporter were transfected with 100 nmol/L siRNA for 72 hours, TGFβ was added for an additional 1 hour, and reporter activity was measured. Fold change in reporter activity was calculated from change in nontargeted siRNA (NSS). Error bars denote SEM. E, cell lysates were prepared and analyzed by Western blot against anti–phospho-(S465–S467) Smad2, total Smad2, luciferase, C-jun, c-myc, and GAPDH. F, A549 BTR-WT cells were serum starved and treated with TGFβ (10 ng/mL) for 72 hours showing epithelial-to-mesenchymal transition. G, cell lysates of A549-BTR WT cells treated with TGFβ for 0.5, 24, 48, 72, and 96 hours were probed with antibodies against N-cadherin, E-cadherin, luciferase, c-jun, c-myc, and GAPDH. H, Western blot analysis of A549 BTR-WT cells treated with 10 μmol/L SB431542 or 10 ng/mL TGFβ for 0, 1, 3, and 6 hours with antibodies against phospho-(S465–S467) Smad2, c-myc, and c-jun.
was observed (Fig. 2G), which is a characteristic of cells undergoing EMT.

Two early regulatory genes shown to be downstream targets of R-Smad activation are the oncogenes c-jun and c-myc (29, 30). Expression levels of c-myc decreased over 96 hours and c-jun levels increased during the same time period (Fig. 2G). We further elucidated the connection of c-myc and c-jun expression to TGFβ signaling in A549 cells by treating with TGFβ alone and in the presence of SB431542. With TGFβ, c-myc levels remain constant for up to 6 hours, but when TGFβ signaling was inhibited, c-myc levels increased (Fig. 2H). Conversely, c-jun expression levels increase within 1 hour after TGFβ treatment and are abolished in the presence of SB431542 (Fig. 2H).

In vivo imaging and analysis of TGFβ signaling in BTR expressing tumor xenografts

Having established the specificity and sensitivity of the BTR reporter in vitro, we next investigated reporter utility for monitoring changes in TGFβ signaling in a tumor mouse model. We implanted A549 BTR-WT cells into the flanks of 4- to 6-week-old female athymic CD-1 nu/nu mice. Images of in vivo BTR reporter activity are shown following intraperitoneal injection of SB431542 (10 mg/kg body weight) or vehicle control (DMSO) at 1, 4, 8, and 24 hours. Bioluminescence activity in tumor-bearing mice before treatment and in response to treatment was measured and expressed as fold induction. Values represent the mean ± SEM. C, tumor xenografts were sectioned 4 hours posttreatment with SB431542 (10 mg/kg body weight) or vehicle control (DMSO) and immunohistochemical analysis was done using pSmad2(S465–S467)-specific antibody. The scale bar is 2 mm and a 10-fold magnification of selected section. D, quantification of number of cells staining positive for nuclear pSmad2 for control- (n = 3) and SB431542-treated (n = 3) tumors in 3 random fields. A decrease in pSmad2-stained nuclei in SB431542-treated tumors (761 nuclei, 37.03%) was observed when compared with DMSO-treated tumors (1294 nuclei, 62.97%). Statistical analysis: *, P = 0.06 calculated by a 2-sided Student t test.
elevated through 8 hours followed by lower, but sustained, reporter activity at 24 hour (Fig. 3A and B). Bioluminescence was unchanged over the 24-hour period in vehicle-treated mice (Fig. 3A and B).

In a similar experiment, mice (n = 5) bearing xenografts of A549 BTR-WT and MUT reporters were treated with 10 mg/kg SB431542 and DMSO control. Bioluminescence was acquired 4 hours after treatment and plotted as fold change over control (Supplementary Fig. S3). BTR-WT expressing tumors showed around 8-fold induction in bioluminescence activity with SB431542, whereas MUT tumors exhibited an attenuated response (around 3.5-fold).

