

RESEARCH ARTICLE

IGFBP-3 and TGF- β inhibit growth in epithelial cells by stimulating type V TGF- β receptor (T β R-V)-mediated tumor suppressor signaling

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Abstract

The TGF- β type V receptor (T β R-V) mediates growth inhibition by IGFBP-3 and TGF- β in epithelial cells and loss of T β R-V expression in these cells leads to development of carcinoma. The mechanisms by which T β R-V mediates growth inhibition (tumor suppressor) signaling remain elusive. Previous studies revealed that IGFBP-3 and TGF- β inhibit growth in epithelial cells by stimulating T β R-V-mediated IRS-1/2-dependent activation and cytoplasm-to-nucleus translocation of IGFBP-3- or TGF- β -stimulated protein phosphatase (PPase), resulting in dephosphorylation of pRb-related proteins (p107, p130) or pRb, and growth arrest. To define the signaling, we characterized/identified the IGFBP-3- and TGF- β -stimulated PPases in cell lysates and nucleus fractions in Mv1Lu cells treated with IGFBP-3 and TGF- β , using a cell-free assay with ³²P-labeled casein as a substrate. Both IGFBP-3- and TGF- β -stimulated PPase activities in cell lysates are abolished when cells are co-treated with TGF- β /IGFBP-3 antagonist or RAP (LRP-1/T β R-V antagonist). However, the IGFBP-3-stimulated PPase activity, but not TGF- β -stimulated PPase activity, is sensitive to inhibition by okadaic acid (OA). In addition, OA or PP2A_c siRNA reverses IGFBP-3 growth inhibition, but not TGF- β growth inhibition, in Mv1Lu and 32D cells. These suggest that IGFBP-3- and TGF- β -stimulated PPases are identical to PP2A and PP1, respectively. By Western blot/phosphorimager/immunofluorescence-microscopy analyses, IGFBP-3 and TGF- β stimulate T β R-V-mediated IRS-2-dependent activation and cytoplasm-to-nucleus translocation of PP2A_c and PP1_c, resulting in dephosphorylation of p130/p107 and pRb, respectively, and growth arrest. Small molecule TGF- β enhancers, which potentiate TGF- β growth inhibition

Abbreviations: A549 cells, human Caucasian lung carcinoma cells; CDK, cyclin-dependent kinase; CHO cells, Chinese hamster ovary epithelial cells; D32 cells, murine 32D myeloid cells; EMT, epithelial mesenchymal transition; IGF-1, insulin-like growth factor-1; IGF-2, insulin-like growth factor-2; IGFBP-3, insulin-like growth factor-binding protein-3; IR, insulin receptor; IRS-1/2, insulin receptor substrate-1/2; LRP-1, low density lipoprotein receptor-related protein-1; Mv1Lu cells, mink lung epithelial cells; OA, okadaic acid; p107, p130, pRb-related proteins; PAI-1, plasminogen activator inhibitor-1; PP1, protein phosphatase 1; PP1_c, 36-kDa PP1 catalytic subunit; PP2A, protein phosphatase 2A; PP2A-B56, a 56-kDa substrate-recognition B subunit of PP2A; PP2A_c, 37-kDa PP2A catalytic subunit; PPase, protein phosphatase; pRb, retinoblastoma protein (p105); RAP, receptor-associated protein; siRNA, small interfering RNA; TGF- β , transforming growth factor- β ; T β R-I, type I TGF- β receptor; T β R-II, type II TGF- β receptor; T β R-III, type III TGF- β receptor; T β R-V, type V TGF- β receptor; β_1 ²⁵, TGF- β peptide antagonist containing amino acid residues 41st to 65th of human TGF- β_1 .

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by enhancing T β R-I–T β R-II-mediated canonical signaling and thus activating T β R-V-mediated tumor suppressor signaling cascade (T β R-V/IRS-2/PP1/pRb), could be used to prevent and treat carcinoma.

