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## TGF- $\beta$ receptor levels regulate the specificity of signaling pathway activation and biological effects of TGF- $\beta$

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### Abstract

TGF- $\beta$  is a pluripotent cytokine that mediates its effects through a receptor composed of TGF- $\beta$  receptor type II (TGFBR2) and type I (TGFBR1). The TGF- $\beta$  receptor can regulate Smad and nonSmad signaling pathways, which then ultimately dictate TGF- $\beta$ 's biological effects. We postulated that control of the level of TGFBR2 is a mechanism for regulating the specificity of TGF- $\beta$  signaling pathway activation and TGF- $\beta$ 's biological effects. We used a precisely regulatable TGFBR2 expression system to assess the effects of TGFBR2 expression levels on signaling and TGF- $\beta$  mediated apoptosis. We found Smad signaling and MAPK-ERK signaling activation levels correlate directly with TGFBR2 expression levels. Furthermore, p21 levels and TGF- $\beta$  induced apoptosis appear to depend on relatively high TGFBR2 expression and on the activation of the MAPK-ERK and SMAD pathways. Thus, control of TGFBR2 expression and the differential activation of TGF- $\beta$  signaling pathways appears to be a mechanism for regulating the specificity of the biological effects of TGF- $\beta$ .

### Keywords

TGF- $\beta$ ; Smad; nonSmad; p21; apoptosis; signaling

### 1. Introduction

Transforming growth factor  $\beta$  (TGF- $\beta$ ) is a secreted multifunctional cytokine that regulates a variety of cellular processes including growth inhibition secondary to G1/S phase arrest, differentiation, apoptosis, immunosuppression, stimulation of connective tissue deposition, secondary induction of angiogenesis, and probably maintenance of genomic stability. TGF- $\beta$  induces these effects by activating the TGF- $\beta$  receptor, a heterodimeric complex composed of type I (TGFBR1) and type II (TGFBR2) subunits, which are both serine-threonine kinases with single transmembrane domains [1]. Both TGFBR1 and TGFBR2 appear to be essential for

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TGF- $\beta$  induced effects on the cell [2,3]. The TGF- $\beta$  receptor is activated through a sequence of events that is initiated by the TGFBR2 subunit binding to the activated TGF- $\beta$  ligand. The activated TGFBR2 associates with TGFBR1, and phosphorylates a glycine-serine rich sequence in TGFBR1. TGFBR1 is then activated and can phosphorylate downstream targets [4]. The TGF- $\beta$  receptor complex activates both the Smad signaling pathway, which includes Smad2, Smad3, and Smad4, and the nonSmad signaling cascades (e.g. PI3K/AKT, p38MAPK, MAPK-ERK, JNK, etc.) to produce the full spectrum of TGF- $\beta$  responses [5-7].

With regard to the nonSmad signaling pathway, TGF- $\beta$  can activate a variety of nonSmad signaling pathways, including the MAPK, JNK, p38MAPK, NF $\kappa$ B, RhoA, and PI3K pathways. TGF- $\beta$  can regulate the nonSmad signaling pathways both directly and indirectly. For instance, direct physical interactions between the TGF- $\beta$  receptor and TAB1 (TAK1-binding protein 1), which can regulate TAK1 (TGF- $\beta$  Activated Kinase 1) and p38MAPK, and PI3K/AKT, have been shown [8]. Other nonSmad signaling pathways are also clearly TGF- $\beta$  responsive but, in most cases, it has not been shown whether the effect is a direct or indirect effect of TGF- $\beta$ . The nonSmad signaling pathways have been implicated in regulating TGF- $\beta$  mediated apoptosis [8,9], extracellular matrix production [10-12], and differentiation [11,13].

The Smad signaling pathway is more completely understood than the nonSmad signaling pathways that are activated by the ligand bound TGF- $\beta$  receptor. Ligand binding to the TGF- $\beta$  receptor complex results in TGFBR1-mediated phosphorylation of Smad2 and Smad3 on two serine residues in a conserved Ser-Ser-X-Ser motif located at the C-terminus of the R-Smads [14,15]. Phosphorylation of these serine residues activates the signaling pathway and is required for the function of Smads in transcription factor complexes [16,17]. The phosphorylated Smad2 and Smad3 bind to Smad4, and the complex can then translocate into the nucleus to function as a transcription factor complex. The details of this pathway have been the subject of a recent review (see [18]).

Although initially perceived as a binary (i.e. “on” or “off”) and linear mechanism, it is now clear that the TGF- $\beta$  signaling pathways' effects on the biological responses of a cell depend on the intensity and duration of the activation of the pathways [19]. Indeed, a substantial number of studies have demonstrated that the TGF- $\beta$  signaling pathways are regulated by a variety of mechanisms that control the cellular localization, phosphorylation state, and expression levels of the post-receptor signaling elements [19,20]. Moreover, these studies have provided a plausible explanation for why the cell state and cell type, referred to as the “cellular context” can have a profound effect on the way in which TGF- $\beta$  affects a cell [21-23]. Recent data has shown that the degree of activation of the TGF- $\beta$  signaling pathways is subject to regulation by a large number of intracellular and extracellular agonists and antagonists, including decorin, Smad7, Smurf, SARA, etc [24,25].

The discovery of these regulatory mechanisms demonstrates that not only is the absolute activation state of the pathway important for determining TGF- $\beta$ 's effects on cells but that the degree of pathway activation and the duration of pathway activation is also likely important. In fact, it is plausible that the paradoxical effects of TGF- $\beta$  on tumor cells could be at least partly explained through differences in the intensity of activation of the pathways or by differential activation of Smad vs. nonSmad signaling pathways. Regulation of the expression levels of TGFBR1 and TGFBR2 may be a mechanism through which the activation state of the TGF- $\beta$  signaling pathways can be regulated [8]. Consistent with this model is the recent demonstration that levels of TGFBR1 have been shown to correlate with an increased risk of colorectal cancer [26].

In order to assess whether the regulation of TGFBR2 expression could be a mechanism for determining the response of cells to TGF- $\beta$ , we generated a system using the RheoSwitch®

inducible gene expression system to regulate the expression of *TGFBR2*. We have found that the level of *TGFBR2* affects the ability of TGF- $\beta$  to induce p21 and apoptosis in the V-400 colorectal cancer cell line. In addition, TGF- $\beta$ 's ability to activate the MAPK pathway is directly dependent on the expression level of *TGFBR2*. These results demonstrate that the regulation of the receptor level for TGF- $\beta$  is a potential mechanism for determining the specificity of the response of a cell to TGF- $\beta$ .

## 2. Materials and Methods

### 2.1. Cell Lines and inhibitors

V-400 is a microsatellite stable (MSS), TGF- $\beta$  resistant colon cancer cell line, which was kindly provided by James K.V. Willson (UT Southwestern Medical School, Dallas, TX). It carries biallelic missense *TGFBR2* mutations that inactivate the endogenous receptor, and it has an intact Smad signaling pathway [27]. The V-400 cell line was grown in Dulbecco's Modified Eagle Medium (Gibco, Grand Island, NY). The media was supplemented with 10% FBS (Cambrex, East Rutherford, NJ). The MEK1/2 inhibitor U0126 (cat# 662005), and the PIK3 inhibitor LY294002 (cat# 440204) were obtained from Calbiochem (San Diego, CA). The Smad7 plasmid was kindly provided by Neil Bhowmick (Vanderbilt University, Nashville, TN).

### 2.2. Inducible gene expression system

The inducible system used in this project was developed by RheoGene, Inc. (Norristown, PA), now Intrexon Corporation (Blacksburg, VA), and is a highly engineered system based on a modified version of the ecdysone receptor (EcR) [28,29]. Important modifications of this system include mutations in the ecdysone receptor to increase potency to synthetic diacylhydrazine agonists; trans-positioning of the VP16 activation domain and the Gal4 DNA binding domain as fusions to the RXR and EcR components, respectively; and development of potent and bioavailable synthetic agonists. Two agonists, RG-102240 and RG-115819, were used in these studies. They are closely related diacylhydrazine analogs, with the latter being slightly more potent. These modifications significantly improve the precision of the transgene expression regulation by the synthetic agonist.

