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Retinoids regulate TGFβ signaling at the level of Smad2 phosphorylation and nuclear accumulation

Loretta L. Hoover, Elizabeth G. Burton, Megan L. O'Neill, Bonnie A. Brooks, Shilpa Sreedharan, Nineveh A. Dawson, Steven W. Kubalak *

Department of Cell Biology and Anatomy, Cardiovascular Developmental Biology Center, Medical University of South Carolina, 173 Ashley Avenue Suite 601BSB Charleston, SC 29425, USA

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ABSTRACT

Indirect regulation of transforming growth factor (TGF)-β signaling by retinoids occurs on a long-term timescale, secondary to transcriptional events. Studies by our group show loss of retinoid X receptor (RXR) alpha results in increased TGFβ2 in the midgestational heart, which may play a role in the cardiac defects seen in this model [S.W. Kubalak, D.R. Hutson, K.K. Scott and R.A. Shannon, Elevated transforming growth factor beta2 enhances apoptosis and contributes to abnormal outflow tract and aortic sac development in retinoic X receptor alpha knockout embryos, Development 129 (2002) 733–746.]. Acute and direct interactions between retinoid and TGFβ signaling, however, are not clearly understood. Treatment of dispersed hearts and NIH3T3 cells for 1 h with TGFβ and retinoids (dual treatment) resulted in increased phosphorylated Smad2 and Smad3 when compared to treatment with TGFβ alone. Of all dual treatments, those with the RXR agonist Bexarotene, resulted in the highest level of phosphorylated Smad2, a 7-fold increase over TGFβ2 alone. Additionally, during dual treatment phosphorylation of Smad2 occurs via the TGFβ type I receptor but not by increased activation of the receptor. As loss of RXRα results in increased levels of Smad2 phosphorylation in response to TGFβ treatment and since nuclear accumulation of phosphorylated Smad2 is decreased during dual treatment, we propose that RXRα directly regulates the activities of Smad2. These data show retinoid signaling influences the TGFβ pathway in an acute and direct manner that has been unappreciated until now.

1. Introduction

TGFβ is a pleiotropic cytokine which affects cell growth, differentiation, apoptosis, immune modulation and epithelial to mesenchymal transformation [2]. With or without the assistance of the TGFβ type III receptor (TβRIII) [3] TGFβ isoforms bind to the constitutively active type II receptor (TβRII) promoting its heterodimerization with and activation of the type I receptor (TβRI) [4]. TβRI then acts as a serine/threonine kinase that phosphorylates the C-terminal SXS motif of the most immediate downstream signal, TGFβ-activated receptor Smads, Smad2 and Smad3 (Smad2/3) [4]. Smad2/3 can be phosphorylated at alternate motifs by other kinases outside of the canonical TGFβ signaling scheme [5] modifying the downstream events normally ascribed to TGFβ-mediated Smad signaling [6]. Once phosphorylated by TβRI, Smad2/3 heterodimerize with Smad4, i.e. the common Smad, aptly named because it is common to both the TGFβ and bone morphogenetic protein (BMP) pathways [4]. The Smad4/Smad2/3 complex then accumulates in the nucleus [7] where it interacts with co-activators and -repressors to regulate the transcription of specific target genes [8].

There are several points of control between TGFβ binding its receptor and affecting downstream transcriptional targets. SARA, the Smad anchor for receptor activation, regulates the subcellular localization of Smad2 and its availability to the kinase action of TβRI [9]. The inhibitory Smads, Smad6 and Smad7, block receptor Smad phosphorylation via competitive inhibition at the level of the receptor [10]. The expression of Smad7 is positively regulated by activation of the canonical TGFβ cascade and therefore serves as a negative feedback mechanism to blunt TGFβ signaling [11]. Smurfs, Smad-ubiquitin regulatory factors, are E3 ligases that mediate Smad and TGFβ receptor ubiquitination and proteasomal degradation. Smurf2 has been shown to ubiquitinate newly activated Smad2 that has accumulated in the nucleus [12]. Smurfs also interact with Smad7 to regulate ubiquitination and proteasomal degradation of TβRI [13].

Once activated and in the nucleus, the transcriptional effects of TGFβ-activated receptor Smads can be blunted by binding the transcriptional co-repressors Ski and Sno [14]. Additionally, the phosphatase activity of protein phosphatase 1A (PPM1A) is known to regulate the duration of TGFβ's transcriptional effects by decreasing the amount of SXS-motif-phosphorylated Smad2 (pSmad2) in the nucleus [15].

The retinoid derivatives of vitamin A show their own canonical signaling cascade, which ultimately regulates developmental

* Corresponding author.
E-mail address: kubalaks@musc.edu (S.W. Kubalak).

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patterning and apoptosis. Retinoids are enzymatically converted from vitamin A and act as the ligands which agonize retinoid receptors promoting their nuclear import [16], dimerization, and transcriptional effects [17–19]. Retinoid receptor hetero- (RXR/RAR) or homo- (RXR/RXR) dimers bind to retinoic acid response elements (RARE) or retinoid X response elements (RXRE), respectively, within the promoter regions of retinoid target genes such as Hox family members, the metabolic regulator phosphoenolpyruvate carboxykinase, and retinoid receptors themselves [20]. As such, retinoid receptors are transcriptional regulators, however, there is evidence to suggest that they serve additional purposes in the cell. It is known that retinoid signaling can affect TGFβ signaling at the level of the receptor Smads. Cao et al. proposed that all-*trans* retinoic acid (atRA) mediates the activity of an unidentified Smad2 phosphatase leading to lower levels of activated Smad2 [21]. It is unclear if this purported phosphatase and PPM1A are one and the same. It has also been reported that antagonist-treated and therefore unliganded RAR binds Smad3 and increases Smad3-driven transcription [22]. Additionally the transcriptional corepressor TGFβ-induced factor homeobox (TGIF) has been shown to regulate Smad2- and RXR-driven transcription [22,23]. During high levels of TGFβ signaling indicated by increased nuclear pSmad2, TGIF binds the Smads and prevents their interaction with transcriptional co-activators CBP and p300, effectively blunting Smad-mediated transcription [24,25]. Under conditions of decreased nuclear pSmad2, however, TGIF binds RXREs, blocking the transcriptional activity of RXR homodimers [26].