Because BTR reporter bioluminescence represents decreased levels of pSmad2, we next evaluated pSmad2 levels in tumor xenografts harvested 4 hours posttreatment when peak bioluminescence was observed. Immunohistologic staining was done using pSmad2-specific antibody (Fig. 3C and D). Total numbers of pSmad2-positive nuclei were counted for each group and plotted, revealing a significant decrease (P = 0.06) in levels of pSmad2-stained nuclei in SB431542-treated tumors (761 nuclei, 37.03%) as compared with control tumors (1294 nuclei, 62.97%; Fig. 3D).

High-throughput screening of BTR reporter assay against a kinase-inhibitor compound library reveals cross-talk with multiple kinase signaling pathways

Having confirmed the sensitivity of the BTR reporter to both direct and indirect inhibition of TGFβ-stimulated phosphorylation of the C-terminal serines of Smad2, we examined its suitability for high-throughput screening. Cellular activity of the BTR reporter was screened against a kinase inhibitor library in the presence of TGFβ. Of the 84 small molecular weight kinase inhibitors screened, 13 compounds increased reporter activity. The active compounds included 5 protein tyrosine kinase (PTK) inhibitors, (tyrphostin analogs, AG1478, AG 1288, AG 825, and RG14620, and an Erlotinib analog, BML-265); a selective JNK inhibitor (SP600125); 2 p38 MAP kinase inhibitors (SB203580, SB202190); a phosphoinositide 3-kinase (PI3K) inhibitors (LY294002); 2 inhibitors of Akt signaling (BML257, triciribine); and finally, staurosporine, an apoptosis inducer and potent pan-specific protein kinase inhibitor, and quercetin, a common dietary flavonoid believed to have antioxidant and anticancer properties. No significant change in reporter activity was observed when the screen was repeated using A549 BTR-MUT cells, confirming assay specificity for phosphorylation of the C-terminal SXS motif (Fig. 4A). In addition, the HTS screen was repeated using the 1833 BTR-WT reporter cell line, to independently validate the obtained hits (Fig. 4C). Most of the hits between 2 cell lines were similar, although the reporter fold induction varied (Fig. 4A and C; Table 1). The EGFR-directed inhibitor AG1478 was a hit in A549 and 1833 cells (8.2-fold and 12.7-fold). Not surprisingly, the Akt inhibitor (BML257) and the PI3K inhibitor (LY294002) were also hits in the A549 cells (5.0-fold and 5.4-fold, respectively). In 1833 cells, LY294002 yielded a 12.3-fold increase in bioluminescence, whereas BML257 yielded 3.1-fold increase. A JNK inhibitor (SP600125) and p38MAPK inhibitors (SB203580 and SB202190), important regulators of TGFβ signaling, were also hits in both cell lines (see Table 1). Interestingly, GW5074 was a hit in 1833 cells but not in the A549 cells.

To evaluate the effect of these inhibitors on TGFβ-induced Smad2 phosphorylation, select active compounds, along with 2 clinically important receptor kinase inhibitors, erlotinib and lapatinib, underwent further analysis. A549 BTR-WT cells were treated with inhibitors in the presence of TGFβ, lysates were prepared, and Western blots probed with pSmad2 antibodies. The results (summarized in Table 1) confirm the previously described inhibition of TGFβ-activated Smad2 phosphorylation by the direct TGFβR1 inhibitor SB431542 and indirect inhibition with src-kinase inhibitor, PP2. Of the selected kinase inhibitors, lapatinib, AG1478, and LY294002 completely block TGFβ-induced Smad2 phosphorylation, whereas erlotinib, AG825, SP600125, SB203580, SB202190, and staurosporine show only moderate reduction in measurable pSmad2 levels. BML257 and quercetin treatments resulted in no measurable reduction in pSmad2 levels despite increased reporter activity (Fig. 4B). In addition Western blots were probed with pSmad3 antibody to access the potential role of non-TGFβ receptor kinase inhibitors in inhibiting TGFβ-mediated Smad3 phosphorylation. Lapatinib and AG1478 moderately block TGFβ-induced Smad3 phosphorylation, whereas SB203580, SB202190, and staurosporine show only marginal reductions in measurable pSmad3 levels.