A single construct carrying both the RheoSwitch® receptor genes and an HA-tagged *TGFBR2* transgene was made. The *TGFBR2* transgene was inserted into the cloning site present in the lentiviral vector p5004, provided by RheoGene. (Figure 1) The *TGFBR2* transgene was obtained from the vector pRC/CMV kindly provided by Dr. Sanford Markowitz (Case Western Reserve University, Cleveland, OH) [27]. The *TGFBR2* gene was released from its original backbone using the enzymes NotI and EcoRI and then treated with Klenow. After cloning the *TGFBR2* transgene into the p5004 vector, plasmids were screened for inserts with the correct orientation and were confirmed to carry a wild-type *TGFBR2* transgene by sequencing

A lentivirus carrying the *TGFBR2* transgene was produced in the Gene Therapy core lab at the Fred Hutchinson Cancer Research Center using a 293T-based packaging cell line. V-400 cells were infected with 0.5 ml of viral supernatant. The cells were incubated for 24 hours with the viral supernatant in presence of polybrene. The viral supernatant was then removed and Blasticidin (6  $\mu$ g/ml) was added into the complete media (DMEM in presence of 10% FBS). The transduced cells were then subjected to cloning by limiting dilution. An evaluation to identify clones that displayed minimal expression in the baseline state but showed adequate induction of *TGFBR2* after treatment with RG-115819 was performed in different clonal populations. The control cells were treated with DMSO vehicle alone.

### 2.3. Luciferase reporter assays

In order to evaluate TGF- $\beta$ -mediated transcription, the cell lines were transiently transfected with the p3TP-lux reporter (kindly provided by Joan Massagué, Memorial Sloan-Kettering Cancer Center, New York, NY) or with the CAGA reporter assay (kindly provided by Bert Vogelstein, Johns Hopkins University, Baltimore, MA) concomitantly with the pRL-TK reporter construct (Promega, Madison, WI). The cells were treated with TGF- $\beta$ 1 (10ng/ml), and luciferase activity was evaluated 48h after transfection using the Dual Luciferase Reporter Assay System (Promega, Madison, WI) with a Veritas luminometer (Turner Biosystems, Sunnyvale, CA). The p21 expression was evaluated using a luciferase reporter assay (kindly provided by Dr. Xiao-Fan Wang's Laboratory) [30]. The luciferase activity was evaluated 24 and 48 hr post-transfection as noted above. All the transfections were performed using FuGENE 6 (Roche, Basel, Switzerland) following the protocol provided by the manufacturer. A 3:1 FuGENE-DNA ratio was used.

### 2.4. FACS analysis

V-400R2 and V-400 cells treated for 48 hours with RG-115819 and TGF- $\beta$ 1 were used for these studies. The cells were collected after being briefly treated with trypsin (0.125%) to detach them from the flasks, washed in media with 20% FBS (4°C) followed by 1X PBS (4°C), and then fixed in 3% formaldehyde for 15 minutes. The cells were then incubated with 10% normal horse serum for 30 minutes, washed with PBS 3 times, and incubated with an anti-HA antibody (1:100, Cat # 2367 Cell Signaling, Danvers, MA), followed by incubation with a goat-anti-mouse secondary antibody labeled with fluorescein (FITC) (1:100, Cat#: 115-095-146, Jackson ImmunoResearch, West Grove, PA). The cells were then washed with PBS (3 times) and subjected to FACS analysis using FACScan (Becton Dickinson, Franklin Lakes, NJ). The results were analyzed using CellQuest software (Becton Dickinson, Franklin Lakes, NJ).

### 2.5. Western blotting

Cell lysates were prepared using RIPA buffer supplemented with a complete protease inhibitor cocktail (Roche) and phosphatase inhibitor cocktails 1 and 2 (Sigma-Aldrich, St. Louis, MO). The lysates were then used for SDS-PAGE (10% polyacrylamide gels and PVDF membranes, Pierce, Rockford, IL). Immunoblots were performed following the manufacturer's recommendations. The following antibodies were used: pERK1/2 (Cat # 9101 Cell Signaling, Danvers, MA 01923), ERK1/2 (Cat # 9102 Cell Signaling, Danvers, MA 01923), p21 (Cat # OP68 EMD San Diego California), anti-TGFBR1 ((H-100 antibody; sc-904, 1;200), HRP-goat anti-mouse (Cat # SC 2031) and donkey anti-rabbit (Cat # SC 2317) (Santa Cruz Biotechnology). A commercial ECL kit (RPN 3004, GE Healthcare, Piscataway, NJ) was used to detect the HRP-labeled secondary antibodies and the signal was detected using autoradiography. Reagents used in conjunction with this kit included n-butyric acid sodium salt (Cat# B-5887), Luminol (Cat# A8511), and p-coumaric acid (Cat# C9008) (Sigma-Aldrich, St. Louis, MO).

### 2.6. PIK3/AKT specific ELISA

Phosphorylated AKT was detected using a commercially available ELISA assay (SuperArray, SABiosciences, Frederick, MD). Ten thousand cells/well were seeded in a 96 well plate, and were treated with different concentrations of RG-115819 in order to differentially induce the expression of the TGFBR2 transgene. TGF- $\beta$  (10 ng/ml) was then added to the cells and the cells were harvested after 48 hours. The ELISA was performed following the manufacturer's recommendations (cat # FE-001 SuperArray), and read in a plate reader at 450 nm (VERSAmax, Molecular Devices, Sunnyvale, CA).

## 2.7. Quantitative RT-PCR

One  $\mu\text{g}$  of RNA extracted from V-400 and V-400R2, was reverse transcribed using oligo d(T) priming and Superscript-II reverse transcriptase (Invitrogen) and 2 nM dNTPs following the manufacturer's protocol. TaqMan On-Demand primers and probes were used to determine the relative expression levels of *CDKN1A/p21* (Assay number Hs99999142\_m1, Applied Biosystems, Foster City, CA) and 18S in all samples (Assay number Hs99999901\_s1, Applied Biosystems,). The reactions were run in triplicate in the ABI Prism 7700 detection system (Applied Biosystems) and results were analyzed with SDS 2.1 software.

## 2.8. Apoptosis assays

Apoptosis was measured with two different assays: the Cell Death ELISA assay (Roche, Cat# 11 774 425 001), which detects histone-complexed DNA fragments, following the manufacturer's protocol, and the Caspase-Glo Luciferase-based reporter assay, which measures caspase 3 and 7 activity (Promega, Catalog number TB323) following the manufacturer's protocol.

## 3. Results

### 3.1. Generation of a colorectal cancer cell line with precisely regulatable TGFBR2 expression

In order to assess the effect of the expression level of TGFBR2 on the activation of TGF- $\beta$  signaling pathways, we employed the RheoSwitch $\text{\textcircled{R}}$  gene regulation system that can precisely regulate the transgene of interest [28,29,31]. An HA-tagged *TGFBR2* transgene was cloned into a lentiviral expression vector and used to generate lentivirus for transducing the target cell line, V-400. This cell line lacks a functional TGF- $\beta$  receptor and becomes TGF- $\beta$  responsive when it is reconstituted with *TGFBR2* [27]. (See Materials and Methods section for information on the plasmids used.) The V-400 cells were transduced with *TGFBR2* and clones expressing the RheoSwitch $\text{\textcircled{R}}$  receptor and *TGFBR2* transgene were selected and then assessed for TGF- $\beta$  responsiveness using the 3TP-lux or CAGA reporters. Two clones displayed a linear dose response of 3TP-Lux activity in relation to RG-102240 concentration. (Figure 2)