Clearly there is growing evidence that TGFβ and retinoid pathways reciprocally regulate each other on a long-term time scale secondary to transcriptional and translational changes. Our findings herein represent the first report examining the acute effect of retinoids on TGFβ-activated receptor Smad phosphorylation and nuclear accumulation.

2. Materials and methods

2.1. Cell lines and treatments

NIH3T3 cells were obtained from ATCC and maintained according to supplier's recommendations. RXRα knockout (RXRα−/−) and matched wild type littermate mouse embryonic fibroblasts (MEFs)

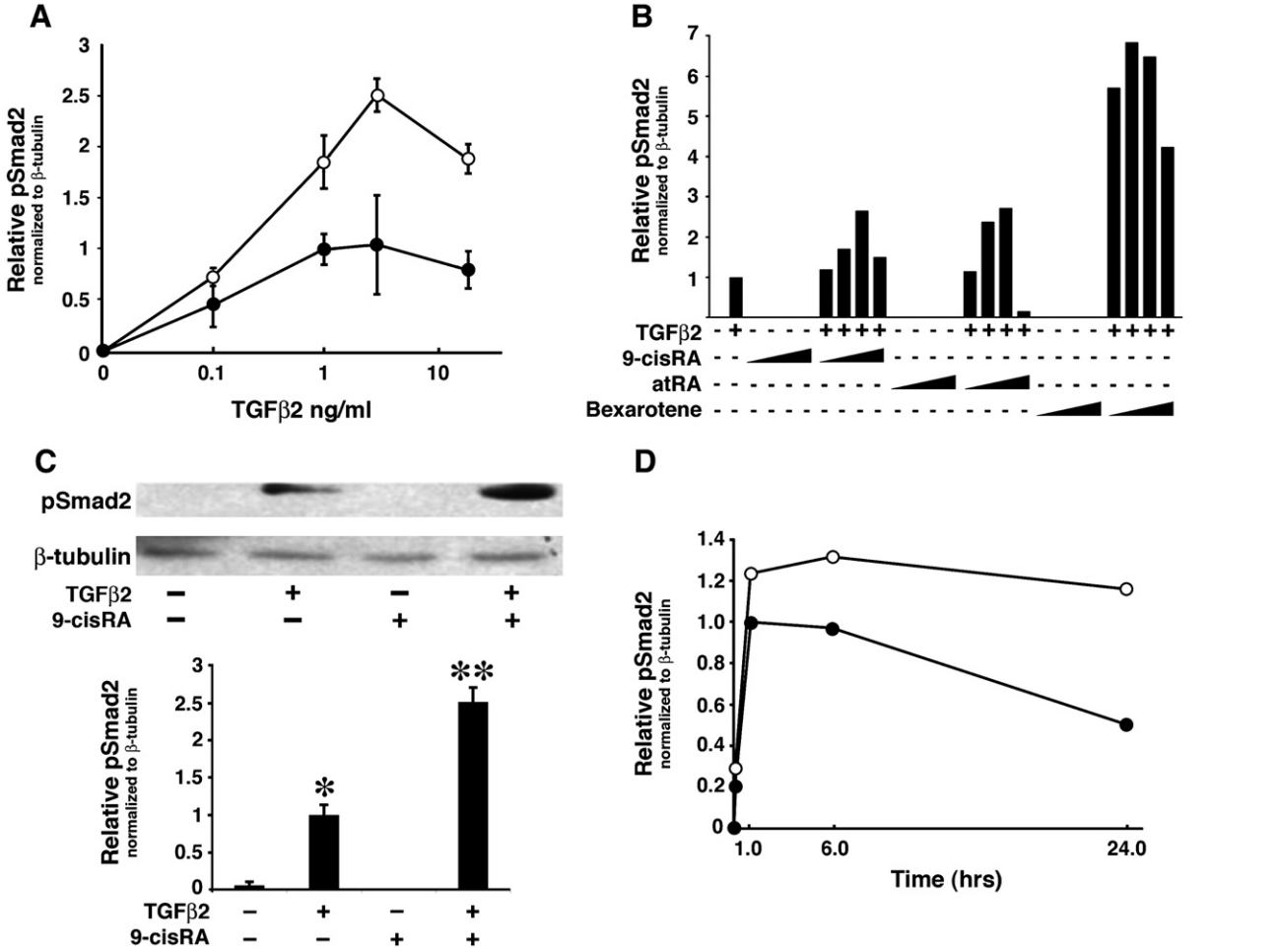

Fig. 1. Retinoids, in the presence of TGFβ2, increase pSmad2 levels above those seen after a one-hour treatment with TGFβ2 alone. (A) NIH3T3 cells were treated for 1 h with 0.1–20 ng/ml of TGFβ2 in the absence (closed circles) or presence (open circles) of 75 nM 9-*cis*RA. Total cellular proteins were subjected to Western blot and pSmad2 levels were densitometrically normalized to β-tubulin. The 1.0 ng/ml TGFβ2 treatments were averaged and assigned a value of one and all other values were further normalized to this data point. Results are mean ± one S.E. (B) NIH3T3 cells were treated for 1 h with varying concentrations of three retinoid species in the absence or presence of 3 ng/ml TGFβ2. Retinoid concentration ranges: atRA — 10 nM, 50 nM, 100 nM, 400 nM; Bexarotene — 25 nM, 100 nM, 200 nM, 400 nM; 9-*cis*RA — 10 nM, 25 nM, 75 nM, 400 nM. Total cellular proteins were subjected to Western blot and pSmad2 levels were normalized to β-tubulin. To demonstrate relative differences between treatments, normalized pSmad2 levels obtained from TGFβ2 treatment were assigned a value of one. (C) NIH3T3 cells were treated with diluent, 3 ng/ml TGFβ2, 75 nM 9-*cis*RA or both for 1 h. Phosphorylated Smad2 and β-tubulin were detected using Western blot. To demonstrate relative differences between treatments, pSmad2 normalized to β-tubulin obtained from TGFβ2 treatment was assigned a value of one. Results are one S.E. of the mean. * indicates a *p*-value of .002 between control and TGFβ2 groups. ** Represents a *p*-value of .001 between TGFβ2 and TGFβ2 plus 9-*cis*RA groups. (D) NIH3T3 cells were treated for 5 min, 1 h, 6 h and 24 h with 3 ng/ml TGFβ2 in the absence (closed circles) or presence (open circles) of 75 nM 9-*cis*RA and total cellular proteins were subjected to Western blot. Phosphorylated Smad2 levels were normalized to β-tubulin, and further normalized to the one-hour TGFβ2 treatment. One representative time course of three separate experiments is shown.

were isolated from E12.5 embryos generated from timed RXRα+/− matings and maintained as previously described [27]. Embryonic tails were genotyped as described [1]. For dispersed heart cultures, hearts minus atria were dissected from E12.5 embryos. Hearts were placed in 0.05% trypsin EDTA (Gibco) and incubated at 37 °C with gentle trituration every 2 min until minimal intact tissue remained, approximately 6 min total. Dispersed heart media (DMEM+10% rat serum) was added to the mixture at a 1:1 concentration and cells were pelleted via centrifugation (200 ×g for 5 min). Supernatant was removed and pellets were resuspended in 100 μl of media per heart followed by micromass plating in 35 mm cell culture dishes (Corning). Cultures were maintained at 37 °C, 5% CO_2. Twelve hours later, 900 μl of media was added to each dish and cells were allowed to