To delineate other signaling events affecting TGFβ receptor activity, we probed the Western blot with antibodies to target proteins (see Table 1 for references). The PTK inhibitors erlotinib, lapatinib, and tyrphostin AG1478 reduce levels of pHER2 and pEGFR, while increasing the expression of the inhibitory-Smad, Smad7. The tyrphostin analog AG825 had no measurable effect on activation of HER2 or EGFR kinase. AG825 is an ATP-competitive inhibitor of HER2 and EGFR kinase activity in cell-free assays but, due to high intracellular ATP levels, it has minimal activity in whole cells (31). Treatment with JNK inhibitor SP600125 resulted in a moderate decrease in TGFβ-mediated Smad2 phosphorylation but levels of pJNK, as measured with pJNK (T180/Y182) antibody, remain unchanged with TGFβ or SP600125 treatment. TGFβ treatment increases phosphorylated p38 MAP kinase (p-p38MAPK) levels which is unchanged following treatment with the p38MAPK inhibitors SB203580 and SB202190; p-p38MAPK levels are attenuated by tyrphostin AG1478, SB431542, and PP2 treatment. Levels of phosphorylated PI3K were partly reduced by LY294002, a PI3K inhibitor, as well as, with lapatinib, tyrphostin AG1478, and SP600125. A slight decrease in luciferase expression was observed with PP2, erlotinib, lapatinib, tyrphostin AG1478, staurosporine, and LY294002 treatments with no corresponding decrease in GAPDH levels, suggesting that results are not due to differences in protein loading (Fig. 4B). Staurosporine treatment caused only a small increase in BTR activity and a moderate decrease in Smad2...
phosphorylation, while completely blocking activation of PI3K and causing a marked increase in p-p38MAPK, along with reduced expression of most of the other proteins measured, including luciferase. It has been reported that staurosporine treatment of Mv1Lu cells, 100 ng/mL, increased pSmad2 and enhanced TGFβ-stimulated apoptosis (32) suggesting that a 5-hour exposure to a 10-fold higher concentration of staurosporine, as seen in our experiment, most likely initiated cell death, thus decreasing protein expression (Fig. 4B).

Quercetin, a common dietary flavanoid believed to have antioxidant and anticancer properties, was observed to decrease levels of HER2 protein and inhibit downstream PI3K signaling in breast cancer cells (33) and suppress basal expression of TGFBR1/2, Smad2/3/4, and pSmad2/3 after 72-hour treatment of keloid fibroblasts (34). In this study, quercetin treatment stimulated BTR reporter bioluminescence and showed a moderate inhibition of TGFBR1 and pEGFR levels but did not show any measurable inhibition of pSmad2 levels, PI3K signaling, or increased expression of...
inhibitory Smad7. These results suggest that the reporter response may be a result of an immeasurable change in pSmad2 level due to moderate changes in TGFBR1 and pEGFR expression (Fig. 4B). Lysates were also probed with c-Jun and c-Myc antibodies to evaluate the effect of each inhibitor on TGFβ-Smad-mediated transcription.

Two RTK inhibitors which abrogated TGFβ-induced activation of Smad2 (lapatinib and AG1478) were selected for further analysis. Lapatinib activated the BTR-WT reporter at a dose of 1 μmol/L (~4-fold) but failed to activate the mutant reporter significantly at doses as high as 10 μmol/L (Fig. 5A). Tyroshostin AG1478 also activated the WT reporter similarly and did not alter the activity of the MUT reporter substantially (Fig. 5B). These data show that lapatinib and AG1478 were valid hits in the screen.