The expression levels of the *TGFBR2* transgene were then assessed by FACS analysis in order to determine the amount of TGFBR2 expressed on the surface of the transduced cells. (Immunoblotting of total protein lysates from the cells for the HA-tagged *TGFBR2* transgene confirmed the results of the FACS analysis but does not demonstrate the same linear response to the RG-115819 because of the lower precision of immunoblotting to detect small differences in protein expression. (Figure 3A-B). Clones 1 and 2 showed a linear increase in 3TP-Lux induction and *TGFBR2* transgene expression after treatment with increasing concentrations of RG-115819. (Figure 3A) A linear dose response for TGFBR2 expression between 0.04  $\mu\text{M}$  to 1  $\mu\text{M}$  RG-115819 was demonstrated, and thus this range of RG-115819 was used for the subsequent experiments. We did not observe any change in TGFBR1 expression with increasing concentrations of RG-115819, which led us to interpret that any RG-115819 dependent phenomenon were strictly the result of changes in the expression level of TGFBR2. (Figure 3C)

### 3.2. The regulation of TGFBR2 expression levels affects the TGF- $\beta$ mediated expression of *CDKN1A/p21*

TGF- $\beta$  can regulate the expression of a variety of genes that control a myriad of cell responses including proliferation, apoptosis, differentiation, etc [30,32,33]. A significant question that arises is how the specificity of the response is determined. One of the TGF- $\beta$  regulated genes that highlights the issues related to control of the specificity of the TGF- $\beta$  response is the CDK inhibitor *CDKN1A/p21*, which plays an important role in several cellular processes including

apoptosis, cell cycle arrest, induction of cell differentiation, and cellular senescence [32,34]. Thus, we assessed the regulation of CDKN1A/p21 expression in the setting of different levels of TGFBR2 expression to determine if the regulation of the expression of the TGF- $\beta$  receptor may be a mechanism through which TGF- $\beta$  mediated responses are regulated. In order to determine how changes in the expression levels of TGFBR2 affect the expression of *CDKN1A/p21*, V-400R2 cells were treated for 24 and 48 hours with four different concentrations of RG-115819 (0.04, 0.08, 0.4 and 1  $\mu$ M) and TGF- $\beta$  (10ng/ml). The induction of *CDKN1A/p21* expression was assessed using a *CDKN1A/p21* luciferase reporter [35]. *CDKN1A/p21* reporter activity was increased at 24 hours but no correlation between increasing TGFBR2 expression and reporter activity was observed at that time point. At 48 hours, a direct relation between TGFBR2 expression level and *CDKN1A/p21* reporter activity was apparent (Figure 4A). Importantly, no basal *CDKN1A/p21* reporter luciferase activity was detected in the V-400R2 without RG-115819 or in the parental cell line V-400. (Figure 4A) *CDKN1A/p21* protein expression also correlated with the *CDKN1A/p21* luciferase reporter activity. (Figure 4B) Localization of p21 was also assessed in light of the possibility that MAPK signaling could regulate p21 nuclear localization in these cells. We observed increased nuclear p21 in association with increased p21 expression induced by increased TGFBR2 expression. These results suggest that the increased p21 is being retained in the nucleus despite the presence of increased activated ERK and that p21 is acting to affect the behavior of the cell through actions on gene transcription (Supplemental Figure 1).

### 3.3. TGFBR2 levels can differentially affect MAPK-ERK pathway activation and Smad pathway activation

The expression of *CDKN1A/p21* is regulated by several signaling pathways and transcription factors, including the Smad signaling pathway, ERK signaling pathway, myc, etc. [23, 36-38]. In light of the differing effect of low and high levels of TGFBR2 expression on *CDKN1A/p21* expression, we assessed the effect of different levels of TGFBR2 expression on the activation status of Smad and nonSmad signaling pathways. V-400R2 cells were treated with RG-115819 (0.4-1.0 $\mu$ M) and assessed for MAPK-ERK activation at 24 and 48 hours. ERK activation was not present at 24 hours but was present at 48 hours after treatment with RG-115819 and TGF- $\beta$ . (Figure 5A) The level of phosphorylated ERK directly correlated with the expression level of TGFBR2 as well as with *CDKN1A/p21* expression (Figure 5A). Of interest, we also assessed the effect of TGFBR2 expression on PI3K pathway activation and found that phosphorylated AKT is present in the parental cell line and that TGF- $\beta$  does not induce an increase in phosphorylated AKT in the *TGFBR2* reconstituted cell line at any level of TGFBR2 expression (data not shown). After assessing the effect of TGFBR2 on the MAPK-ERK pathway, we next assessed the activation state of the Smad signaling pathway. Unlike with the MAPK-ERK pathway, Smad2 phosphorylation was increased with moderate to high levels of TGFBR2 but not with low expression levels of the receptor. There was also a modest increase in phosphorylated Smad2 with increasing levels of TGFBR2 expression, but the range of increased phosphorylated Smad2 was more limited than that for phosphorylated ERK1/2. (Figure 5B) The functional effect of the increase in phosphorylated Smad2 can be observed by a linear increased in CAGA luciferase reporter activity, which is specific for Smad mediated transcription. (Figure 8)

### 3.4. TGF- $\beta$ mediated regulation of *CDKN1A/p21* expression is dependent on Smad and MAPK-ERK activation

It has been previously shown in other cell line systems that the MAPK-ERK pathway can control the expression of *CDKN1A/p21* and that TGF- $\beta$  can regulate *CDKN1A/p21* through the MEK pathway [30,39,40]. Consequently we assessed the effect of inhibition of ERK activation on the ability of TGF- $\beta$  to induce *CDKN1A/p21* expression in the V-400R2 cell line in order to confirm that this was also true in this colorectal cancer cell line. TGF- $\beta$  mediated

activation of ERK was inhibited with the selective MAPK inhibitor U0126 (5 $\mu$ M) in V-400R2 cells treated with RG-115819 (0.04-1.0  $\mu$ M) and TGF- $\beta$  (10 ng/ml). The induction of TGFBR2 expression induced the expression of CDKN1A/p21, but blockade of the MAPK-ERK pathway prevented the differential increase in CDKN1A/p21 expression observed in the prior studies. These results suggest that CDKN1A/p21 expression is regulated by both MAPK-ERK and nonMAPK-ERK signaling pathways. (Figure 6A) We next assessed the effect of TGF- $\beta$  mediated Smad pathway activation on the expression of CDKN1A/p21 using the inhibitory Smad, Smad7. Transfection of the V-400R2 cells with *SMAD7* almost completely blocked the induction of the CAGA luciferase reporter and modestly suppressed the induction of *CDKN1A/p21* reporter activity, but did not prevent the linear increase in *CDKN1A/p21* expression observed with increasing TGFBR2 expression indicating the TGF- $\beta$  mediated regulation of the precise expression levels of *CDKN1A/p21* in V-400R2 is regulated predominantly by nonSmad signaling. (Figure 6B-C)

### 3.5. TGFBR2 expression levels regulate the ability of TGF- $\beta$ to induce CDKN1A/p21 mediated apoptosis

In light of our results showing that TGFBR2 expression directly correlates with increased ERK activation and CDKN1A/p21 expression, we carried out a series of studies to determine whether the expression level of TGFBR2 can directly affect the ability of TGF- $\beta$  to induce apoptosis. The V-400R2 cell line was treated with RG-115819 in order to induce low and high levels of TGFBR2 and then assessed for TGF- $\beta$  mediated apoptosis. A direct relationship between TGFBR2 expression and apoptosis was demonstrated showing that high levels of TGFBR2 are permissive for TGF- $\beta$  mediated apoptosis. (Figure 7) These results suggest that TGF- $\beta$  can mediate apoptosis in the V-400R2 cell line through the induction of the MAPK-ERK pathway and subsequent increase in CDKN1A/p21 expression. Thus, TGFBR2 levels may be a mechanism for determining whether cells are sensitive or resistant to TGF- $\beta$  mediated apoptosis.