KEY WORDS

IGFBP-3, IRS-1/2, PP1 $_c$, PP2A $_c$, TGF- β , T β R-V

1 | INTRODUCTION

Insulin-like growth factor-binding protein-3 (IGFBP-3) is a growth regulator which exhibits IGF-dependent and -independent growth inhibitory activities in target cells.¹ In the IGF-dependent activity, IGFBP-3 inhibits cell growth by binding IGF-1 and IGF-2 and preventing them from binding to their receptor, the IGF-1 receptor (IGF-1R), in these cells. IGFBP-3 is also capable of inhibiting growth of cells by directly interacting with its own specific receptor in cells. This specific IGFBP-3 receptor in responsive cells has been identified as the type V TGF- β receptor (T β R-V) which was discovered in our lab in 1991.^{2–7} It is identical to low density lipoprotein receptor-related protein 1 (LRP-1).⁸ IGFBP-3 inhibits the growth of wild-type mink lung epithelial cells (Mv1Lu cells), which express type I, type II, type III, and type V TGF- β receptors (T β R-I, T β R-II, T β R-III, and T β R-V), T β R-I-deficient Mv1Lu cells (R1B cells), and T β R-II-deficient Mv1Lu cells (DR26 cells).^{7,9,10} Mv1Lu cells have been a model normal epithelial cell system to study TGF- β activity and signaling.⁷ All of these wild-type and mutant cells express T β R-V. IGFBP-3 does not bind to T β R-I, T β R-II, and T β R-III in these cells.^{4,5} The half maximal concentration of IGFBP-3 for inhibiting growth of these cells is close to its K_d (0.3 μ g/ml or 10 nM) for binding to T β R-V,^{4,5,7} suggesting that IGFBP-3-induced growth inhibition is mainly mediated by T β R-V in target cells. IGFBP-3 maximally inhibits growth in these wild-type and mutant cells by ~30%–60%.^{4,5} The T β R-V is absolutely required for growth inhibition by either IGFBP-3 or TGF- β in target normal epithelial cells.^{2–7,9,10} IGFBP-3 and TGF- β are non-covalent and covalent homodimers, respectively, containing a minimal active site motif of WS/CXD.^{2–4,11,12} They bind to the cell surface subdomains of T β R-V at distinct sites. IGFBP-3 and TGF- β bind to cell surface subdomains II and IV, and a site between subdomains I and II of T β R-V, respectively.^{4,5,7} TGF- β at 50 pM mildly and moderately inhibits growth in cells expressing T β R-V but lacking T β R-I or T β R-II such as R1B and DR26 cells by ~15 and ~30% growth inhibition, respectively.^{9,10} However, TGF- β at 1–5 pM potently inhibits growth (~100% inhibition) in wild-type Mv1Lu cells by stimulating T β R-V-mediated growth inhibition (tumor suppressor) signaling in concert with canonical TGF- β signaling (T β R-I/T β R-II/Smad2/3/4)¹³ in wild-type Mv1Lu cells.^{7,9} Canonical TGF- β signaling potentiates T β R-V-mediated growth inhibition from 15 or 30% in mutant R1B and DR-26 cells (at 50 pM TGF- β)

to ~100% TGF- β (at 1–5 pM) growth inhibition by transcriptional activation of cyclin-dependent kinase (CDK) inhibitors in wild-type Mv1Lu cells.¹⁴ These suggest that T β R-V mediates mild or moderate TGF- β growth inhibition in mutant Mv1Lu cells (R1B and DR26 cells) lacking T β R-I or T β R-II, whereas T β R-I–T β R-II-mediated canonical signaling is required for potent TGF- β growth inhibition mediated by T β R-V in wild-type Mv1Lu cells. Absence of TGF- β -stimulated canonical signaling (T β R-I/T β R-II/Smad2/3/4) in R1B cells results in complete loss of TGF- β (at \leq 5 pM) growth inhibition activity in these cells.⁹

