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TGF β -induced Lung Cancer Cell Migration is NR4A1-dependent

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Abstract

Transforming growth factor β (TGF β) induces migration of lung cancer cells (A549, H460 and H1299), dependent on activation of c-Jun N-terminal kinase (JNK1), and is inhibited by the JNK1 inhibitor SP600125. Moreover, TGF β -induced migration of the cells is also blocked by the nuclear export inhibitor leptomycin B (LMB) and the orphan nuclear receptor 4A1 (NR4A1) ligand 1,1-bis(3'-indolyl)-1-(*p*-hydroxyphenyl)methane (CDIM8) which retains NR4A1 in the nucleus. Subsequent analysis showed that the TGF β /TGF β receptor/PKA/MKK4 and -7/JNK pathway cascade phosphorylates and induces nuclear export of NR4A1 which in turn forms an active complex with Axin2, Arkadia (RNF111) and RNF12 (RLIM) to induce proteasome-dependent degradation of SMAD7 and enhance lung cancer cell migration. Thus, NR4A1 also plays an integral role in mediating TGF β -induced lung cancer invasion, and the NR4A1-ligand CDIM8 which binds nuclear NR4A1 represents a novel therapeutic approach for TGF β -induced blocking of lung cancer migration/invasion.

Implications: Effective treatment of TGF β -induced lung cancer progression could involve a number of agents including the CDIM/NR4A1 antagonists which block not only TGF β -induced migration but several other NR4A1-regulated pro-oncogenic genes/pathways in lung cancer cell lines.

Keywords

TGF β ; lung cancer; progression; NR4A1; antagonists

INTRODUCTION

Lung cancer is the leading cause of cancer deaths in the United States and it is estimated that in 2017, 222,500 new cases of lung cancer will be diagnosed in this country and 155,780 patient deaths will be observed (1). Greater than 85% of lung cancers are classified as non-small cell lung cancer (NSCLC) and despite significant advances in treatment regimens, the overall survival rate of NSCLC patients is 15.9% and this rate has not significantly improved over the past decade (2). Smoking is the major risk factor for lung cancer and exposure to secondary smoke, various occupational exposures, air pollution, and genetic factors also

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contribute to the high incidence of this disease. NSCLC is a highly complex disease with multiple subtypes and histologies that are accompanied by mutations of oncogenes (i.e. EGFR, KRAS, EML4-ALK) and tumor suppressor genes (i.e. p53) (3–6) and lung cancer therapy is driven, in part, by the tumor type and its pathological and molecular characteristics and traditional surgery, radiation and combinations of cytotoxic and mechanism-based drugs are extensively used (3–7). Targeted therapies for treating lung cancer have had limited success, and the more recent development and applications of immunotherapeutics that target programmed cell death ligand 1 (PD-L1) and programmed cell death 1 (PD-1) are promising new approaches (8,9). Despite the advances in lung cancer chemotherapy, the improvement in patient survival remains low and most therapies are accompanied by unwanted side-effects and drug resistance. Thus, it is critical to develop new therapeutics which target multiple pro-oncogenic pathways and can be used in combination therapies.

The transforming growth factor β family of ligands and receptors play an important and somewhat paradoxical role in cancer in which TGF β act as an inhibitor of early stage cancers but acts as a tumor promoter for later stage cancers. Several studies report that TGF β induces lung cancer cell migration/invasion and EMT, and this involves multiple kinases and downstream targets (10–19). A recent study showed that TGF β -induced migration/invasion of triple negative breast cancer cells was also NR4A1-dependent where NR4A1 interacts with Arkadia, AXIN2 and RNF12 to induce proteasome-dependent degradation of SMAD7, resulting in TGF β R1/TGF β R2 homodimerization and activation (20). We have also confirmed that NR4A1 plays a key role in breast cancer invasion where TGF β induces nuclear export of NR4A1 which interacts with E3 ligase complex proteins to induce SMAD7 ubiquitination and degradation (20,21). We previously reported that NR4A1 was a negative prognostic factor for lung cancer patient survival and NR4A1 was a pro-oncogenic factor regulating lung cancer cell proliferation and survival (22), and this has also been observed in cell lines derived from other solid tumors (23–30). Structure activity studies among a series of 1,1-bis(3'-indolyl)-1-(substituted phenyl)methane compounds showed that some of these analogs bound NR4A1 and in cancer cell lines, acted as NR4A1 antagonists (22–30). The most active compound 1,1-bis(3'-indolyl)-1-(*p*-hydroxyphenyl)methane (CDIM8; DIM-C-pPhOH) which acts as a nuclear NR4A1 antagonist (29) in lung and other cancer cell lines inhibited NR4A1-dependent pro-oncogenic genes/pathways (22–30). We hypothesized that DIM-C-pPhOH would also inhibit TGF β -induced lung cancer cell migration/invasion, and our results show for the first time that TGF β -induced invasion of lung cancer cells is due to JNK1-dependent phosphorylation and nuclear export of NR4A1 that is inhibited by NR4A1 antagonists.

MATERIALS AND METHODS

Cell lines, reagents and plasmids.