In order to establish if the induction of increased CDKN1A/p21 resulting from the increased TGFBR2 expression and ERK activation is a central mechanism for TGF- $\beta$  mediated apoptosis in the V-400R2 cells, *CDKN1A/p21* expression was inhibited with p21-siRNA in the presence of low and high levels of TGFBR2 and TGF- $\beta$  (10ng/ml). (Figure 8A) Interestingly we found that inhibition of CDKN1A/p21 prevented the increase in apoptosis observed with increased TGFBR2 levels providing support for CDKN1A/p21 regulation being a mechanism for TGF- $\beta$  mediated apoptosis in colon cancer. (Figure 8B) However, we also observed that inhibition of Smad signaling with *SMAD7* impaired TGF- $\beta$  mediated apoptosis presumably through a p21 independent mechanism, given that we did not observe an effect of *SMAD7* on p21 expression. These results demonstrate TGFBR2 can regulate apoptosis through both p21 dependent and independent mechanisms. (Figure 8C).

## 4. Discussion

TGF- $\beta$  can induce a variety of biological responses in cells, and these responses appear to be dependent on the cell type and context of the cell [41]. Some of the mechanisms that appear to play a role in determining the specificity of the response of a cell to TGF- $\beta$  include signal pathway cross-talk, regulation of the expression levels of cytoplasmic and nuclear antagonists, and control of the composition of transcription factor complexes [20,33]. In addition, over a decade ago, Derynck and Feng proposed the possibility of a receptor threshold model for determining the specificity of TGF- $\beta$ 's effects. This model proposes that there is a critical expression level of the TGF- $\beta$  receptor that determines the specific TGF- $\beta$  responses of a cell [42,43]. Several lines of indirect evidence have supported this model, but to date there has been no direct assessment of this hypothesis [8,43-47]. We now provide direct evidence that the

regulation of the expression level of TGFBR2 can affect the specificity of the TGF- $\beta$  response. We have shown that the activation of the Smad and nonSmad signaling pathways can be modulated by the expression level of TGFBR2 and that the activation state of the nonSmad signaling pathway principally determines whether TGF- $\beta$  can induce CDKN1A/p21 mediated apoptosis in an epithelial cell line.

TGF- $\beta$  and the TGF- $\beta$  receptors are expressed in nearly all cell types and in developing and adult organisms. In embryonic development there is clear evidence that the concentration of active TGF- $\beta$  and the extent of TGF- $\beta$  signal pathway activation is tightly regulated and creates a gradient of responses that determines cell fate, among other things [41]. The majority of these studies have provided indirect and correlative evidence that the concentration of the ligands, receptors, and intracellular Smad proteins can determine the specific response of a cell to TGF- $\beta$ . Further indirect evidence that the expression level can affect the specificity of the TGF- $\beta$  response comes from studies of dominant negative *TGFBR2* transgenes and chimeric receptors [22,24,48-50]. Our studies now provide direct evidence that the TGFBR2 expression level can regulate the intensity of activation of the Smad and nonSmad signaling pathways. Furthermore, the differences in activation of the Smad and MAPK-ERK pathways at different TGFBR2 expression levels suggests that not only can the level of TGFBR2 determine the intensity of pathway activation but also which pathways are activated [33]. The differential regulation of the post-TGF- $\beta$  receptor pathways would be expected to lead to differences in transcription factor activation and transcription factor complex formation, which could then induce different patterns of gene expression.

Although the model above predicts that TGFBR2 expression levels will cause the differential expression of a variety of genes, we chose to specifically study *CDKN1A/p21* because of its well-demonstrated regulation by TGF- $\beta$  and because of its clear biological role in the inhibition of proliferation and induction of apoptosis. In addition, *CDKN1A/p21* is regulated by TGF- $\beta$  both by Smad and nonSmad signaling pathways and thus allows an assessment of the effect of Smad and nonSmad signaling on gene regulation [10,51]. Our data suggest that *CDKN1A/p21* is regulated by both the MAPK-ERK and Smad signaling pathways, which is true of other genes, such as *FURIN* [32]. These pathways may crosstalk at the level of Smad phosphorylation, nuclear localization of Smads, or at the level of transcription factor complex formation [33,52,53]. The differential activation of MAPK-ERK and Smad pathways suggests that at low levels of TGF- $\beta$  receptor activation the nonSmad signaling pathways predominate over the Smad signaling pathways in determining the effect of TGF- $\beta$  on the cell. The differences in activation of the MAPK and Smad pathways likely reflects the fact that the MAPK signaling pathway is a catalytic pathway whereas the Smad pathway is non-catalytic [8]. An interesting observation related to this explanation for the differential activation of the Smad and nonSmad pathways is the fact that in V-400R2 the MAPK-ERK pathway appears to be the predominant pathway regulating the level of *CDKN1A/p21* expression whereas the Smad pathway has a more modest effect. These results are consistent with those of Hu *et al* who observed a similar phenomenon in the HaCaT keratinocyte cell line, but are in contrast to other studies that have shown that both Smad and SP1 mediated signaling are necessary for *CDKN1A/p21* expression [20,30,54,55]. These differences may reflect cell line specific differences in the expression of other regulators of *CDKN1A/p21*, such as c-Myc or MIZ-1 [56,57].

There are several limitations related to our studies of the effect of TGFBR2 expression levels on TGF- $\beta$  signaling. At this time, it is not clear whether the effects we have observed involve direct or indirect mechanisms for regulating the Smad and nonSmad signaling pathways. This limitation reflects our incomplete understanding of the mechanisms through which TGF- $\beta$  affects the nonSmad signaling pathways [20]. In addition, we have not assessed the effect of TGFBR2 expression levels on the regulation of other signaling pathways and cannot exclude



the possibility that these pathways are also playing a role in the regulation of *CDKN1A/p21* and apoptosis that is affected by the expression level of the TGF- $\beta$  receptor. Nonetheless, our results demonstrate that TGF- $\beta$  receptor levels can regulate MAPK-ERK activation and that the control of this pathway has a central role in regulating *CDKN1A/p21* expression and CDKN1A/p21 mediated apoptosis in the V-400R2 cell line.