IGFBP-3 and TGF- β do not inhibit growth in cells lacking T β R-V, such as homozygous LRP-1-deficient mouse embryonic fibroblasts (PEA-13 cells), CHO cells deficient in LRP-1 (CHO-LRP-1^{–/–} cells) and H1299 human non-small cell lung carcinoma cells.^{7,10} PEA-13, H1299, and CHO-LRP-1^{–/–} cells express both T β R-I and T β R-II, and respond to TGF- β -stimulated T β R-I/T β R-II-mediated transcriptional activation of extracellular matrix (ECM)-related genes, such as PAI-1.¹⁰ Wild-type mouse embryonic fibroblasts (MEF) are sensitive to growth inhibition by either TGF- β or IGFBP-3.¹⁰ H1299 and CHO-LRP-1^{–/–} cells exhibit a spindle-shaped fibroblastoid morphology, frequently observed in invasive carcinoma cells.^{7,10,15} Stable transfection of H1299 and CHO-LRP-1^{–/–} cells with T β R-V/LRP-1 cDNA confers sensitivity to either TGF- β or IGFBP-3 growth inhibition and restores normal squamous epithelial morphology.^{10,15} These results suggest that T β R-V is essential for IGF-independent growth inhibition by IGFBP-3 and potent growth inhibition by TGF- β in epithelial cells. These results also support the notion that T β R-V acts as a tumor suppressor gene which causes cancer when it is inactivated or turned off.⁷ This notion is also supported by the recent findings that primary tumors from a few hundred human patients with liver, colon and prostate cancers in China, France and Argentina, respectively, exhibit loss or very low levels of LRP-1 (T β R-V) expression.^{16–18} Understanding of the mechanisms whereby the T β R-V mediates growth inhibition (tumor suppressor) signaling stimulated by IGFBP-3 and TGF- β should be important to elucidate the molecular basis of IGFBP-3 and TGF- β actions and to understand their roles in human cancers.^{19,20} IGFBP-3 and TGF- β are moderate and potent growth inhibitory cytokines for epithelial cells, respectively. IGFBP-3 acts as a tumor suppressor gene in several human carcinoma cancers examined.²⁰ TGF- β acts as a tumor suppressor at the

early stage of carcinogenesis and a tumor promoter in late-stage cancer.¹⁹ As a tumor suppressor, TGF- β suppresses carcinogenesis by potently inhibiting growth in epithelial cells for maintaining normal squamous epithelial morphology and physiology.²¹

We previously demonstrated that IGFBP-3 and TGF- β inhibit growth in epithelial cells by stimulating T β R-V-mediated tumor suppressor signaling which involves IRS-1/2-dependent activation and cytoplasm-to-nucleus translocation of IGFBP-3- or TGF- β -stimulated protein phosphatase (PPase), and dephosphorylation of retinoblastoma family proteins in the nucleus, resulting in cell growth arrest.^{7,10,22,23} In this communication, we demonstrate the identification of IGFBP-3- and TGF- β -stimulated PPases as PPase 2A (PP2A) and PPase 1 (PP1), which are the master regulators of the eukaryotic cell cycle, respectively, based on the distinct sensitivity of these PPase activities to okadaic acid (OA) and PP2A_c siRNA. By [³H] thymidine incorporation/Western blot/phosphorimager/immunofluorescence-microscopy analyses, we also demonstrate that IGFBP-3 and TGF- β stimulate IRS-2-dependent activation and cytoplasm-to-nucleus translocation of PP2A_c and PP1_c, resulting in dephosphorylation of pRb-related proteins (p130 or p107) and pRb (p105) in the nucleus, respectively, in epithelial cells and growth arrest.