To further evaluate the validity of the 2 RTK inhibitors as modulators of TGFβ signaling, their impact on Smad2/3–Smad4 complex formation was evaluated. Formation of a Smad2/3–Smad4 complex is a critical event for nuclear translocation of Smad2/3. Lysates from A549 cells treated with SB431542, PP2, AG1478, and lapatinib in the presence of TGFβ were immunoprecipitated using a Smad3 antibody and coimmunoprecipitation of Smad4 was evaluated (Fig. 5C). SB431542 and PP2 completely blocked Smad3–Smad4 complex formation compared with TGFβ-treated cells, whereas AG1478 substantially and lapatinib marginally blocked complex formation between Smad3–Smad4 in A549 cells. The reduced Smad complex formation was due to blockade of TGFβ-induced phosphorylation and activation of Smad2/3 as seen in the input lysates (Fig. 5C). In addition to elucidate whether inhibition of Smad2/3–4 complex formation by PP2 and RTK inhibitors have any effect on downstream transcriptional responses mediated by TGFβ, we carried out transcriptional response assay using a TGFβ-responsive reporter SBE4-Luc (ref. 17; Fig. 5D). TGFβ strongly activated the SBE4-Luc reporter in A549 cells which was significantly blocked by the ALK5 inhibitor SB431542 and moderately blocked by PP2, AG1478, and lapatinib. These results suggest that PP2, AG1478, and lapatinib partially inhibit the activation of Smad pathway resulting in inhibition of TGFβ-induced transcriptional activity.

<table>
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<th>Kinase-inhibitors (10 μmol/L)</th>
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<th>1833 (Fold response)</th>
<th>pSmad2 levels</th>
<th>Kinase-inhibitor target(s)a</th>
<th>References: inhibitors and C—terminal pSmad2/3</th>
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aTarget information from product inserts.

Table 1. Summary of kinase inhibitors and their effects on bioluminescent TGFBR1 reporter activity and phosphorylated Smad2 levels.
The dual modality of TGFβ, both as a potent tumor suppressor and a stimulator of tumor progression, invasion, and metastasis, make it a critical target for therapeutic intervention in human cancers. The ability to conduct real-time, noninvasive imaging of TGFβ-activated Smad signaling in live cells and animal models would significantly enhance our understanding of TGFβ function and potential therapeutic strategies.

Discussion

Figure 5. A, A549 cells stably expressing the BTR-WT and MUT reporter were treated with increasing concentrations of RTK inhibitor lapatinib (0.1, 1, 2, 5, and 10 μmol/L) and tyrphostin AG1478. B, bioluminescence was measured after 1 hour. The change in bioluminescence activity over mock treatment (DMSO) levels was calculated and plotted as fold induction. Bioluminescent measurements were done in 8 wells. C, A549 cells were treated with TGFβ (10 ng/mL) or TGFβ and SB431542, PP2, AG1478, and lapatinib (10 μmol/L) for 1 hour, cell lysates were prepared and immunoprecipitated with Smad3-specific antibody. The level of Smad2/3-Smad4 complex formation was determined by Western blot analysis with Smad4 antibody. The input lysate was probed against pSmad2, total Smad2, pSmad3, total Smad3, Smad4, and GAPDH. D, A549 cells were transiently transfected with SBE4-Luc reporter plasmid and GLuc plasmid as internal control. Cells were treated with 10 μmol/L SB431542, PP2, AG1478, and lapatinib in presence of 10 ng/mL TGFβ for 22 hours. Firefly luciferase activity was normalized to Gaussia luciferase, and fold induction (over mock/DMSO treated) is expressed as the mean ± SEM of triplicate measurements.
improve our ability to develop targeted therapeutics for cancer and to monitor their effects in vivo. To study TGFβ-mediated phosphorylation of the C-terminal serines of R-Smads, we constructed a TGFBR1 bioluminescent molecular imaging reporter, described in Fig. 1A and B, based on split-luciferase technology (14). We validated the specificity and responsiveness of the BTR reporter through direct inhibition of TGFBR1 kinase activity and, indirectly, through inhibition of src-kinase, a downstream modulator of TGFβ-stimulated Smad2 phosphorylation (25). Reporter bioluminescence activity shows a robust response following inhibition of TGFBR1 kinase activity with SB431542. Time- and concentration-dependence of SB431542 inhibition of TGFBR1 kinase was confirmed by increased reporter activity and a concomitant decrease in levels of pSmad2 (Fig. 1C and E; Supplementary Fig. S2).