Our results have several implications for the role of TGF- $\beta$  in regulating normal cellular responses and for the role of TGF- $\beta$  in cancer. The relevance of the effect of TGFBR2 expression levels on the regulation of TGF- $\beta$ 's effects on cells is appreciated in light of prior studies that have shown decreased TGFBR2 expression in a variety of cancers and in some inflammatory states [58]. In fact, many cancers have suppressed *TGFBR2* expression without detectable mutations in any of the TGF- $\beta$  signal pathway genes [59] [60-62]. In these cancers with an intact TGF- $\beta$  receptor that is expressed at a low level, TGF- $\beta$  appears to have the potential to act as an oncogene as well as a tumor suppressor gene [63]. This paradoxical role of TGF- $\beta$  has been associated with its ability to activate Smad independent pathways (MAPK, PI3K and Rho) and with the modulation of TGF- $\beta$  signaling pathway activation through interactions with other signaling pathways induced by mutated oncogenes and tumor suppressor genes [8,18,21,22]. Our results suggest that an additional mechanism by which TGF- $\beta$  may mediate paradoxical effects on cells is through the down-regulation of the expression of TGFBR2, which could differentially activate signaling pathways and alter the gene expression patterns of the cells. Thus, our results not only demonstrate that the expression level of TGFBR2 can influence the pathway activation status of TGF- $\beta$  signaling pathways and the specific response of cells to TGF- $\beta$ , but also that the regulation of the expression level is a plausible mechanism for the paradoxical effects of TGF- $\beta$  on cancer cells. In summary, regulation of TGFBR2 expression levels is an additional mechanism for controlling the specificity of TGF- $\beta$ 's effects on cells and is a potential mechanism for the paradoxical effects of TGF- $\beta$  observed in cancer.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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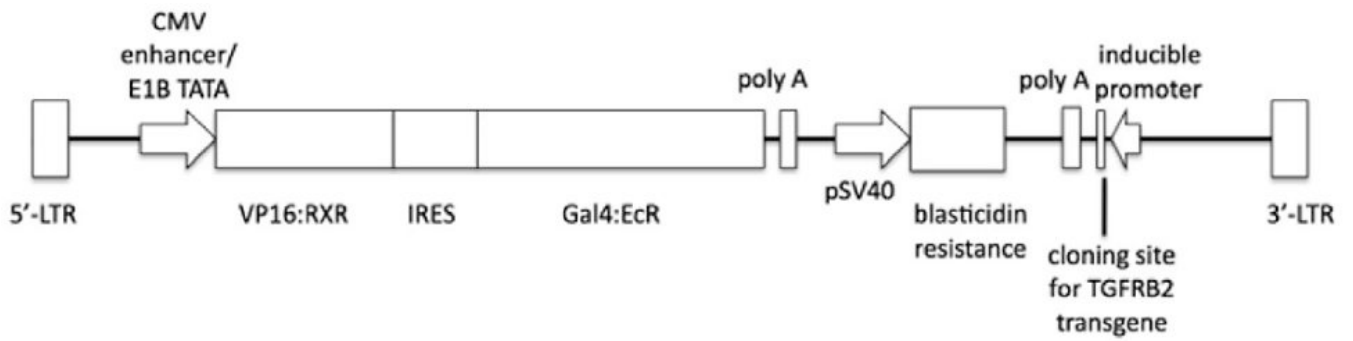
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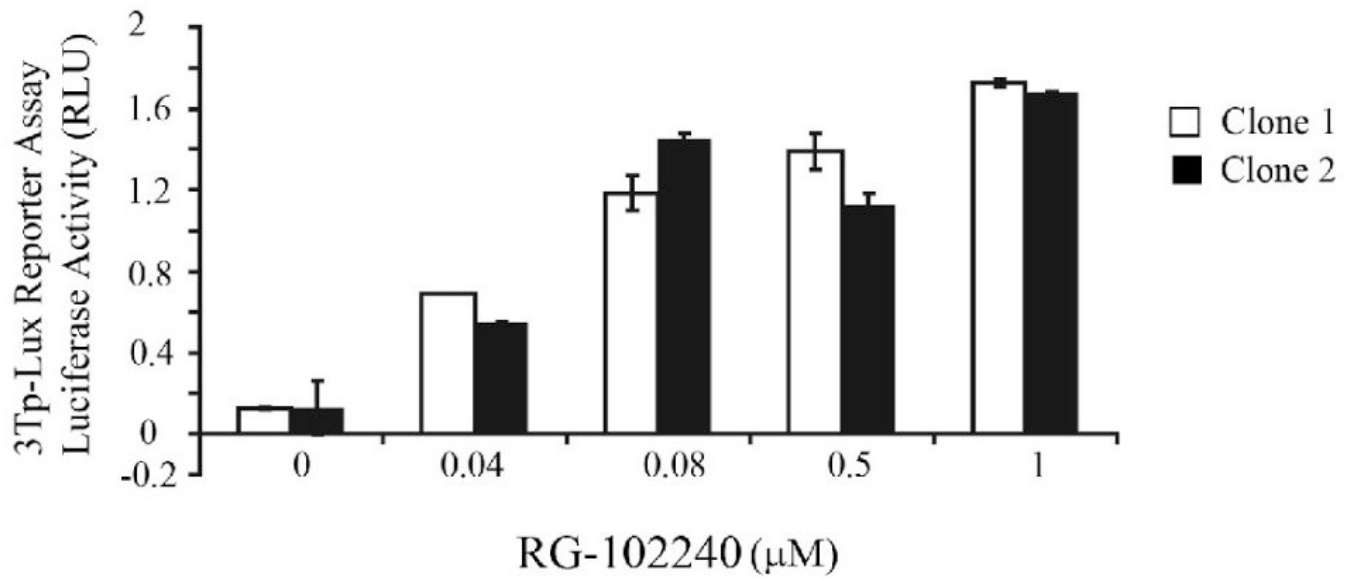
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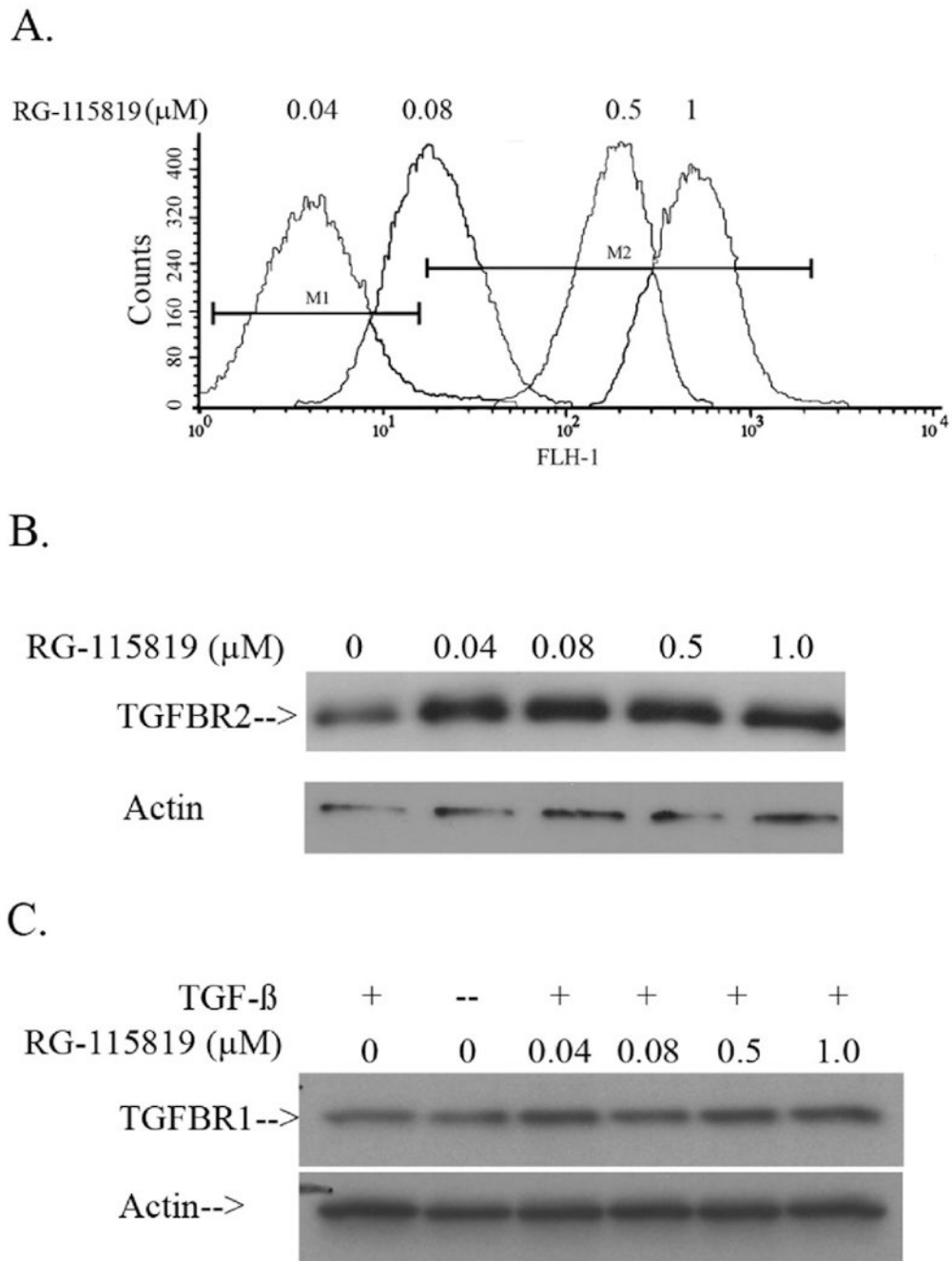
**Figure 1. Schematic representation of the p5004 vector**

The functional elements in the vector are shown, including the location of the *TGFRB2* transgene.