incubate for an additional 60 h. For one-hour treatments, 300,000 MEFs or NIH3T3 cells were plated in 35 mm dishes 24 h prior to use. All cells and dispersed hearts were serum starved for 2 h before addition of atRA (Sigma), 9-*cis*-retinoic acid (9-*cis*RA) (Sigma), Bexarotene (Toronto Chemical Company), TGFβ1 and TGFβ2 (R&D). Retinoids were diluted in DMSO (Sigma) and TGFβ isoforms were diluted in 4 mM HCl with 0.1% bovine serum albumin (BSA). All "diluent" (i.e. control) treatments received appropriate volumes of DMSO and TGFβ diluent. For TβRI kinase inhibition, cells were serum starved prior to addition of SB431452 reconstituted in DMSO. Cells were incubated for 30 min with inhibitor prior to addition of TGFβ and retinoids.

2.2. Immunoblotting

Rabbit anti-phosphorylated Smad2 (Ser465/467, cat. #3108), mouse anti-Smad2 (#3103), rabbit anti-phosphorylated Smad2 (Ser245/250/255, #3108), rabbit anti-phosphorylated Smad3 (Ser425/425, #9520) and rabbit anti-Histone H3 (#9175) were obtained from Cell Signaling Technologies; mouse anti-β-tubulin (Accurate Chemical Company) and HRP-conjugated-anti-phosphorylated threonine (Santa Cruz Biotechnology) were used for Western blotting. Total proteins were harvested using a standard RIPA buffer (20 mM Tris pH 7.5, 100 mM NaCl, 0.5% NP-40, 0.5 mM EDTA, 0.5 mM PMSF) containing protease inhibitors (Complete Mini, Roche, Palo Alto, CA) or fractionated with the Ne-Per Cytoplasmic Nuclear Extraction Kit according to manufacturers recommendations (Pierce). Equal volumes were boiled with denaturing loading buffer (200 mM Tris–HCl pH 6.8, 50% Glycerol, 8% SDS, 400 mM DTT, 0.4% Bromophenol Blue) and run on a 10% denaturing polyacrylamide gel for 120 min at 130 V. Membrane protein enrichment for TβRI analysis was accomplished with a two-step RIPA protocol. Briefly, for step one, cells were lysed in RIPA, pelleted via centrifugation and the supernatant was reserved. For step two, the resulting pellet was resuspended in RIPA and subject to constant gentle rocking at 4 °C for 1 h with 10 s of maximum speed vortexing every 10 min. Following centrifugation the supernatants from step one and two were combined and utilized for further assays.

2.3. Immunocytochemistry

Five thousand NIH3T3 cells were seeded into 4-well chamber slides (Lab-Tek) and incubated overnight. Cells were serum starved for 2 h prior to a one-hour treatment with diluent or combinations of 3 ng/ml TGFβ2 and 75 nM 9-*cis*RA. Total Smad2 immunolocalization was performed using total Smad2 antibody (Cell Signaling Technologies # 3122) according to the manufacturer's suggested protocol. Anti-rabbit Alexa Fluor® 488 secondary antibody (Invitrogen) was applied at a concentration of 1:100 prior to coverslipping. Photographic documentation was performed with a Leica TCS SP2 AOBS confocal microscope. Relative nuclear Smad2 localization was determined by maintaining constant laser/gain settings throughout microscopic documentation and by counting nuclear pixel density using ImageJ software (freeware).

2.4. Immunoprecipitation of TβRI

Twenty microliters of agarose-conjugated rabbit anti-TβRI (Santa Cruz Biotechnology) was incubated overnight at 4 °C with 500 μg of membrane-enriched proteins harvested from treated or untreated NIH3T3 cells. Agarose beads plus bait and prey proteins were pelleted, washed four times in RIPA buffer and resuspended in 2× denaturing loading buffer. SDS PAGE was performed as described above.