The EC₅₀ derived using the reporter (for SB431542) was estimated to be 2.09 µmol/L, whereas the pSmad2 Westerns yielded an EC₅₀ of 1.6 µmol/L in A549 cells. In 1833 cells, the values were 15.68 µmol/L using the reporter and 0.36 µmol/L using pSmad2 Westerns. Strong positive correlation (Fig. 1C; Supplementary Fig. S2A), goodness of fit, and statistical significance between reporter fold induction and SB431542 concentration clearly show the robustness of the BTR reporter. A strong negative correlation between reporter fold induction and pSmad2 levels (Fig. 1E; Supplementary Fig. S2B, bottom panel) further substantiated the effectiveness of the reporter. Inhibition of downstream modulation of pSmad2 levels by src-kinase also showed time- and concentration-dependent increases in reporter bioluminescence, along with a decrease in measurable levels of pSmad2 (Supplementary Fig. S1). These results strongly support the relationship between increased reporter activity and both direct and indirect inhibition of Smad2 phosphorylation.

Direct confirmation that TGFβ is a biological transducer of BTR reporter activity is shown by the reduction of reporter bioluminescence following TGFβ treatment of A549 BTR-WT cells (Fig. 2A) and the corresponding increase in pSmad2 (Fig. 2B). The sustained decrease in reporter activity and prolonged elevation of pSmad2 are consistent with continuous shuttling of Smad proteins between cell nucleus and cytoplasm shown to exist during TGFβ signaling (23). Immunoprecipitation of the reporter, followed by analysis of phosphoserine content, confirms that the reporter was phosphorylated in a TGFβ-dependent manner (Fig. 2C; Supplementary Fig. S2D). In addition, suppression of the upstream kinases by siRNA knockdown of TGFβ1 and TGFβ2 verifies the direct link between reporter activity and TGFβ receptor signaling. Knockdown TGFβ1 and TGFβ2 cells have reduced levels of pSmad2 and show a significant increase in reporter bioluminescence (Fig. 2D and E; Supplementary Fig. S2E and F).

TGFβ signaling contributes to tumor progression and metastasis through activation of EMT, a process in which epithelial cells convert to fibroblastoid-like cells which generate an invasive and metastatic phenotype associated with poor clinical outcomes for cancer patients (35). TGFβ-activation of Smad2 signaling induces EMT in A549 BTR-WT cells, resulting in the loss of cell-to-cell contact and the cellular conversion to an elongated, spindle-shaped morphology (Fig. 2F). Protein analysis reveals the characteristic EMT downregulation of E-cadherin expression, a critical caretaker of epithelial integrity, and a corresponding upregulation of N-cadherin (Fig. 2G; ref. 35). Activated Smad2 has also been shown to regulate expression of the proto-oncogenes c-myc (30) and c-jun (29). TGFβ downregulation of c-myc is critical to growth inhibition (30) in which the upregulation of c-jun is important for cell survival (29). TGFβ stimulation of A549 BTR-WT cells suppresses c-myc expression with a corresponding upregulation of c-jun (Fig. 2G). Cell treatment with TGFBR1 kinase inhibitor SB431542 results in increased expression of c-myc and eliminates c-jun expression (Fig. 2H). A TGFβ increase in total c-jun levels is consistent with previous findings showing an effective downstream signaling cascade initiated by activated R-Smads in response to TGFβ (29).