2 | MATERIALS AND METHODS

2.1 | Materials

All chemicals used in the experiments were prepared as a 10 mM stock solution in DMSO. The final concentration of DMSO in all experiments was 0.1% or lower, which had no effect on IGFBP-3 and TGF- β activity. Human receptor-associated protein (RAP) was provided by Dr. Dudley K. Strickland (Department of Vascular Biology, American Red Cross). [γ -³²P]ATP, [³²P]-orthophosphate and [methyl-³H] thymidine (67 Ci/mmol) were purchased from ICN Biochemicals (Irvine, CA, USA). Okadaic acid (OA) was purchased from Tocris. IGFBP-3 and TGF- β 1 (TGF- β) were purchased from Peprotech. Insulin (A11382II) was purchased from Gibco. Primary antibodies against IRS-1 (sc-398), IRS-2 (sc-390761), PP1_c (37-kDa catalytic subunit) (sc-7482), pRb (p105) (sc-65230), p107 (sc-250), p130 (sc-374521), phosphorylated Smad2 (P-Smad2) (sc-135644), Ser 270-phosphorylated IRS-1/2 (P-IRS-1/2) (sc-17192), β -actin (sc-47778), and lamin B (sc-6216) were purchased from Santa Cruz Biotechnology. Rabbit antibodies against N- and C-terminal of human LRP-1 (T β R-V) were purchased from Sigma Chemical Co. and Abcam, respectively. Rabbit polyclonal antibodies against hyperphosphorylated Rb (P-Rb) (#8516) and PP2A_c (36-kDa PP2A catalytic subunit) (#2038) were purchased from Cell Signaling Technology.

Alexa Fluor 488- and 594-conjugated secondary antibodies were purchased from Thermo Fisher. Secondary antibodies conjugated with horseradish peroxidase (Millipore, USA) and enhanced chemiluminescence (ECL) kit (Perkin-Elmer Life Sciences) were used to develop immunoblots. TGF- β peptide antagonist [β ₁²⁵], a dual TGF- β /IGFBP-3 antagonist, was synthesized as previously described.¹¹

2.2 | Cell culture

Mv1Lu cells (CCL-64) and human lung adenocarcinoma cell line A549 (CCL-185) were purchased from ATCC. 32D cells (murine 32D myeloid cells stably expressing human insulin receptor (IR) and IRS-2)^{22,23} were provided by Dr. Martin G. Myers, Jr. (Joslin Diabetes Center, Harvard University). CHO-K1 cells were purchased from American Type Culture Collection (Rockville, MD, USA). CHO-LRP-1^{-/-} cell²⁴ were provided by Dr. Guejun Bu, Department of Pediatrics and Cell Biology and Physiology, Washington University School of Medicine. CHO-LRP-1^{-/-} cells were generated from CHO-K1 cells by ethyl methane sulfate mutagenesis followed by pseudomonas exotoxin (PE)-mediated selection of LRP-1-deficient cells.²⁴ 32D cells stably expressing IR and IRS-2 were grown in RPMI 1640 medium containing 10% fetal bovine serum and 5% WEHI conditioned medium according to the procedure provided by Dr. Martin G. Myers. CHO Cells were grown in DMEM/Ham's F-12 medium containing 10% fetal bovine serum. Other cell lines used in this study were maintained in DMEM containing 50 U/mL each of penicillin and streptomycin and 10% fetal bovine serum (Invitrogen) in humidified incubators at 37°C and 5% CO₂.

2.3 | PPase activity assay

³²P-labeled casein was prepared by incubation of casein (21.6 mg) in 50 mM Tris-HCl, pH 7.0, containing 10% glycerol, 1 mM benzamidine, 0.1 mM PMSF, 14 mM mercaptoethanol, 0.2 mM [γ -³²P] ATP (200 cpm/pmol), 10 mM MgCl₂, and 1.5 Unit/ml of the catalytic subunit of protein kinase A in a final volume of 3 ml. After overnight incubation at room temperature, the solution was filtered on a column (1.5 × 20 cm) of Sephadex 50G equilibrated in 50 mM Tris-HCl containing 10% glycerol and 1 mM benzamidine. Before stimulation with IGFBP-3 or TGF- β , cells were treated with or without 25 μ g/ml of RAP (receptor-associated protein) and 30 μ g/ml of TGF- β peptide antagonist (β ₁²⁵) in serum-free DMEM or DMEM/Ham's F-12 medium for 10 min. The cells were stimulated with IGFBP-3 (0.6 μ g/ml) or TGF- β (40 pM) for 3 hr. The cells were washed with cold phosphate-buffered saline (PBS), detached with 50 mM Tris-HCl

pH 7.0 containing 0.25 M sucrose, 5 mM EDTA, and pelleted at 1,500 rpm for 5 min at 4°C. The cells were then lysed in 50 µl of homogenization buffer (50 mM Tris-HCl, pH 7.0 containing 150 mM NaCl, 1% Triton X-100, and 0.1 mM PMSF).