2.5. Luciferase assay

Mink lung epithelial cells (MLEC) stably transfected with a PAI-1-luciferase reporter construct were obtained from Dr. Daniel Rifkin and were maintained as previously described [28]. In 96-well format,

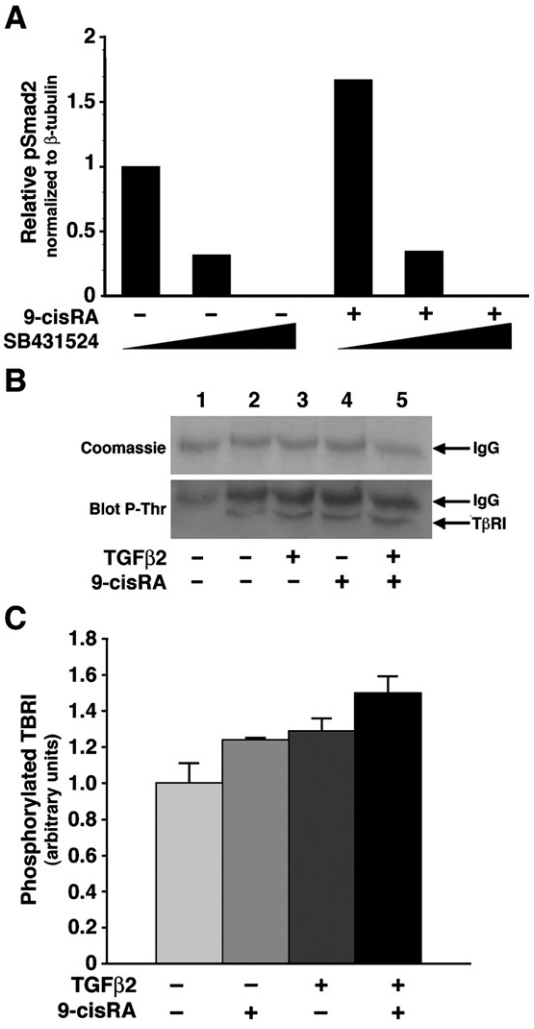

Fig. 2. Phosphorylation of Smad2 SXS motif occurs solely via TβRI during co-treatment with TGFβ2 and 9-*cis*RA. (A) NIH3T3 cells were pretreated for 30 min with 0, 1.0 or 10 μM of TβRI inhibitor (SB431542) prior to addition of 3 ng/ml TGFβ2 in the absence or presence of 75 nM 9-*cis*RA. Total cellular proteins were subjected to Western blot and pSmad2 levels were normalized to β-tubulin levels using densitometry values obtained from Image J Software. For further normalization, TGFβ2 treatment in the absence of inhibitor was assigned a value of 1. (B) TβRI was immunoprecipitated from membrane fraction enriched proteins of NIH3T3 cells treated with diluent (Lane 2), 3 ng/ml TGFβ2 (Lane 3), 75 nM 9-*cis*RA (Lane 4) or both (Lane 5) for 1 h. Lane 1 shows quiescent NIH3T3 cells that have experienced no media change. Phosphorylation of TβRI was detected using HRP-conjugated anti-phospho-threonine primary antibody (P-Thr). (C) Densitometry values obtained from Image J Software were used to normalize phosphorylated TβR1 levels to IgG. Diluent treatment was assigned an arbitrary value of 1.0 and results are shown as one S.E. of the mean.

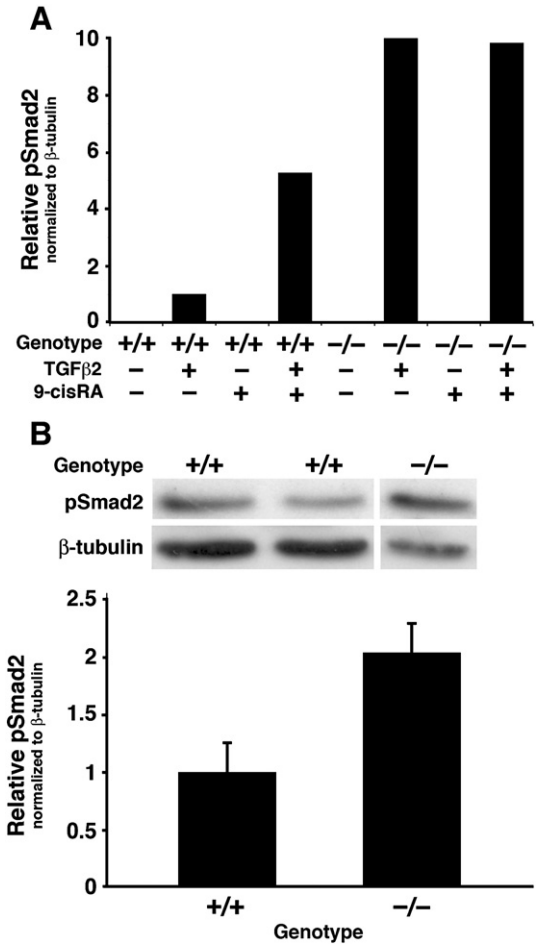

Fig. 3. Loss of RXRα leads to increased TGFβ2 mediated-Smad2 phosphorylation. (A) Wild type (+/+) and RXRα−/− (−/−) MEFs were treated for 1 h with 3 ng/ml TGFβ2, 75 nM 9-*cis*RA or both. Total cellular proteins were subjected to Western blot analysis and pSmad2 levels were densitometrically normalized to β-tubulin. Treatment of wild type cells with TGFβ2 alone was assigned a value of one for further normalization. (B) Wild type (+/+) and RXRα−/− (−/−) dispersed whole hearts were treated for 1 h with 3 ng/ml TGFβ2. Total cellular proteins were subjected to Western blot analysis and pSmad2 levels were normalized to β-tubulin levels using densitometry values obtained from Image J Software. To demonstrate relative differences between genotypes, normalized pSmad2 levels obtained from treatments of wild type hearts were assigned a mean value of one. Results are shown as one S.E. of the mean.