Lung cancer cell lines (A549, H460, and H1299) were purchased from American Type Culture Collection (Manassas, VA). A549 cells were maintained 37°C in the presence of 5% CO₂ in Dulbecco's modified Eagle's medium/Ham's F-12 medium with 10% fetal bovine serum with antibiotic H460, and H1299 lung cancer cells were maintained in RPMI-1640

medium with 10% fetal bovine serum and antibiotic. Alexa Fluor 488 and 455, Hoechst 33342, leptomycin B, SP600125, SB202190, LY294002 and PD98059 were obtained from Cell Signaling Technologies (Manassas, VA), and TGF β was purchased from BD Biosystems (Bedford, MA). Dulbecco's Modified Eagle's Medium, 14–22 Amide PKA inhibitor, ALK5i inhibitor (LY-364947) and 36% formaldehyde were purchased from Sigma-Aldrich (St. Louis, MO), and hematoxylin was purchased from Vector Laboratories (Burlingame, CA). The antibodies and their sources are summarized in Supplemental Table 1. FLAG-NR4A1, FLAG-NR4A1-(A-B), and FLAG-NR4A1-(C-F) were synthesized in the lab using site directed mutagenesis (31); pcDNA3-FLAG-MKK4WT, pcDNA3-FLAG-MKK7-JNK1A1WT [MKK7(CA)] and pcDNA3-FLAG-MKK7-JNK1A1APF [(MKK7(DN))] were purchased from Origene Technologies (Rockville, MD). pCMV5-FLAG-SMAD7 was a gift from Lin SC et. al. (Department of Biochemistry, Hong Kong University of Science and Technology, Kowloon, Hong Kong, China).

Boyden Chamber Assay.

A549, H460, and H1299 lung cancer cells (3.0×10^5 per well) were seeded in Dulbecco's modified Eagle's medium/Ham's F-12 medium supplemented with 2.5% charcoal-stripped fetal bovine serum and were allowed to attach for 24 hr. After various treatments including knockdown of various genes (48 hr), cells were allowed to migrate for 24 hr, fixed with formaldehyde, and then stained with hematoxylin, and cells migrating through the pores were then counted as described (31).

RT PCR.

RNA was isolated using Zymo Research *Quick-RNA* MiniPrep kit (Irvine, CA). Quantification of mRNA (slug, snail, and NR4A1) was performed using Bio-Rad iTaq Universal SYBER Green 1-Step Kit (Richmond, CA) using the manufacturer's protocol with real-time PCR. TATA Binding Protein (TBP) mRNA was used as a control to determine relative mRNA expression.

Immunoprecipitation and chromatin immunoprecipitation.

A549 cells were transfected with various constructs and, 6 hr after transfection, cells were treated with DMSO or various agents and immunoprecipitation experiments and subsequent analysis were carried out previously described (31).

The chromatin immunoprecipitation (ChIP) assay was performed using the ChIP-IT Express magnetic chromatin immunoprecipitation kit (Active Motif, Carlsbad, CA) according to the manufacturer's protocol. The treatment conditions and analysis were performed as described (31). The primers for detection of the NR4A1 promoter region were 5'-CCTGCCCTCGGGAAGG-3' (forward) and 5'-CAGGCCGCGGGCTGAGG-3' (reverse). PCR products were resolved on a 2% agarose gel in the presence of RGB-4103 GelRed Nucleic Acid Stain.

Nuclear/cytosolic extraction and western blots.

Lung cancer cells were treated with various agents/constructs, and nuclear and cytosolic fractions were isolated using Thermo Scientific NE-PER Nuclear and Cytoplasmic

Extraction Kit (Rockford, IL) according to manufacturer's protocol. Fractions were analyzed by western blots as described (31). GAPDH and p84 were used as cytoplasmic and nuclear positive controls, respectively.

Immunofluorescence.

A549 cells (1.0×10^5 per well) were treated with either DMSO or TGF β (5 ng/ml) was added for 4 hr after $\pm$ pretreatment with various agents or transfection for 48 hr. Cells were then fixed with 37% formalin, blocked, treated with fluorescent NR4A1 primary antibody [Nur77 (D63C5)] XP® for 24 hrs. Cells were then washed with PBS and treated with Anti-rabbit IgG Fab2 Alexa Fluor® 488 secondary antibody for 3 hrs. Cells were then treated with Hoechst (Hoechst 33342) stain and phalloidin (Alexa Fluor® 555 Phalloidin) for 15 min in the dark following manufacturer's protocol and visualized by confocal microscopy (Zeiss LSM 780 confocal microscope, Peabody, MA) as previously described (31) Cells were analyzed by western blot as described previously (24–28).

Small interfering RNA interference assay.