**Figure 2. Assessment of TGF- $\beta$  signaling in representative clones of V-400 after transduction with the inducible *TGFBR2* transgene**

V-400R2 clones 1 and 2 display a linear dose response from 0-1.0  $\mu$ M of RG-102240 with the greatest increase in 3TP-Lux activity occurring between 0  $\mu$ M and 0.8  $\mu$ M. 3TP-Lux activity was detected at low concentrations of RG-102240. Note that clone 1 has a wider dose-response range than clone 2.

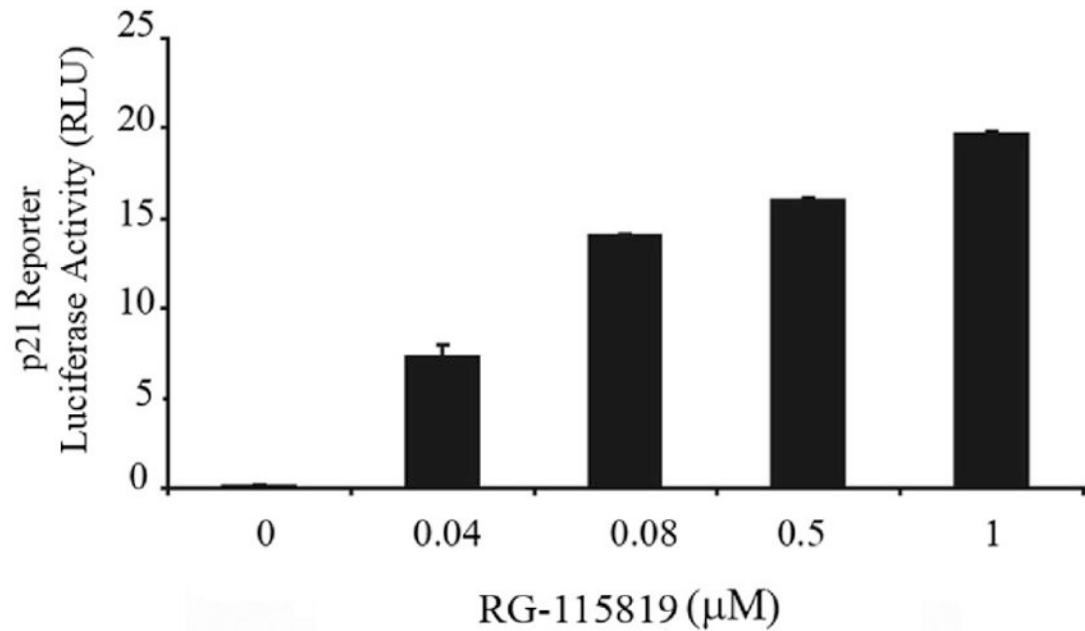


**Figure 3. The expression of cell surface TGFBR2 in V-400 after treatment with RG-115819**  
**A.** FACS analysis using an anti-HA antibody was performed after treatment with different doses of RG-115819 (0  $\mu$ M-1.0  $\mu$ M). An increased amount of cell surface TGFBR2 is detected with the increasing RG-115819 doses. M1 designates the fluorescence signal detected in the parental V-400 cell line. Of note, although no HA-tagged TGFBR2 can be detected with this technique after 0.04  $\mu$ M RG-115819 treatment, as shown in figure 1, TGF- $\beta$  induced gene expression is detected with this concentration of RG-115819 indicating that the transgenic receptor is present on the cell surface with this dose of RG-115819. The untreated V-400R2 clones showed the same level of fluorescence as that seen in the parental V-400 cell line. **B.** Immunoblotting using an anti-HA antibody was performed after treatment with different doses

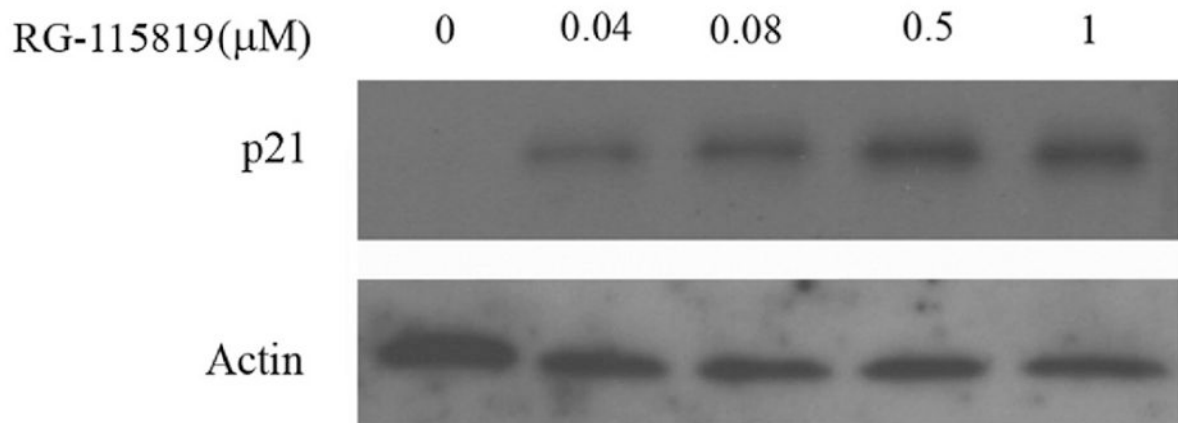
of RG-115819 (0  $\mu$ M-1.0  $\mu$ M). An increased amount of cell surface TGFBR2 is detected when comparing the untreated V-400R2 cell line to the cell line treated with the increasing RG-115819 doses although the resolution of immunoblotting does not reveal the changes in expression level as accurately as does flow cytometry. C. Immunoblotting using an anti-TGFBR1 antibody after treatment with different doses of RG-115819 (0 $\mu$ M-1.0 $\mu$ M) reveals no significant change in TGFBR1 expression with RG-115819 treatment. Expression of actin was assessed to control for protein loading.



A.



B.



**Figure 4. CDKN1A/p21 expression in V-400R2 cells after treatment with different concentrations of RG-115819**

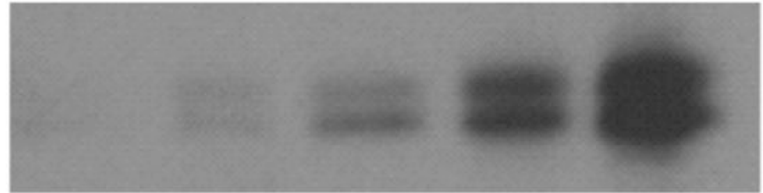
V-400R2 clone 1 was treated with four different concentrations of RG-115819 (0.04; 0.08; 0.4 and 1 μM) and then *CDKN1A/p21* expression was assessed. **A.** *CDKN1A/p21* luciferase reporter activity after 24 and 48 hours of RG-115819. Induction of *CDKN1A/p21* is present at 24 and 48 hours with a linear dose-response being evident at 48 hours. **B.** After 48 hours of treatment with RG-115819, a dose related increase in *CDKN1A/p21* protein expression is present, consistent with the *CDKN1A/p21* reporter assay results. Densitometry was performed on the bands, and these results were normalized for loading using the densitometry values from the corresponding actin immunoblot. The densitometry revealed a linear increase in band

density that correlated with increasing RG-115819 concentration [93 (0  $\mu\text{M}$ ), 90 (0.04  $\mu\text{M}$ ), 101 (0.08  $\mu\text{M}$ ), 121 (0.5  $\mu\text{M}$ ) and 159 (1  $\mu\text{M}$ )]. Image J software (NIH) was used for the densitometry analysis.

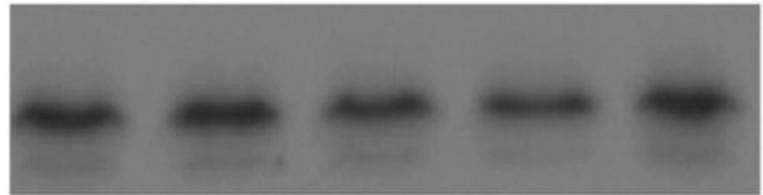
A.