15,000 cells were plated per well in test media (DMEM+0.1% BSA) 3 h prior to use and treated for either 1 or 7 h with combinations of TGFβ (3 ng/ml) and retinoids (75 nM). Following the one-hour treatment, the cells were washed with PBS and incubated for an additional 6 h in test media. Treatments were executed in triplicate and luciferase assay was performed using Bright Glo reagent (Promega) according to the manufacturer's suggested protocol. Luciferase levels were obtained by 30-second well readings on a 96-well format luminometer (Perkin-Elmer).

2.6. Densitometry

Immunoblot band intensities were determined using ImageJ software and pSmad levels were normalized to β-tubulin levels. β-tubulin was utilized as a loading control for densitometry and was considered more appropriate than total Smad2 as dephosphorylated Smad2 undergoes TGFβ-activated proteasomal degradation [12]. This degradation occurs rapidly in response to TGFβ and can affect the apparent levels of total Smad2 (Supplemental Figure 1 and [12]). β-tubulin levels were not affected by a one-hour treatment with the ligands used in these studies (Supplemental Figure 1).

2.7. Statistics

Parametric or non-parametric statistical tests were performed using SPSS software when appropriate. Statistical significance was assumed at a p-value=.05. For non-quantified experiments, representative data from a minimum of three independent trials are shown.

3. Results

3.1. One-hour co-treatment with TGFβ and retinoids enhances pSmad2 accumulation compared to treatment with TGFβ alone

While it is known that downstream interactions between TGFβ and retinoid signaling pathways exist, it is unknown if they affect each other on an acute timescale. To explore potential acute interactions between TGFβ and retinoids we treated NIH3T3, MEFs and dispersed heart cells for 1 h with combinations of TGFβ1 or TGFβ2 and retinoid isoforms of varying receptor affinities [29] and determined the levels of subsequent Smad2 and Smad3 phosphorylation. A one-hour time point was chosen because, as previously reported, a one-hour treatment with TGFβ alone results in maximum accumulation of phosphorylated Smad2 from the undetectable levels seen at time zero [12]. Concentration–response studies with varying TGFβ2 concentrations in the absence or presence of 75 nM 9-*cis*RA resulted in maximum Smad2 phosphorylation at 3 ng/ml TGFβ2 (Fig. 1A). Phosphorylated Smad3 levels were similarly affected as a result of dual treatment (data not shown). Likewise, treatment with varying concentrations of 9-*cis*- or atRA combined with 3 ng/ml TGFβ2 altered the Smad2 phosphorylation profile in a manner that was

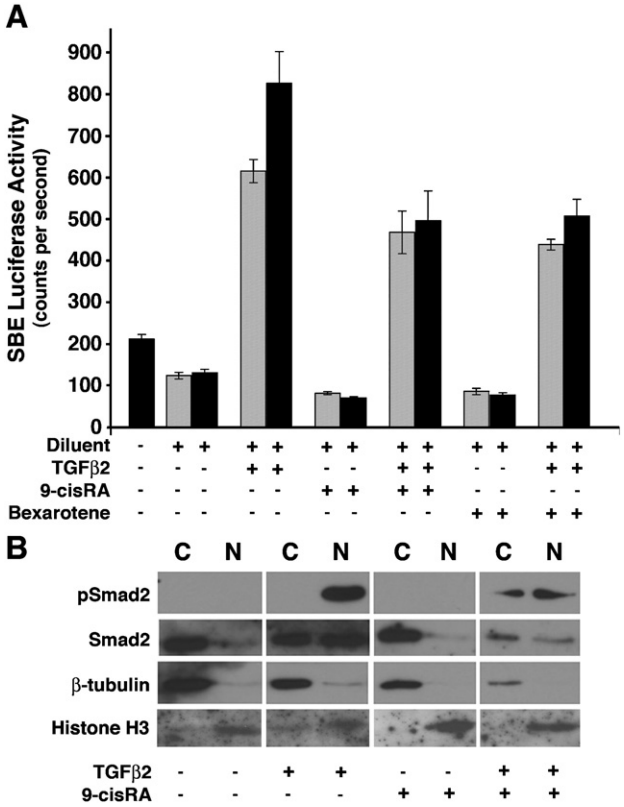

Fig. 4. Retinoid ligands blunt Smad2-nuclear accumulation and transcriptional activity. (A) MLEC cells harboring the PAI-1-luciferase construct were used to infer levels of TGFβ-activated receptor Smad-driven transcription in the absence and presence of retinoids. Cells were treated with indicated treatments for one (light bars) or 7 h (dark bars), washed and assayed for luciferase levels 7 h after initiation of treatment. Results show one S.E. of mean. (B) NIH3T3 cells were treated for 1 h with diluent, 3 ng/ml TGFβ2, 75 nM 9-*cis*RA, or both. Cytoplasmic (C) and nuclear (N) proteins were enriched and subjected to Western blot. Histone H3 and β-tubulin were utilized as nuclear and cytoplasmic loading controls, respectively.

retinoid concentration-dependent (Fig. 1B). At maximally effective concentrations of 9-*cis*- or atRA combined with TGFβ2 (3 ng/ml), pSmad2 levels were nearly three fold increased over those seen with TGFβ2 treatment alone (Fig. 1C). A similar Smad2 phosphorylation profile was observed using TGFβ1 in concert with 9-*cis*RA (data not shown). Dual treatment with the RXR specific ligand, Bexarotene, in a concentration range which solely targets RXR resulted in a seven-fold increase in pSmad2 levels over those seen after treating with TGFβ2 alone (Fig. 1B). Additionally, time course experiments showed that TGFβ2 treatment results in a peak level of pSmad2 at one-hour posttreatment followed by a return toward basal levels 24 h later (Fig. 1D). Adding retinoids to the TGFβ2 treatment results in higher levels of pSmad2, which remain elevated at 24-hour posttreatment (Fig. 1D) demonstrating that retinoids not only potentiate the phosphorylation of Smad2 but also affect the dynamics of its dephosphorylation. Lastly, phosphorylation of the Smad2 linker domain was not affected by any combination of TGFβ or retinoid treatment (Supplemental Figure 2 and data not shown) demonstrating that this potentiated phosphorylation effect is specific to the SXS motif of Smad2.