Recent research has shown growth factors which signal through RTKs such as, hepatocyte growth factor and epidermal growth factor (EGF), mediate Smad2 activity independent of TGFβ1 activation, suggesting intricate cross-talk between RTK and RSK signaling pathways (37, 38). Our results show that EGF receptor (EGFR) and human epidermal growth factor receptor 2 (HER2) kinase inhibitors erlotinib, lapatinib, and tyrphostin AG1478 block TGFβ-mediated increases in pSmad2 (Fig. 4B). Lapatinib and tyrphostin AG1478 marginally reduce TGFβ-mediated phosphorylation of Smad3, whereas erlotinib had no significant effect (Fig. 4B). The reduction in pSmad2 levels coincides with an equally pronounced decrease in expression of the active kinases, phosphorylated HER2 (pHER2), and phosphorylated EGFR (pEGFR). Treatment of A549 BTR-WT cells with SB431542 also reduces basal levels of pEGFR, further supporting signaling cross-talk between RTK and RSK pathways.

An important component of cross-talk between TGFβ and RTK signaling is the increased expression of inhibitory-Smad (I-Smad) proteins (39). The I-Smads, Smad6, and Smad7, modulate signaling of TGFβ and, another TGFβ isoform, bone morphogenetic protein (BMP), by binding to the activated type-1 receptor, thereby preventing recruitment and phosphorylation of Smad3 (38, 39). In some cell types, TGFβ or BMP induce I-Smad expression resulting in a negative feedback mechanism to regulate further TGFβ or BMP signaling. Besides induction by TGFβ or BMP, I-Smad expression can also be induced in a cell-dependent manner by other cytokine signaling pathways and stress.
induction (39, 40). In A549 cells, expression levels of Smad6 and Smad7 did not increase following TGFβ treatment. However, treatment with EGFR and HER2 kinase inhibitors significantly increased expression of Smad7 in A549 BTR-WT cells resulting in interference with recruitment and phosphorylation of Smad2 by active TGFβR1 (Fig. 4B). These data suggest that, at least in part, the RTK signaling pathways increase activation of R-Smads by suppressing Smad7 expression and subsequent interference with Smad2 phosphorylation, providing evidence of the importance of RTK and RSK cross-talk in regulating TGFβ-induced Smad2 signaling.

In addition to cross-talk between RTK and RSK signaling, other kinase pathways have been shown to modulate Smad2 phosphorylation. BTR reporter activity was increased by inhibitors of PI3K, p38MAPK, and JNK signaling pathways. Martin and colleagues (41) showed that kinase inhibitors against PI3K (LY294002), p38MAPK (SB203580), and JNK (SP600125) activity attenuate TGFβ-mediated phosphorylation of Smad2 in lung fibroblasts, in which treatment with inhibitors for other kinase signaling pathways, MEK1 (PD98059), PKC (Ro31-8425), and Erk/MEK (U0126), had no effect. These observations are supported by our screening results, BTR reporter activity was increased for inhibitors shown to reduce R-Smad activation (LY294002, SB203580, and SP600125; Fig. 4A) and was inactive when treated with inhibitors to kinase pathways that do not modulate TGFβ-stimulated phosphorylation of C-terminal serines of Smad2/3 (PD98059, Ro31-8425, and U0126; Data not shown). A parallel screen using the 1833 cell line stably expressing the BTR-WT reporter yielded hits that were overlapping with the hits obtained from the A549 cells (Fig. 4C). Published studies support some of these hits, for example, inhibitors for JNK (SP600125) and p38MAPK (SB203580) have been shown to downregulate phosphorylation of the C-terminal serines in rat mesangial cells (42), and the p38MAPK inhibitor (SB202190) abolishes TGFβ activation of Smad-dependent promoters and attenuate pSmad2 levels in glioblastoma cells (43).