RG-115819 ( $\mu\text{M}$ )                    0    0.04    0.08    0.5    1

pERK



ERK



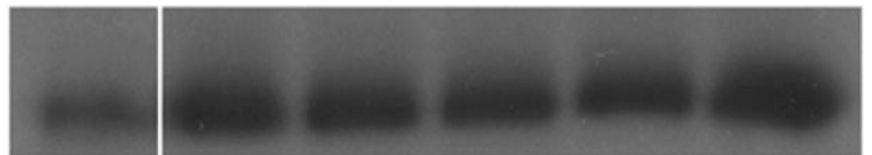
B.

RG-115819 ( $\mu\text{M}$ )                    NR    0    0.04    0.08    0.5    1.0

pSmad2



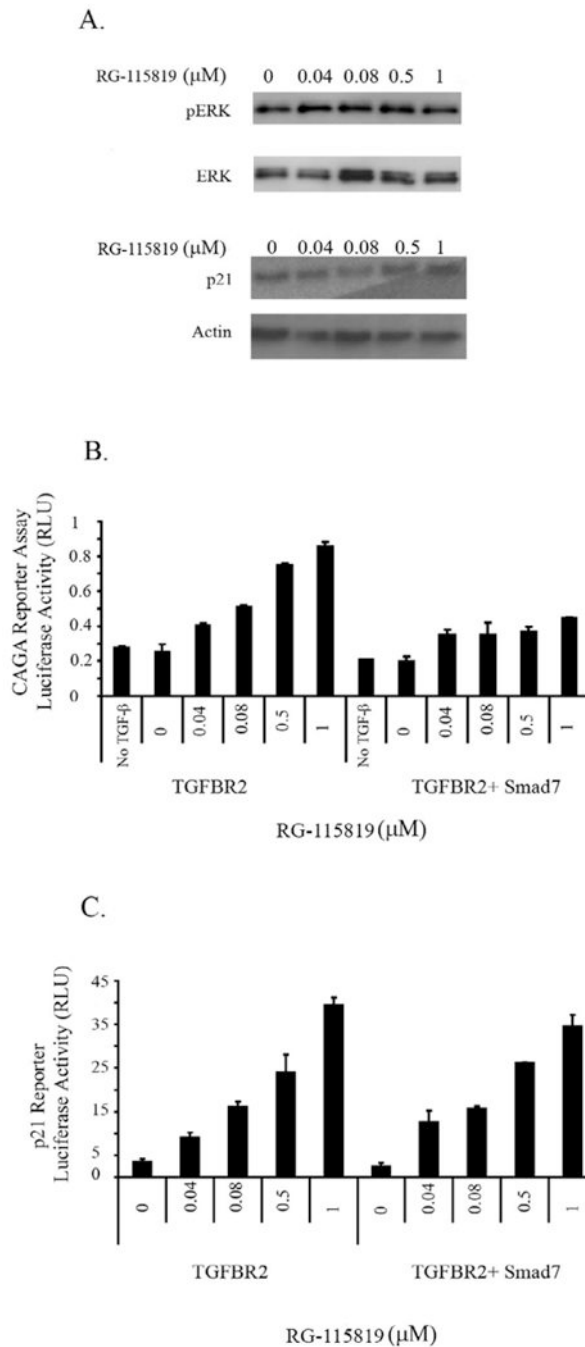
Smad2



**Figure 5. ERK and Smad activation after treatment with RG-115819 demonstrates a direct dose relation of RG-115819 to activation of both pathways**

**A.** Immunoblotting for phosphorylated ERK1/2 (pERK) was performed on V-400R2 treated for 48 hours with RG-115819 (0  $\mu\text{M}$ -1  $\mu\text{M}$ ). A dose-related increase in pERK in the cells is demonstrated. Total ERK levels reveal no differences with RG-115819 treatment. **B.** Immunoblot results of phosphorylated Smad2 (pSmad2) after 48 hours of treatment with RG-115819 also reveal a dose-related increase in the pSmad2 levels with doses greater than 0.08  $\mu\text{M}$ . The amount of increase in pSmad2 is less than that observed for pERK. Densitometry was performed on the bands, and these results were normalized for loading using the densitometry values from the corresponding actin immunoblot. The densitometry revealed a

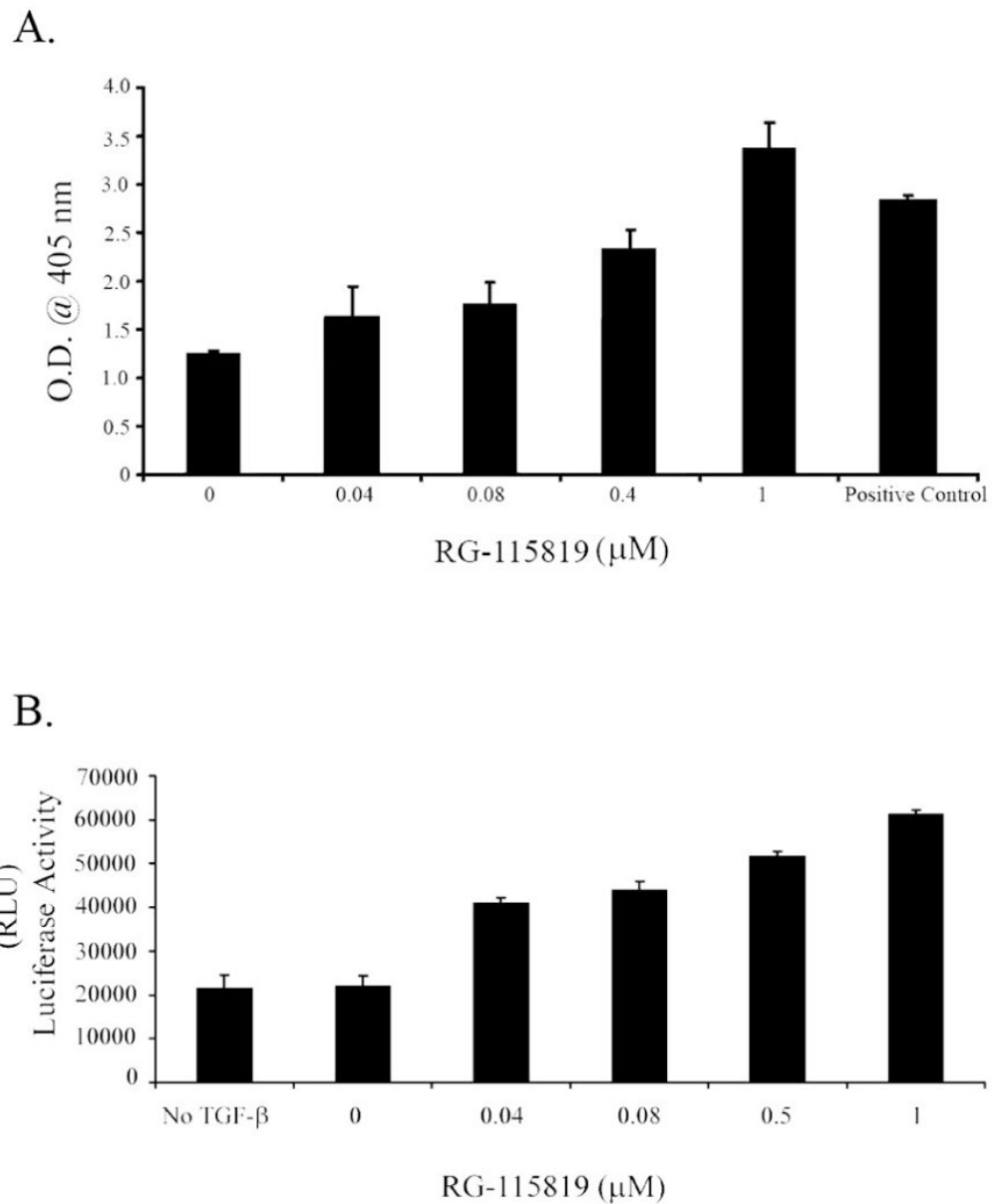
linear increase in band density that correlated with increasing RG-115819 concentration [60 (0  $\mu\text{M}$ ), 90 (0.04  $\mu\text{M}$ ), 101 (0.08  $\mu\text{M}$ ), 121 (0.5  $\mu\text{M}$ ) and 159 (1  $\mu\text{M}$ )]. Image J software (NIH) was used for the densitometry analysis.