3.2. Phosphorylation of Smad2 during dual treatments occurs via TβRI

To begin to investigate the mechanism of the potentiation of Smad2 phosphorylation in the presence of TGFβ and retinoids, NIH3T3 cells were pretreated with SB431542, a specific and potent inhibitor of TGFβ-induced phosphorylation of Smad2 which acts on TβRI [30]. With inhibitor present, cells were treated for 1 h with TGFβ2 alone or in combination with 9-*cis*RA followed by Western blot analysis of pSmad2 levels. Loss of Smad2 phosphorylation was seen in a SB431542 dose-dependent manner for both TGFβ2 and TGFβ2 plus 9-*cis*RA treated cells (Fig. 2A). These data show that all phosphorylation of Smad2 occurs via action of TβRI regardless of the presence of retinoids and importantly rules out the action of alternate kinases. We next asked if retinoids in the presence of TGFβ lead to increased activity of TβRI as assessed by detecting phosphorylation of threonines within TβRI itself. NIH3T3 cells were treated with combinations of 9-*cis*RA and TGFβ2 for 1 h and membrane proteins were extracted. Immunoprecipitation of TβRI was performed followed by Western blot using HRP-conjugated anti-threonine antibodies. In

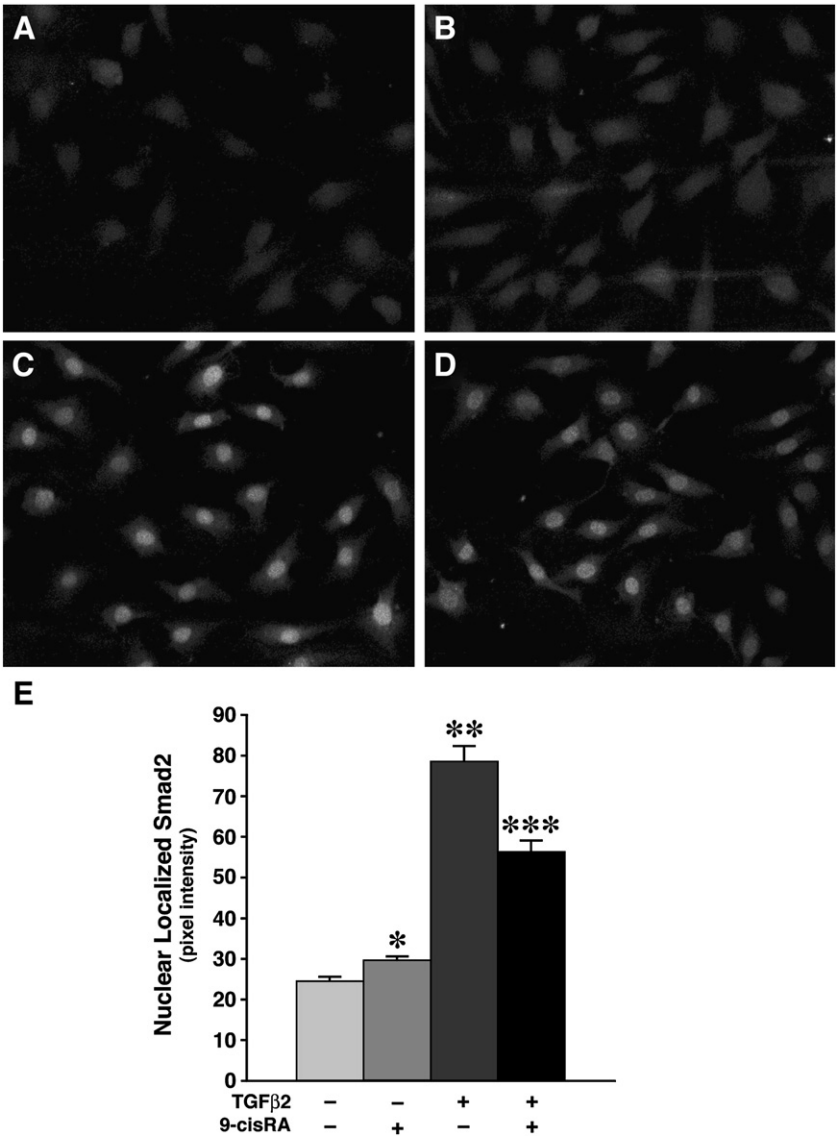

Fig. 5. During TGFβ2 treatment, Smad2 shows less nuclear localization in the presence of retinoids. (A) NIH3T3 cells were treated for 1 h with diluent, (B) 75 nM 9-*cis*RA, (C) 3ng/ml TGFβ2, and (D) a combination of 3 ng/ml TGFβ2 plus 75 nM 9-*cis*RA. Immunocytochemistry of Smad2 was performed and subcellular localization determined by confocal microscopy. (E) Densitometry of nuclear regions was performed using Image J software and average nuclear pixel intensity is shown. Error bars denote one S.E. of the mean. * Represents a *p*-value of 0.001 between RA and diluent. ** Represents a *p*-value of 0.0001 between TGFβ2 and either diluent or RA. *** Represents a *p*-value of 0.0001 between TGFβ2 plus RA and all other treatments.

accordance with the literature [31,32], we found that untreated cells (no media change) show no detectable basal levels of TβRI threonine phosphorylation (Fig. 2B Lane 1) whereas a one-hour treatment with ligands resulted in TβRI phosphorylation (Fig. 2B Lanes 3–5). Phosphorylation in response to TGFβ (Lane 3) was increased above that seen for diluent treatment (Lane 2). Densitometric analysis showed that treatment with 9-*cis*RA plus TGFβ2 did not result in statistically significant elevation (p=.151) of phospho-threonine levels compared to levels seen with TGFβ treatment alone (Fig. 2C) suggesting that increased activity of the type I receptor is not the mechanism by which pSmad2 is elevated during co-treatment with TGFβ and retinoids.