SiRNA experiments were conducted as described previously (21). The siRNA complexes used in the study that were purchased from Sigma-Aldrich are as follows: siGL2–5': CGU ACG CGG AAU ACU UCG A; siNR4A1(1): SASI_Hs02_00333289; siNR4A1(2): SASI_Hs02_00333290; siAxin2(1): SASI_Hs01_00110148; siAxin2(2): SASI_Hs01_00110149; siArkadia(1): SASI_Hs01_00064840; siArkadia(2): SASI_Hs01_00064841; siRNF12 (1): SASI_Hs01_00238255; siRNF12(2): SASI_Hs02_00348888; siTAK1(1): SASI_Hs02_00335227; siTAK1(2): SASI_Hs01_00234777; siTAB1(1): SASI_Hs01_00094398; siTAB1(2): SASI_Hs02_00340933; siTRAF6(1): SASI_Hs01_00116391; siTRAF6(2): SASI_Hs01_00116390; siMKK4(1): SASI_Hs02_00334897; siMKK4(2): SASI_Hs02_00334898; siMKK7(1): SASI_Hs01_00059905; siMKK7(2): SASI_Hs01_00059906; siJNK1(1): SASI_Hs01_00010441; siJNK1(2): SASI_Hs01_00010442; sic-fos (1): SASI_Hs01_00184572; sic-fos(2): SASI_Hs01_00184573; siATF2(1): SASI_Hs01_00147372; siATF2(2): SASI_Hs01_00147373; siElk1(1): SASI_Hs02_00326325; siElk1(2): SASI_Hs02_00326324. The following siRNA complexes that were used in this study were purchased from Santa Cruz Biotechnology: c-jun siRNA(h): sc29223; c-jun siRNA(h2): sc-44201 SRF siRNA: sc-36563; PKA α siRNA: sc-36240.

PKA activity assay.

A549 lung cancer cells (3.0×10^5 per well) were seeded in Dulbecco's modified Eagle's medium/Ham's F-12 medium supplemented with 2.5% charcoal-stripped fetal bovine serum and were allowed to attach for 24 hr. Cells were then treated with above described treatments as used in other assays, then lysed with PKA lysis buffer (made in the laboratory using the manufacturer's recipe). PKA activity assay (Promega, Madison, WI) was performed following manufacturer's protocol, then lysates were resolved on a 2% agarose gel.

Statistical analysis.

Statistical significance of differences between the treatment groups was determined as previously described (31).

RESULTS

1. TGF β -induced nuclear export of NR4A1 is JNK-dependent

In this study, we initially used three NSCLC cell lines (A549, H460 and H1299) to investigate the role of NR4A1 in TGF β -induced migration/invasion using a Boyden Chamber assay. TGF β induced migration of the three cell lines (Fig. 1A) and cotreatment with the NR4A1 antagonist CDIM8, the nuclear export inhibitor leptomycin B (LMB), the TGF β receptor inhibitor ALK5i, or knockdown of NR4A1 by RNA interference (RNAi) (siNR4A1) significantly inhibited the TGF β -induced cell migration. The results also showed that CDIM8 and siNR4A1 also inhibited basal migration of the lung cancer cell lines. TGF β also induced nuclear export of NR4A1 in A549, H460 and H1299 lung cancer cells (Figs. 1B-1D, respectively) which was inhibited by LMB and CDIM8. We also observed that TGF β induced both expression and phosphorylation (S351) of NR4A1 and this was inhibited by cotreatment with CDIM8 and LMB (Fig. 1E). The intracellular location of NR4A1 in these experiments was determined by western blots of nuclear and cytosolic extracts using GAPDH (cytosolic) and P84 (nuclear) as subcellular controls.

We also examined the effects of kinase inhibitors on TGF β -induced cell migration and the JNK inhibitor SP600125 but not p38MAPK (SP202190), p42/44MAPK (PD98059) or PI3K (LY294002) inhibitors blocked TGF β -induced migration of A549, H460 and H1299 cells (Fig. 2A). SP600125 also inhibited TGF β -mediated nuclear export of NR4A1 in A549 (Fig. 2B), H460 (Fig. 2C) and H1299 (Fig. 2D) cells, whereas cotreatment with SB202190, LY294002 or PD98059 did not inhibit nuclear export of NR4A1 in cells treated with TGF β , indicating that TGF β -induced nuclear export of NR4A1 was JNK-dependent in lung cancer cells. TGF β also induced phosphorylation of (S351) NR4A1, JNK1, c-jun and c-fos which was also inhibited by SP600125, demonstrating that TGF β induces JNK and genes downstream from JNK.

Since the TGF β -JNK-NR4A1 (nuclear export) pathway is critical for enhanced migration of lung cancer cells, we used A549 cells as a model to further investigate the role of upstream kinases in this pathway. MKK4 and MKK7 are upstream from JNK1, and overexpression of FLAG-MKK4 (wild-type) enhanced invasion of A549 cells and this was inhibited by LMB, CDIM8 and SP600125 (Fig. 3A). Overexpression of MKK4 also induced nuclear export of NR4A1 and this was inhibited by LMB, CDIM8 and SP600125 (Fig. 3B). Overexpression of FLAG-MKK7(CA) also induced A549 cell migration which was inhibited by LMB, CDIM8 and SP60012, and TGF β -induced migration was inhibited by a dominant negative FLAG-MKK7(DN) (Fig. 3C). MKK7 overexpression also induced nuclear export of NR4A1 which was inhibited by LMB, CDIM8, SP600125, and dominant negative MKK7 (Fig. 3D). We also observed that TGF β -induced migration in A549 cells was inhibited by transfecting a construct expressing MKK7(DN) and also by knockdown of JNK1 (siJNK1) and upstream kinases including MKK4 (siMKK4), and MKK7 (siMKK7), TRAF6 (siTRAF6), TAK1

(siTAK1), and TAB1 (siTAB1) (Fig. 3E). TGF β -induced nuclear export of NR4A1 was also inhibited in A549 cells transfected with siJNK, siMKK4 and siMKK7, confirming that the intact MKK4/7-JNK pathway is required for NR4A1 nuclear export (Fig. 3F). We also observed that knockdown of the upstream kinases TAB1, TAK1 and TRAF6 inhibited TGF β -induced nuclear export of NR4A1 and the loss of TAB1 increased levels of nuclear NR4A1 (Fig. 3G). TRAF6 potentially plays a role in activation of PKA, such as recruitment of PKA to the plasma membrane or enhance dissociation of regulatory subunits of PKA. TRAF6 is K63 polyubiquitinated and forms signaling cascades that activate MAPK (like JNK1). Knockdown efficiencies are indicated in Figure 3H. Knockdown studies were performed using at least two different oligonucleotides (see Materials and Methods).