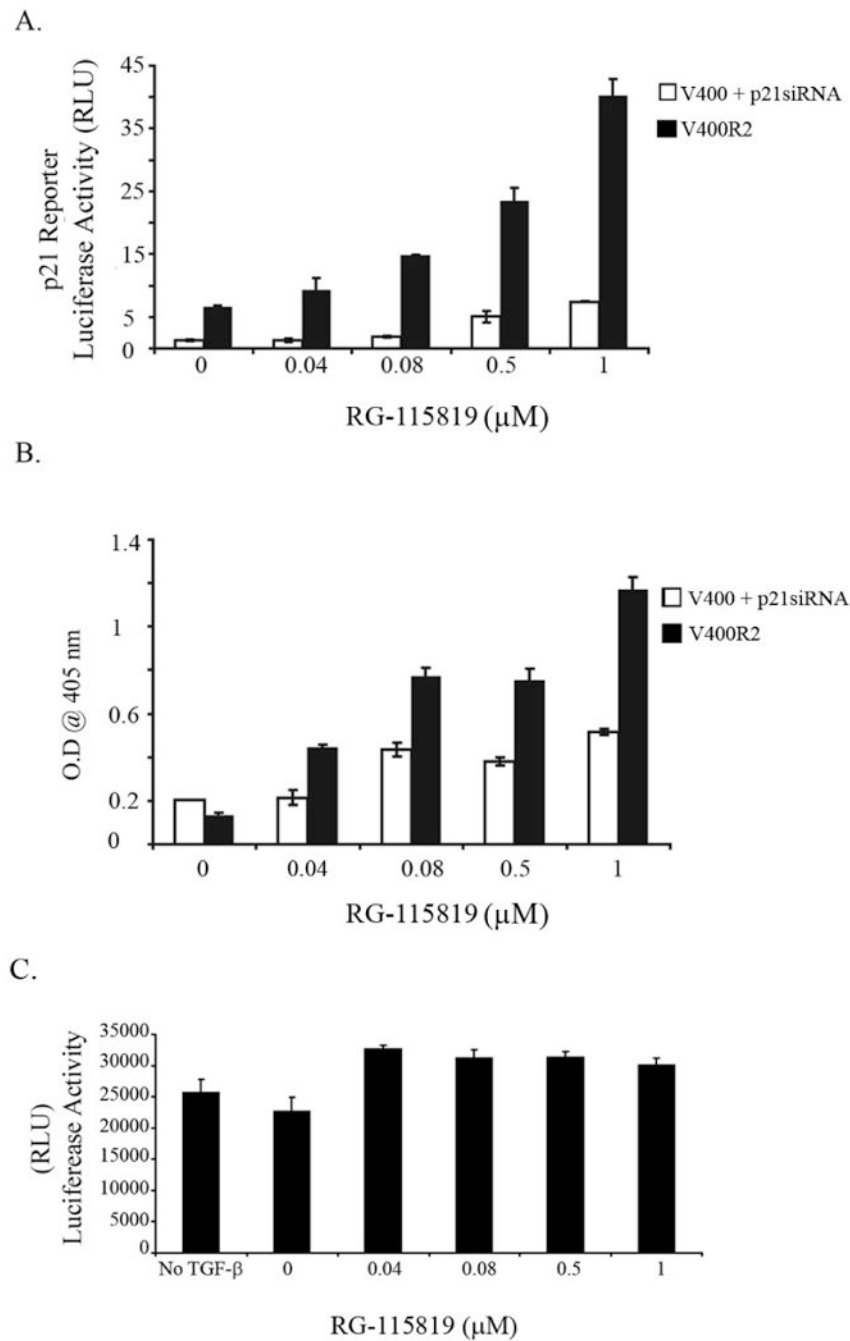
**Figure 6. *CDKN1A/p21* expression levels are regulated by MAPK/ERK in V-400R2 cells**

The V-400R2 cell line was cultured in the presence of RG-115819, TGF- $\beta$  (10ng/ml) and the MAPK/ERK inhibitor U0126 for 48 hours. **A.** No increase in phosphorylated ERK (pERK) or in *CDKN1A/p21* expression is detected in V-400R2 cells treated with the MAPK/ERK inhibitor demonstrating *CDKN1A/p21*'s expression is regulated by ERK in a TGFBR2 dependent manner. The activation of ERK is considerably reduced due to the presence of the inhibitor. Of note, total levels of ERK were not altered. *CDKN1A/p21* and pERK are detectable with vehicle only treatment, which is likely a consequence of a nonspecific effect of the U0126 vehicle. **B.** Inhibition of TGF- $\beta$  mediated 3TPLux reporter activity by Smad7 occurs with all concentrations of ligand. V-400R2 cells were transfected with a plasmid expressing Smad7 in

order to inhibit the Smad dependent pathway, and then transfected with the 3TP-Lux reporter. The dose dependent relation between luciferase activity and TGFBR2 is blocked by Smad7 demonstrating Smad7 is blocking Smad mediated transcription. C. Smad7 does not inhibit TGFBR2 dependent increases in *CDKN1A/p21* luciferase reporter activity. V-400R2 cells were transfected with both Smad7 and the *CDKN1A/p21* reporter assay. Interestingly, no significant changes in the activity of the *CDKN1A/p21* luciferase reporter is detected after Smad7 transfection demonstrating that the expression level of TGFBR2 does not regulate *CDKN1A/p21* expression through Smad signaling. The intensity of the bands was determined using densitometry as described in the prior figure legends. Densitometry for phosphorylated ERK revealed the following values: 120 (0  $\mu$ M), 130 (0.04  $\mu$ M), 126 (0.08 $\mu$ M), 145 (0.5  $\mu$ M) and 131 (1  $\mu$ M) and for p21: 130 (0  $\mu$ M), 135 (0.04  $\mu$ M), 142 (0.08  $\mu$ M), 142 (0.5  $\mu$ M) and 145 (1  $\mu$ M).



**Figure 7. High expression levels of *TGFBR2* induce apoptosis in V-400R2 cells**  
**A.** TGF- $\beta$  mediated apoptosis was assessed using the Cell Death ELISA in V-400R2 treated with RG-115819 and TGF- $\beta$  (10ng/ml). TGF- $\beta$  mediated apoptosis is increased with doses of RG-115819 greater than 0.4  $\mu\text{M}$  indicating that moderate-high levels of *TGFBR2* and *CDKN1A/p21* are needed for TGF- $\beta$  to induce apoptosis in these cells. **B.** TGF- $\beta$  mediated apoptosis assessed by caspase 3 and 7 activity confirms the results obtained with the Cell Death ELISA assay showing apoptosis directly correlates with *TGFBR2* levels.



**Figure 8. TGFBR2 dependent apoptosis is suppressed by *CDKN1A/p21* siRNA**

**A.** siRNA for *CDKN1A/p21* suppresses *CDKN1A/p21* reporter luciferase activity in the V-400R2 cell line. A dose related increase in luciferase reporter activity in the siRNA control treated cell line is demonstrated. **B.** Apoptosis in V-400R2 was measured using the Cell Death ELISA (Roche) after 48 hours of treatment with TGF- $\beta$  (10ng/ml). Treatment with the *CDKN1A/p21* siRNA inhibits the TGFBR2 dependent induction of apoptosis, which is seen in the siRNA control treated cells. **C.** TGF- $\beta$  mediated apoptosis is inhibited by SMAD7. Apoptosis was induced by TGF- $\beta$  in the V-400R2 cell line after transfection with SMAD7. SMAD7 abrogates the increased apoptosis observed in the cells V-400R2 cells treated with increasing concentrations of RG-115819.