Interestingly, our results showed that the act of changing media can result in a detectable level of phosphorylation of the TβRI and though beyond the scope of this study, we suspect that shear fluid stress may play a role in this effect. A 2004 article by Morgera et al., described that adding fluid shear stress in the presence of high glucose media (which is utilized in our experiments) led to a "30% increase in TGFβ release compared to glucose stress alone" in a mesothelial cell model [33]. This released TGFβ could act in an autocrine manner causing some phosphorylation of the TβRI.

3.3. MEFs and dispersed hearts from RXRα−/− embryos show increased pSmad2 following treatment with TGFβ2 in the absence of added retinoids

In order to determine the potential role of RXRα in TGFβ2-mediated Smad2 phosphorylation, wild type and RXRα−/− MEFs and dispersed heart cells were generated from E12.5 mice. Cells were treated for 1 h with TGFβ2 in the absence or presence of 9-*cis*RA and pSmad2 levels were detected using Western blot. RXRα−/− MEFs (Fig. 3A) and dispersed hearts (Fig. 3B) showed higher levels of pSmad2 in response to a one-hour TGFβ2 treatment when compared to matched wild type controls. Moreover, when cells were co-treated with 9-*cis*RA and TGFβ2, pSmad2 levels were not elevated beyond that of TGFβ2 alone (Fig. 3A) suggesting that RXRα likely plays a direct role in controlling the levels of TGFβ2-mediated Smad2 phosphorylation in both the absence and presence of added RA.

3.4. Potentiation of Smad phosphorylation secondary to dual treatment does not yield increased receptor Smad-driven transcription

To determine the downstream signaling potential of the increased pSmad2/3 resulting from dual treatment versus that of treating with TGFβ alone, we utilized MLEC cells stably transfected with PAI-1-luciferase reporter. We treated equal numbers of cells and assayed for luciferase production 7 h after a one- or seven-hour exposure to TGFβ and/or retinoids. A one-hour exposure to TGFβ2 alone followed by a washout and six-hour serum free incubation resulted in similar levels of luciferase production as a seven-hour exposure to TGFβ2 (Fig. 4A). This finding alone demonstrates that the downstream effect of TGFβ2 is determined rapidly, and that continual exposure to ligand is not necessary to yield significant transcriptional changes. Interestingly, at seven-hour posttreatment, the one- and seven-hour dual treatments with TGFβ2 and 9-*cis*RA resulted in less luciferase production than seen after treatment with TGFβ2 alone (Fig. 4A) suggesting that although pSmad2/3 levels are escalated by 1 h after dual treatment, only a fraction of this pSmad pool is positively regulating transcrip-

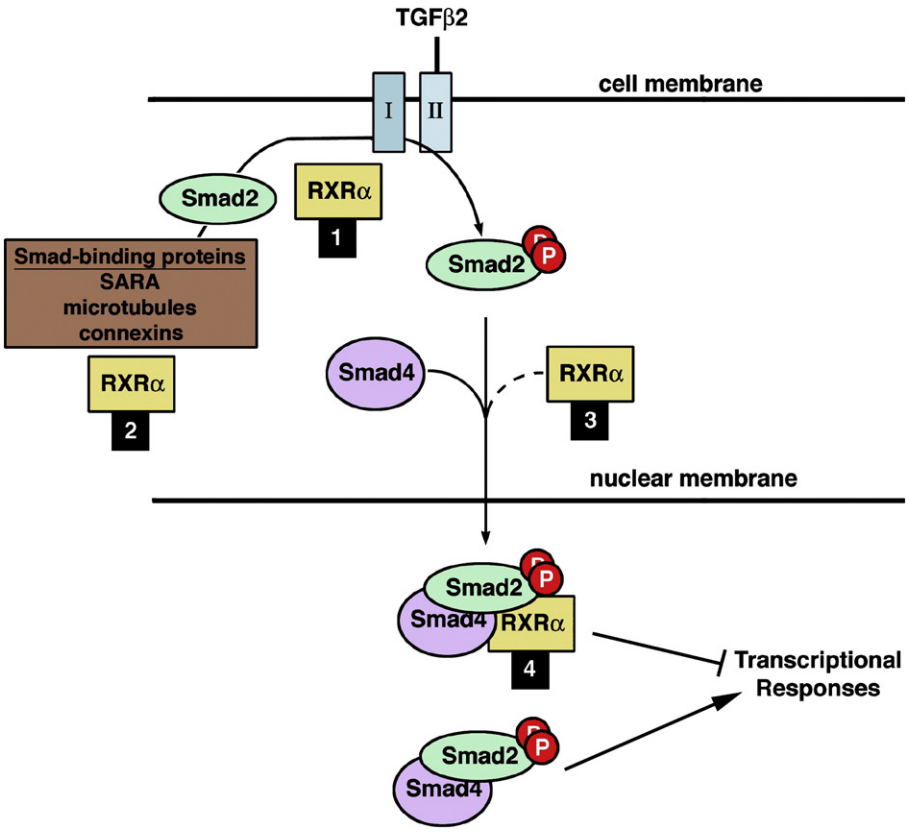

Fig. 6. Schematic of working hypothesis by which retinoid signaling via RXRα may directly regulate Smad phosphorylation and subcellular accumulation. Smad2 is phosphorylated by TβRI upon TGFβ exposure. Retinoid receptors can directly bind receptor Smads (Box 1), creating a mechanism by which they may regulate Smad availability to the TβRI. Alternately Smad2 binding partners that are acutely responsive to retinoids may be responsible for the increased level of TGFβ-mediated Smad2 phosphorylation seen during dual treatments (Box 2). Smad2 nuclear accumulation is blunted in the presence of retinoids suggesting that cytoplasmically localized retinoid receptors may control Smad2 nuclear uptake (Box 3). Likewise, in the presence of retinoids, receptor Smad-driven transcription is decreased. This effect may be secondary to decreased Smad nuclear accumulation or direct, negative effects of retinoid signaling on Smad-mediated transcription (Box 4).