We also investigated the effects of TGF β , MKK4, and MKK7(CA) alone and in various combinations with TGF β , LMB, SP600125 and CDIM8, and also MKK7(DN) alone and in combination with TGF β by immunostaining and confocal microscopy. In DMSO treated cells, NR4A1 was primarily nuclear and this was significantly decreased after treatment with TGF β , whereas TGF β -mediated nuclear export of NR4A1 was inhibited after cotreatment with LMB, CDIM8 and SP600125 or transfected with MKK7(DN) (Suppl. Figs. 1 and 2). Overexpression of FLAG-MKK4 and FLAG-MKK7(CA) also induced nuclear export of NR4A1 as determined by confocal microscopy and this response was inhibited after cotreatment with LMB, CDIM8 and SP600125 (Suppl. Fig. 2).

2. Mechanism of TGF β -induced expression of NR4A1

TGF β induces NR4A1 in breast cancer cells, (31) and this was also observed in lung cancer cells (Fig. 1E) and the mechanism of this response was further investigated. Treatment of A549 cells with TGF β for 5 hr induced a >10-fold increase in NR4A1 mRNA levels and these effects were inhibited after cotreatment with CDIM8, SP600125 or ALK5i (TGF β receptor inhibitor) but not LMB (Fig. 4A) or after transfection with siJNK1, siMKK4 and siMKK7 (Fig. 4B). The effects of TGF β alone on induction of NR4A1 protein were minimal (Figs. 3B and 3F) as were the effects of LMB on this response, and this was in contrast to the induction of NR4A1 gene expression by TGF β (Fig. 4A). Downstream targets of JNK, such as c-jun, c-fos, ATF2, Elk-1 and SRF, bind AP1 (c-jun, c-fos), CRE (c-jun, ATF2) and SRE (Elk-1, SRF) promoter elements, and CRE and SRE sites were identified within the NR4A1 promoter (Fig. 4C). Therefore, we used a ChIP assay to investigate association of c-Jun/ATF2 and Elk-1 and SRF with the CRE/SRE motifs using primers that cover the -807 to -703 region of the NR4A1 promoter. A549 cells were treated with TGF β , transfected with FLAG-MKK4 or FLAG-MKK7(CA) alone and this resulted in recruitment of c-jun, ATF2 and SRF and also Pol II to the promoter and Elk-1 was constitutively bound in control (DMSO) cells (Fig. 4D). CDIM8 and SP600125 but not LMB blocked recruitment of c-jun, ATF2 and SRF to the NR4A1 promoter, and the result for LMB correlated with its effect (or lack thereof) on TGF β -induced levels of NR4A1 mRNA (Fig. 4A). ChIP assay results in cells transfected with FLAG-MKK7(DN) showed that TGF β -induced recruitment of c-Jun, ATF2 and SRF to the promoter was blocked by the DN plasmid (Fig. 4D). We did not detect any c-fos bound to the NR4A1 promoter, which is consistent with the fact that no putative AP1 promoter elements were identified within the promoter. The time-dependent activation of JNK phosphorylation by TGF β was also

accompanied by activation and/or induction of phosphorylated ATF2, SRF, c-jun and Elk-1 (Fig. 4E) and these results are consistent with recruitment of these factors to the NR4A1 promoter as results of the ChIP assay (Fig. 4D). TGF β -induced NR4A1 mRNA (Fig. 4F) and protein (Fig. 4G) expression was inhibited in A549 cells after knockdown of c-jun, ATF2, SRF and Elk-1 but not c-fos (Fig. 4F) and this complemented results of the ChIP assay. There was some off-target variability in this experiment; for example, knockdown of c-jun also resulted in decreased c-fos expression and, loss of Elk-1 and SRF increased levels of c-jun and this may indicate some interactions and crosstalk between of these transcription factors. Since c-jun, ATF2 and SRF are recruited to the NR4A1 promoter and regulate expression of NR4A1, we also observed that their loss (by RNAi) also resulted in decreased TGF β -induced migration (Fig. 4H).