The PPase activity assay mixtures were composed of 50 mM Tris-HCl, pH 7.0 containing 10% glycerol, 1 mM benzamidine, 0.1 mM PMSF, 14 mM mercaptoethanol, 0.1 mg of bovine serum albumin (BSA), PPase-containing sample (cell lysates or nucleus extracts containing 5 µg protein), and ³²P-labeled substrate in a final volume of 0.05 ml. Reactions in triplicates were initiated with the ³²P-labeled casein at 30°C, and after a 10 min reaction period, 0.1 ml of 10% trichloroacetic acid (TCA) was added. The mixture was centrifuged at 12,000 g for 2 min in a microcentrifuge. About 0.1 ml of the supernatant was then added to 1 ml scintillation counting liquid, and radioactivity was determined.

The lysates from cells treated with vehicle only exhibited non-specific PPase activity (IGFBP-3- or TGF-β-independent PPase activity with certain ~10²–10³ cpm; 200 cpm/pmol phosphate). This non-specific PPase activity was subtracted from the total PPase activity in the cell lysates from cells treated with IGFBP-3 or TGF-β in order to estimate IGFBP-3-stimulated or TGF-β-stimulated PPase activity. For this reason, the mean (±SD) of the non-specific PPase activity from triplicates was taken as 0 cpm in cells treated with vehicle only.

2.4 | Immunofluorescence microscopy

One milliliter of culture media containing approximately 5,000–10,000 Mv1Lu cells was added to a 35 mm culture dish containing a square coverslip. Mv1Lu cells grown on coverslips were treated with IGFBP-3 or TGF-β. Cells were then fixed in 4% paraformaldehyde for 15 min followed by permeabilization. Fixed cells were blocked with 5% BSA in PBS for 20 min at room temperature (RT) and then incubated with an appropriate primary antibody solution overnight at 4°C. Fixed cells were incubated with Alexa Fluor-conjugated secondary antibodies for 1 hr at RT. Samples were observed with a Zeiss AxioObserver Z1 microscope (Zeiss), and images were captured using AxioVision Rev 4.6 software. To determine the nuclear localization and the colocalization of PPI_c and hyperphosphorylated pRb (P-Rb), the images were analyzed in three dimensions using an AxioObserver Z1 Apotome microscope (Zeiss). Colocalization was evaluated in single optical planes taken through the entire z-axis of each cell. All images were acquired using identical intensity and photo-detector gain to allow quantitative comparisons of relative levels of immunoreactivity between samples. All images were cropped and sized using ImageJ.

2.5 | Nucleus fractionation for PPase activity assay

Nuclear extracts of the cells were prepared by hypotonic lysis followed by high salt extraction. Briefly, cell pellets were homogenized in 0.5 mL of ice-cold lysis buffer, composed of 10 mM HEPES pH 7.9, 10 mM KCl, 2 mM MgCl₂, 0.1 mM EDTA, 1 mM dithiothreitol (DTT), and 0.5 mM phenylmethylsulfonyl fluoride (all from Sigma Chemical Co.). The homogenates were centrifuged for 30 s at 500 g at 4°C to eliminate any unbroken tissue. The supernatants were incubated on ice for 20 min, vortexed for 30 s after the addition of 50 µL of 10% Nonidet P-40 (Sigma Chemical Co.), and then centrifuged for 1 min at 5,000 g at 4°C. The crude nucleus pellet was suspended in 200 µL of ice-cold extraction buffer (20 mM HEPES pH 7.9, 420 mM NaCl, 1.5 mM MgCl₂, 0.1 mM EDTA, 1 mM DTT, and 0.5 mM PMSF) and incubated on ice for 30 min, mixed frequently, and centrifuged at 12,000 g at 4°C for 15 min. The supernatants were collected as nucleus extracts for Western blot and PPase activity assay. Protein concentration was determined using a bicinchoninic acid assay kit with BSA as the standard (Pierce Biochemicals).