tion. In order to determine the mechanism by which this occurs, we performed cytoplasmic and nuclear fractionation of NIH3T3 cells that were treated with combinations of retinoids and TGFβ (Fig. 4B). Consistent with the above transcription results, in cells treated solely with TGFβ, the entire pSmad2 pool shuttles to the nucleus, whereas with dual treatment, a proportion of the pSmad2 remains cytoplasmic. Similarly, we found that immunocytochemistry of dual-treated NIH3T3 cells (Fig. 5D, E) showed significantly less nuclear Smad2 than did cells treated with TGFβ2 alone (Fig. 5C, E). These findings can be explained by the ability of retinoid receptors to bind and sequester Smads [22] and to act as regulators for the nuclear uptake of its binding partners [34].

4. Discussion

Our data show that retinoids in the presence of TGFβ can potentiate Smad2/3 phosphorylation and this potentiation is likely not due to transcriptional events as it occurs during a one-hour timeframe. Retinoid ligands alone had no effect on Smad2 phosphorylation (Fig. 1B–C) demonstrating that immediate activation of endogenous local, latent TGFβ by retinoids is not contributing to the elevated pSmad2 seen with dual treatment in this system. Further, this work suggests that retinoid signaling controls the availability of Smad2 for phosphorylation by the TβRI as well as nuclear uptake of pSmad2. As it is known that retinoid receptors physically interact with Smads [22] and that unliganded RXR exists both in the cytosol and nucleus [16], we hypothesize that unliganded RXRα sequesters a portion of the total Smad2 pool, preventing it from being phosphorylated by the TβRI in the presence of TGFβ (Box 1 Fig. 6). When RXRα is absent from the cytosol (as is the case during 9-*cis*RA treatment which promotes RXRα nuclear translocation [16] or in RXRα−/− MEFs) Smad2 is not sequestered by RXRα and proportionally more Smad2 can be phosphorylated under TGFβ stimulation (Fig. 3B). Alternately, acute exposure to retinoids may affect other elements which bind Smad2, such as microtubules, SARA, or connexins [9,35,36] (Box 2 Fig. 6). Reorganization of these elements at or near the TβRI could modify the availability of Smad2 to be phosphorylated. An alternate hypothesis supported by the literature is that liganded RXR heterodimerizes with the vitamin D receptor (VDR) [37] lessening known Smad-VDR interplay thus allowing more Smads to be available for canonical TGFβ signaling.

Our data suggest that the increased pSmad2 in dual-treated wild type cells does not escalate downstream pSmad2-mediated events secondary to decreased nuclear accumulation of Smad2 (Box 3 Fig. 6). We employed a Smad binding element reporter assay and showed that a one-hour treatment with TGFβ2 plus 9-*cis*RA results in less Smad2/3-driven transcription than a treatment with TGFβ2 alone. Similar findings were described by Cao et al., albeit after an 18-hour treatment with TGFβ and atRA [21]. The mechanism by which retinoid receptors control Smad nuclear accumulation is still unclear however it may be explained by the fact that RXRα, in the presence of ligand, dimerizes with other nuclear receptors and translocates to the nucleus to mediate transcription thus blunting its purported shuttle function (Figs. 4B and 5). As pSmad2 is dephosphorylated in the nucleus [15], decreased nuclear uptake may also explain the sustained elevation of pSmad2 levels observed over a time course with dual treatment (Fig. 1D). Alternately, in dual-treated cells it is possible that RXRα shuttles to the nucleus and binds a portion of the nuclear pSmad2, preventing its transcriptional activities (Box 4 Fig. 6). Co-immunoprecipitation and live cell imaging studies utilizing fluorescently-tagged Smads and retinoid receptors are currently being undertaken to assess the extent of subcellular co-localization of these players during TGFβ and retinoic acid treatments.

Interactions between retinoid receptors and Smads have important implications for RXRα null cells. In the RXRα−/− embryo we have noted elevated levels of proteins (i.e. fibronectin) [38] and events (i.e. apoptosis) [1] that are positively regulated by TGFβ. We suspect that in addition to elevated TGFβ2 levels detected in the RXRα−/−, the loss of RXRα-mediated negative regulation of pSmad2 driven transcription is responsible for the upregulation of events downstream of TGFβ signaling and possibly many of the developmental defects seen in this mouse.

To determine the effects of Smad2 dosage in vivo, crosses between RXRα+/− and Smad2+/− mice are currently being performed. As decreased dosage of TGFβ2 results in a partial rescue of the cardiac phenotype seen in the RXRα−/− [1], we hypothesize that Smad2 heterozygosity will show a similar effect on the RXRα knockout mouse if TGFβ's effects are Smad-dependent. As TGFβ can signal through Smad-independent pathways, it is important to determine the role of canonical signaling during cardiac development. It is clear that retinoids can influence canonical TGFβ signaling on a long-term timescale dependent on transcriptional events and this study demonstrates that retinoids directly regulate Smad activity on a truncated timescale independent of retinoid-mediated transcription. This effect may be particularly relevant to the rapid growth and remodeling required by the developing embryo. Our observations contribute to the growing list of known interactions between retinoid and TGFβ signaling and future studies will lead us to a better understanding of such crosstalk.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbamcr.2008.07.028.

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