3. TGF β -induced PKA is also involved in nuclear export of NR4A1

TGF β -induced activation of CRE and CRE binding factors suggests that protein kinase A (PKA) may also be activated by TGF β , and we therefore used a fluorescent peptide (kempTide) with multiple PKA phosphorylation sites and show that TGF β alone or in combination with LMB, CDIM8 and SP600125 induced phosphorylation (activity), whereas this response was not observed in cells cotreated with TGF β plus ALK5i, the TGF β receptor inhibitor (Fig. 5A). In cells transfected with FLAG-MKK4-WT or FLAG-MKK7(CA) alone or in combination with CDIM8, LMB and SP600125 or FLAG-MKK7(DN) $\pm$ TGF β , phosphorylation of PKA was not observed. As a positive control, we also observed increased phosphorylation of PKA in cells overexpressing the PKA catalytic subunit (Fig. 5A). Treatment of cells with the PKA inhibitor 14–22 Amide or knockdown of PKA-C α (siPKA-C α) by RNAi inhibited TGF β -induced A549 cell migration but did not affect MKK4/7-induced migration which are downstream from PKA (Figs. 5B and 5C). We also investigated the effects of 14–22 Amide and siPKA-C α (Figs. 5D and 5E) on TGF β /MKK4/7-induced nuclear export of NR4A; only the TGF β -induced effect was inhibited and MKK4/7 differentially enhanced nuclear export of NR4A1 independent of PKA inhibition. Results obtained for MKK4 $\pm$ 14–22 Amide (Fig. 5D) were somewhat inconsistent; however, results in Supplemental Figure 3 confirm these observations using confocal microscopic analysis. We also examined the effects of 14–22 Amide and siPKA-C α (Figs. 5F and 5G) on NR4A1 and the JNK1 pathway in A549 cells treated with TGF β and show that phosphorylation of NR4A1, JNK1 and jun were inhibited and SMAD7 levels were increased compared to that observed in cells treated with TGF β alone.

4. TGF β induces proteasome-dependent degradation of SMAD7

Previous studies show that TGF β induces proteasome-dependent SMAD7 degradation via an NR4A1/RNF12/Arkadia/Axin2 complex (21,31,32) and treatment of A549 cells with TGF β or transfection with FLAG-MKK4 and FLAG-MKK7(CA) followed by immunoprecipitation with NR4A1 antibodies showed that NR4A1 interacts with Axin2 and SMAD7 but not RNF12 or Arkadia (Suppl. Figs. 4A-4C). Cotreatment with LMB, CDIM8 or SP600125 significantly decreased these interactions and transfection with FLAG-MKK7(DN) blocked TGF β -induced interactions of NR4A1 with Axin2 and SMAD7 (Suppl. Fig. 4C). The same treatment groups were used and A549 cells were also transfected with FLAG-NR4A1-LBD (containing the LBD region of NR4A1) and immunoprecipitated with

FLAG antibodies, and results showed that SMAD7 interacted with the ligand binding domain of TGF β /MKK4/MKK7(CA)-activated NR4A1 (Suppl. Figs. 4D-4F). Using a SMAD7-FLAG construct in 549 cells treated with TGF β , we also showed that SMAD7 interacts with Axin2, RNF12, Arkadia and NR4A1, and these interactions are blocked by LMB, CDIM8 and SP600125. Treatment of A549 cells with TGF β or transfection with MKK4 or MKK7(CA) followed by immunoprecipitation by SMAD7 antibodies showed that a broad band of ubiquitinated SMAD7 proteins were formed (Figs. 6A-6C). Moreover, the intensity of the TGF β -induced ubiquitinated SMAD7 was inhibited by LMB, CDIM8 and SP600125. In addition, TGF β -induced ubiquitination was blocked after transfection with FLAG-MKK7(DN) and minimal ubiquitinated SMAD7 was observed in the control IgG lane (Fig. 6C). TGF β -induced ubiquitination of SMAD7 was inhibited after knockdown of Axin2, Arkadia and RNF12 (Fig. 6D) and TGF β -induced ubiquitination of SMAD7 was also inhibited by 14–22 Amide or after transfection with siPKA-C α (Fig. 6E). We also observed that MKK4/7 enhanced ubiquitination of SMAD7 (Fig. 6F) and these responses were not blocked by inhibition of PKA since both kinases are downstream from PKA. In contrast, both 14–22 Amide and siPKA-C α inhibited TGF β -induced interactions of NR4A1, Axin2, Arkadia and RNF12 with SMAD7 (Fig. 6G). The importance of the ubiquitin ligase complex members in mediating TGF β -induced migration of A549 cells is consistent with results in Figure 6H showing that knockdown of Axin2, Arkadia or RNF12 inhibits the TGF β -induced response. Figure 6I illustrates the specificity of the ubiquitin ligase complex proteins after knockdown by RNA interference. These results demonstrate the critical role of NR4A1, Axin2, Arkadia and RNF12 in mediating degradation of SMAD7 and TGF β -induced cell migration and identify several inhibitors of this pathway including the NR4A1 antagonist CDIM8.

Since TGF β and elements of the TGF β signaling pathway and the ubiquitin ligase complex proteins (including NR4A1) play a role in SMAD7 expression and ubiquitination, we further examined their role in SMAD7 degradation. A549 cells were treated with TGF β (Fig. 7A) or transfected with FLAG-MKK4 (Fig. 7B), FLAG-MKK7(CA) or FLAG-MKK7(DN) $\pm$ TGF β (Fig. 7C) plus or minus the proteasome inhibitor MG132. Kinase activation alone decreased expression of SMAD7 which is consistent with activation of TGF β signaling; however, cotreatment with LMB, CDIM8 or SP600125 $\pm$ MG132 prevented SMAD7 degradation and this was consistent with their resulting blockade of TGF β -induced signaling and cell migration (Figs. 1B and 2B). The critical effects of SMAD7 degradation on TGF β -induced migration are illustrated in Figure 7D in which TGF β -, MKK7(CA)- and MKK4-induced migration of A549 cells is blocked by cotreatment with MG132 which increases SMAD7 levels due to inhibition of proteasome-dependent degradation of SMAD7 (Fig. 7E). We also confirmed the critical role of TGF β -induced SMAD7 degradation by showing that TGF β -induced invasion can be inhibited by overexpression of SMAD7 (Fig. 7F). Figure 7G illustrates the unique TGF β -NR4A1-SMAD7 interactions in lung cancer cells and the role of PKA-MKK4/7-JNK in mediating the phosphorylation of NR4A1 and its nuclear export. Although TGF β -induced nuclear export of NR4A1 and its role in degradation of SMAD7 are common in breast and lung cancer cells, there are significant cell context-dependent differences in TGF β -induced kinase pathways and PKA-dependent induction of NR4A1 in lung vs. β -catenin/TCF/LEF-mediated induction of NR4A1 in breast cancer cells (31).