However, the extent of signaling cross-talk between TGFβ and mitogen-activated protein kinase (MAPK) pathways has been shown to be dependent on cell type and context. Where the previous studies show JNK and p38MAPK modulation of pSmad, TGFβ-mediated phosphorylation in mouse mammary epithelial (NMuMG) cells was shown to mediate TGFβ responses independent of Smad phosphorylation (44). The cell-specific differences in cross-talk between MAPK and Smad pathways are also evident with the JNK pathway. Where active JNK enhances or maintains TGFβ-mediated expression in fibroblasts (41) and mesangial cells (42), through phosphorylation of C-terminal Smad2, inhibiting JNK activity in embryonic lung explants increases both endogenous and TGFβ-induced Smad phosphorylation (45), suggesting that active JNK antagonizes TGFβ-induced pSmad2. In yet another study, TGFβ-activated JNK was shown to only phosphorylate Smad3 outside the C-terminal SXS motif in Mv1Lu cells (46).

Besides cross-talk between p38MAPK and JNK with TGFβ signaling pathways, our data suggests that the PI3K- Akt signaling pathway may also provide significant regulation of TGFβ-induced cellular responses. Treatment with the PI3K inhibitor LY294002 results in a robust activation of the BTR reporter and a nearly complete inhibition of TGFβ-mediated phosphorylation of the C-terminal series of Smad2 (Fig. 4A and B). Inhibition of Akt1/2/3, a PI3K target protein, with BML257 also activates the BTR reporter but did not yield a measurable decrease in pSmad. PI3K signaling has been shown to be critical for Smad2-dependent activity in fibroblasts (41) and transcriptional responses, EMT and cell migration of NMuMG cells (47).

Although C-terminal SXS phosphorylation by the TGFβ type-1 receptor is the key event in TGFβ signaling, phosphorylation of the linker region and, to a lesser extent, the MH1 domain of Smad proteins by intracellular protein kinases can also positively and negatively regulate R-Smad activity (2). Inhibitors for many of these kinases were screened with the BTR reporter and, as expected, did not increase bioluminescence, confirming the specificity of the BTR reporter assay to phosphorylation of the critical SXS motif. MEK1 inhibitor, PD98059, which inhibits RTK phosphorylation of Smad2 through activation of ras proteins does not affect TGFβ-mediated Smad2 phosphorylation (37) and did not activate the BTR reporter in our kinase inhibitor screen. Protein kinase inhibitors, known to modulate pSmad in the linker region, were also inactive in the high-throughput screen, including the Ca2+-calmodulin-dependent protein kinase II (CaMKII)-specific inhibitor KN93 (48) and inhibitors of ROCK, PKA/PKG, and MLCK (Y027632, HA-1077, and ML-7, respectively; ref. 49).

In summary, we have designed and validated a sensitive and highly specific bioluminescence imaging tool to monitor TGFβ-mediated phosphorylation of the C-terminal series of R-Smads in live cells and tumor xenografts. The BTR reporter is adaptable to high-throughput screening in which it can be used to aide in identification and evaluation of therapeutic compounds targeting TGFβ–Smad2 signaling. Following compound identification, this bioluminescent imaging technique provides a repetitive, noninvasive, dynamic method to monitor in vivo TGFβ signaling to evaluate drug-target interaction and pharmacodynamics of therapeutic compounds that directly or indirectly effect TGFβ-stimulated Smad2 phosphorylation providing new insights into TGFβ signaling and help in identifying new drug therapeutics for cancer treatment.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Molecular Imaging of TGFβ Kinase Activity

Correction: Molecular Imaging of TGFβ-Induced Smad2/3 Phosphorylation Reveals a Role for Receptor Tyrosine Kinases in Modulating TGFβ Signaling

In this article (Clin Cancer Res 2011;17:7424–39), which was published in the December 1, 2011, issue of Clinical Cancer Research (1), the middle initial of one of the coauthors was omitted. The correct listing is Mukesh K. Nyati. The authors regret this error.

Reference


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Molecular Imaging of TGF\(\beta\)-Induced Smad2/3 Phosphorylation Reveals a Role for Receptor Tyrosine Kinases in Modulating TGF \(\beta\) Signaling

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