DISCUSSION

In lung cancer cells, several reports demonstrate that TGF β induces cell migration, invasion and EMT through modulation of multiple genes/pathways (10–19) and these pro-oncogenic functions of TGF β have been observed in many other tumor types (32–36). Recent studies in breast cancer cells show that TGF β -induced migration involves the orphan nuclear receptor NR4A1 which is part of an ubiquitin ligase complex required for proteasome-dependent degradation of SMAD7, an inhibitor of TGF β -activated signaling (20,31). Studies in this laboratory previously showed the pro-oncogenic functions of NR4A1 in lung cancer cells and NR4A1 was overexpressed in tumors from lung cancer patients and inversely correlated with their survival (22). Based on these observations, we hypothesized that NR4A1 may also play a role in TGF β -induced lung cancer migration/invasion and that this pathway can be inhibited by DIM-C-pPhOH/CDIM8, a compound that binds nuclear NR4A1 and acts as an NR4A1 antagonist in cancer cells (24).

We initially used three lung cancer cell lines as models and show that TGF β -induced migration was blocked by knockdown of NR4A1 or treatment with CDIM8, LMB or the TGF β receptor inhibitor Alk5i (Fig. 1). These data confirm that TGF β -dependent activation of the TGF β receptor is important for cell migration, and western blot analysis confirmed that the TGF β -induced response requires nuclear export of NR4A1 which is blocked by LMB and CDIM8. Results of kinase inhibitor studies show that the JNK1 inhibitor SP600125 also blocked TGF β -induced cell migration, nuclear export of NR4A1 (Fig. 2) and inhibitors of nuclear export (LMB and CDIM8), and JNK also inhibited phosphorylation of NR4A1 (Figs. 1B-1D and Fig. 2). These results are consistent with previous studies showing that selected apoptosis-inducing agents also induce phosphorylation-dependent nuclear export of NR4A1 through activation of JNK1 or other kinases (37–40). In addition, we also investigated both MKK4 and MKK7 which are upstream from JNK and demonstrate that overexpression of MKK4 or MKK7 recapitulated the effects observed with TGF β in terms of enhanced cell migration and nuclear export of NR4A1 and inhibition of these responses by LMB, CDIM8 and SP600125 (Fig. 3 and Suppl. Figs. 1 and 2). Moreover, MKK7(DN) also inhibited MKK7 and TGF β -induced responses and thus, linking the upstream effects of TGF β with MKK4/7-mediated activation of JNK.

Our previous studies in breast cancer cells (31) demonstrated that TGF β -dependent phosphorylation and nuclear export of NR4A1 was due to activation of MKK3/MKK6 and p38 but not MKK4/7 and JNK. Moreover in breast cancer cells, we observed that TGF β induced expression of both β -catenin and NR4A1, and the mechanism of NR4A1 expression involved β -catenin/TGF/LEF interactions with the NR4A1 promoter (31). In contrast, we did not observe induction of β -catenin in lung cancer cells treated with TGF β , whereas TGF β -induced expression of NR4A1 protein (1.5- to 2-fold) (Figs. 1B-1D) and RNA (> 10-fold) (Fig. 4A). We identified upstream CRE and SRE sites in the NR4A1 promoter at –807 and –703 (Fig. 4C) that bind c-jun/ATF2 and Elk1/SRF which are among some of the genes induced by JNK1 and these were induced by TGF β in A549 cells (Fig. 4E). Moreover, like the upstream kinases, knockdown of c-jun, ATF2, Elk1 and SRF inhibited induction and nuclear export of NR4A1 and migration of A549 cells treated with TGF β (Fig. 4).

Previous studies report that induction of NR4A1 expression in multiple cell types is associated with PKA, cAMP or cAMP inducers (41–46) and cadmium induction of NR4A1 in A549 cells is both PKA- and MAPK-dependent (41). These reports, coupled with the identification of PKA-activated transcription factors interacting with the NR4A1 promoter (Fig. 4D), suggested that PKA may be a potential kinase downstream from TGF β /TGF β receptor in lung cancer cells. Moreover, there is prior evidence demonstrating that TGF β /TGF β receptor induces PKA (47–49). Data illustrated in Figure 5 confirm that TGF β induces PKA activity and the PKA inhibitor 14–22 Amide or transfection with siPKA-C α inhibits TGF β -induced migration and NR4A1 expression, nuclear export of NR4A1 and transcription factors associated with induction of NR4A1. TGF β also induces NR4A1 in fibroblasts through activation of a SMAD3/SMAD4/Sp1 complex bound to GC-rich sites in the NR4A1 promoter (50), thus illustrating cell context-dependent differences in regulating NR4A1 expression.

Thus, TGF β activates the TGF β receptor–PKA–MKK4/7–JNK1 pathway which in turn phosphorylates NR4A1 and subsequently undergoes nuclear export (Fig. 7G). Although TGF β /kinase-dependent nuclear export of NR4A1 is necessary for A549 cell migration, this process also involves subsequent responses associated with extranuclear NR4A1 since TGF β -induced A549 cell migration is also inhibited by LMB and CDIM8 (Fig. 1). Previous studies in breast cancer cells showed that phosphorylated NR4A1 was a necessary component of a RNF12/Arkadia/Axin2/SMAD7 complex that induced ubiquitination and proteasome-dependent degradation of SMAD7 which in turn activated TGF β /TGF β receptor signaling (31,36). Results illustrated in Figure 6 and Supplemental Figure 4 confirm that this same complex is also functional in A549 cells and is necessary for ubiquitination and subsequent degradation of SMAD7. Thus, another major difference between breast and lung cancer cells is TGF β -dependent activation of p38 (breast) vs. PKA/JNK (lung) which is required for nuclear export of NR4A1 and subsequent activation of proteasome-dependent degradation of SMAD7. The importance of SMAD7 degradation in mediating TGF β -induced A549 cell migration is also supported by results showing that overexpression of SMAD7 inhibits the TGF β -induced effect (Fig. 7F). Figure 7G illustrates that the mechanism of TGF β -induced migration is a cyclic rather than a linear process since inhibition is observed by TGF β receptor inhibitors (Alk5i), kinase inhibitors, NR4A1 antagonists, nuclear export and proteasome inhibitors. Thus, effective treatment of TGF β -induced lung cancer progression could involve a number of agents including the CDIM/NR4A1 antagonists which block not only TGF β -induced migration but several other NR4A1-regulated pro-oncogenic genes/pathways in lung cancer cell lines (22).

Supplementary Material

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Figure 1.

Role of NR4A1/CDIM8 in TGFβ-induced lung cancer cell migration. (A) A549, H460 and H1299 lung cancer cells were treated 5 ng/ml TGFβ (for 5 hr) and various reagents including siNR4A1 oligonucleotide (for NR4A1 knockdown), and cell migration was determined in a Boyden chamber assay. A549 (B), H460 (C) and H1229 (D) cells were treated with 5 ng/ml alone and in combination with LMB (20 nM) or CDIM8 (20 μM), and cytosolic and nuclear (B-D) or whole cell lysates (E) were analyzed by western blots. Results (A) are expressed as means ± SE for 3 separate determinations, and significant ($p < 0.05$) induction of migration compared to solvent control (DMSO/CTL) is indicated (*). Bands in western blots (B-E) were quantitated relative to β-actin, and control values of NR4A1 were 1.0. The LMB and CDIM8 concentrations indicated above were used in subsequent experiments.

Figure 2.

Effects of kinase inhibitors on TGFβ-induced migration and nuclear export of NR4A1. (A) Cells were treated with TGFβ alone or in combination with kinase inhibitors SP600125 (30 μM), SB202190 (30 μM), LY294002 (30 μM) and PD98059 (30 μM), and effects on cell migration were determined. A549 (B), (H460 (C) and H1299 (D) cells were treated with TGFβ alone and in combination with kinase inhibitors, and nuclear and cytosolic extracts were analyzed for NR4A1 expression by western blots. (E) Lung cancer cells were treated with TGFβ and SP600125 alone or in combination, and whole cell lysates were analyzed by western blots. Significant ($p < 0.05$) induction of TGFβ-induced cell migration is indicated (*). Bands in western blots (B-D) were quantitated relative to β-actin, and DMSO control values of NR4A1 were 1.0. Relative intensities of p-NR4A1 are given in (E). The kinase concentrations indicated above were used in subsequent experiments.

Figure 3. Role of upstream kinases and CDIM8 and other inhibitors on TGFβ/kinase-induced responses in lung cancer cells. Overexpression of FLAG-MKK4 on A549 cell migration (A) and nuclear export of NR4A1 (B) and effects of LMB, CDIM8 and SP600125 were determined in a Boyden chamber assay and by western blot analysis of nuclear and cytosolic extracts, respectively. Overexpression of FLAG-MKK7(CA) alone or in combination with various inhibitors or expression of FLAG-MKK7(DN) (± TGFβ) on cell migration (C) and nuclear export of NR4A1 (D) were determined in a Boyden chamber assay or by western blot analysis of nuclear and cytosolic extracts, respectively. Kinase knockdown by RNA interference on TGFβ-induced migration (E) and nuclear export of NR4A1 (F and G) were determined in a Boyden chamber assay and by western blot analysis of nuclear and cytosolic extracts, respectively. (H) Various oligonucleotides targeting kinases were transfected into A549 cells and, after 72 hr, whole cell lysates were analyzed by western blots. Results in A, C and E are means ± SE for 3 separate determinations, and significantly (p<0.05) enhanced

migration (*) and inhibition of this response (**) are indicated. Relative concentrations of NR4A1 (B, D, F, G) were relative to β -actin were determined.

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Figure 4.

NR4A1 regulation in A549 cells. A549 cells were treated with TGFβ alone or in combination with various inhibitors (A) or after knockdown of JNK1 pathway kinases (B), and NR4A1 mRNA levels were determined by real time PCR. (C) Identification of *cis*-elements on the NR4A1 gene promoter. (D) Cells were treated with TGFβ or transfected with MKK4(WT), MKK7(CA) and MKK7(DN) alone or in combination with various agents for 6 hr and then analyzed in a ChIP assay using multiple antibodies and primers targeting the -869 to -853 (F) and -669 to -86 (R) regions of the NR4A1 promoter. (E) A549 cells were treated with TGFβ (5 ng/ml) for 0, 3, 6, 9, 12 and 24 hr, and whole cell lysates were analyzed by western blots. A549 cells were transfected with oligonucleotides targeted to factors that regulate NR4A1 expression, and their effects on NR4A1 mRNA levels (F) and protein knockdown efficiencies (G) were determined by real time PCR and western blot analysis of whole cell lysates, respectively. The effects of knockdown of these same factors on cell migration (H) and intracellular location (nucleus vs. cytosol) of NR4A1 (I) was determined in Boyden chamber assays and western blots, respectively. The NR4A1 band relative to β-actin in western blot (G) was quantitated, and control values of NR4A1 were set at 1.0.

Figure 5.

Role of PKA in TGF β -NR4A1 interactions. (A) The Promega PKA activity assay kit was used to investigate PKA activation by TGF β , MKK4(WT) and MKK7(CA) alone and in combination with various agents and by TGF β plus MKK7(DN). A549 cells were treated with TGF β or transfected with MKK7(CA) and MKK4(WT) alone or in combination with 14–22 Amide or cotransfected with siPKA-C α (knockdown) and effects on cell migration (B and C) and intracellular location (nucleus vs. cytosol) of NR4A1 (D and E) were determined by Boyden chamber and western blot assays, respectively. The effects of 14–22 Amide (F) and siPKA-C α (G) on the time-dependent expression of TGF β -induced proteins was determined by western blot analysis of whole cell lysates. Significant ($p < 0.05$) inhibition of induced migration (determined in triplicate) by 14–22 Amide or siPKA-C α is indicated (*). The NR4A1 band relative to β -actin in western blots (D, E) was quantitated, and control values of NR4A1 were set to 1.0.

Figure 6.

Role of kinase pathways and proteasome complex proteins on SMAD7 ubiquitination and cell migration. A549 cells were transfected with FLAG-SMAD7 and treated with TGFβ (A), transfected with MKK4(WT) (B), transfected with MKK7(CA) or MKK7(DN) (± TGFβ) (C), and treated with various agents. Whole cell lysates were immunoprecipitated with FLAG antibodies and analyzed for ubiquitinated FLAG-SMAD7 by ubiquitin antibodies. A549 cells were transfected with FLAG-SMAD7, treated with TGFβ alone or in combination with oligonucleotides that knockdown Axin2, arkadia and RNF12 (D), treated with TGFβ alone or in combination with 14–22 Amide or siPKA-Cα (transfected) (E) or MKK4(WT)/MKK7(CA) (transfected) alone or in combination with 14–22 Amide or siPKA-Cα (transfected) (F), and whole cell lysates were immunoprecipitated FLAG antibodies and analyzed for ubiquitinated FLAG-SMAD7 using ubiquitin antibodies. (G) A549 cells were transfected with FLAG-SMAD7 and treated with TGFβ alone or in combination with 14–22 Amide or siPKA-Cα (transfected), immunoprecipitated with FLAG antibodies, and the immunoprecipitate was analyzed by a western blot. (H) A549 cells were treated with TGFβ and transfected with siAxin2, siArkadia and siRNF12 oligonucleotides and effects on cell migration were determined in a Boyden chamber assay.

(I) The efficiency of siAxin2, siArkadia and siRNF12 on protein knockdown was determined by western blot analysis of whole cell lysates.

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Figure 7.

TGFβ induces proteasome-dependent degradation of SMAD7 that is inhibited by NR4A1 ligand CDIM8 (DIM-C-pPhOH). A549 cells were treated with TGFβ (A) and MKK4(WT) (alone, transfected) (B) alone or in combination with MG132 and various agents. Whole cell lysates were analyzed for SMAD7 expression by western blots. (C) A549 cells were transfected with MKK7(CA) alone or in combination with MG132 and various agents and transfected with MKK7(DN) ± TGFβ, and whole cell lysates were analyzed for SMAD7 expression by western blots. A549 cells were treated with DMSO, TGFβ, transfected with MKK7(CA) and MKK4(WT) alone or in combination with MG132 and effects on cell migration (D) and SMAD7 expression (E) were determined in Boyden chamber and western blot assays, respectively. (F) Cells were treated with TGFβ alone or transfected with pCMV6 (empty vector), pCMV6-SMAD7 alone or in combination with TGFβ, and effects on A549 cell migration were determined; cell lysates from these treatment groups were also analyzed for SMAD7 expression by western blots. Results (D and F) are means ± SE for 3 separate determinations, and significantly (p<0.05) enhanced migration (*) and inhibition of this response (**) are indicated. (G) Summary of TGFβ-PKA-MKK4/7-JNK phosphorylation

and nuclear export of NR4A1 and inhibition by CDIM8/NR4A1 antagonist. The NR4A1 band intensities relative to β -actin in the western blots (A-C, E) were determined.

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