2.6 | siRNA interference

Murine PP2A_c siRNA oligonucleotide corresponding to nucleotide sequence 5'-xxx-3' (ON-TARGETplus SMARTpool Cat #: L-040657-00) and negative control siRNA were obtained from Dharmacon. PP2A_c siRNA and negative control siRNA were resuspended in RNase-free water and stored at -80°C. Transfection of siRNA was carried out using electroporation (Bio-Rad Gene Pulser Xcell Total System). Three million cells in 600 µl of RPMI 1640 were incubated with siRNA in a 0.4 cm cuvette for 5 min on ice before electroporation (260 V, 950 µF). After additional 5-min incubation on ice, cells were re-suspended in 12 ml of RPMI 1640 supplemented with glutamine and 10% FCS (fetal calf serum) without antibiotic. Antibiotics (1% penicillin/streptomycin) were added at 6 hr after electroporation. All measurements were performed at 24 or 72 hr after transfection.

2.7 | [Methy-³H] thymidine incorporation

Growth of OA-treated Mv1Lu cells and PP2A_c siRNA knocked-down 32D cells were determined by the measurement of [methyl-³H] thymidine incorporation into cellular DNA as described previously.^{4,9,10,12} Briefly, cells grown to near confluence in 48-well dishes were treated with several concentrations of OA at 37°C for 1 hr in serum-free DMEM. The final concentration of DMSO was 0.2%. Treated cells were then incubated with 0.1 and 0.2 µg/ml IGFBP-3, or 40 pM TGF-β in DMEM

containing 0.1% FCS at 37°C for 18 hr. The [methyl-³H] thymidine incorporation into cellular DNA was determined by incubation of cells with [methyl-³H] thymidine for 6 hr.

2.8 | Western blot

Seventy-two hours after siRNA transfection, 32D and Mv1Lu cells (3×10^6 cells) were lysed with 100 μ l, 50 mM Tris-HCl, pH 7.0 containing 1% Triton X-100, 150 mM NaCl, 5 mM EDTA, and 0.1 mM PMSF. Cell lysates were subjected to 7.5% SDS-PAGE and Western blotting using specific antibodies (Santa Cruz Biotechnology) as described previously.^{22,23} The antigens on the blots were visualized using horseradish oxidase-conjugated anti-rabbit IgG antibody and ECL system.

2.9 | Metabolic labeling and immunoprecipitation

Mv1Lu and 32D cells (3×10^6 cells) grown in 6-well plate were washed and incubated in phosphate-free DMEM for 1 hr to deplete intracellular phosphate. After 2 hr of incubation with [³²P] orthophosphate at 37°C in a CO₂ incubator, cells were treated with 1 μ g/ml of IGFBP-3 and/or OA (and RAP) for 16 hr. Cell lysates were prepared by suspending cells in 600 μ l of lysis buffer and p130 or p107 was immunoprecipitated with a rabbit polyclonal antibody against the N-terminal domain of p130 or p107. The p130 or p107 antibody complex was captured with a protein G-coated agarose beads. The immunoprecipitated proteins were resolved using 7.5% SDS-PAGE. The gel was dried and autoradiographed by a phosphorimager.

2.10 | Statistical analysis

Two-tailed unpaired Student's *t*-test was used for determining the significance of a difference between two (vehicle only and sample) means. It was mainly used to compare the means between two groups (vehicle only and one specific concentration of IGFBP-3 or TGF- β). The values were presented as mean \pm SD. *p* < 0.05 was considered significant.

3 | RESULTS

3.1 | IGFBP-3- and TGF- β -stimulated PPase activities are distinct in the sensitivity to OA inhibition in Mv1Lu cells

We previously proposed a model for the mechanisms by which IGFBP-3 and TGF- β inhibit growth in epithelial cells

by stimulating T β R-V/IRS-1/2/PPase signaling.⁷ However, in this model, the identity of IGFBP-3- or TGF- β -stimulated PPase was unknown. To characterize and identify the IGFBP-3- and TGF- β -stimulated PPases, we developed a cell-free PPase activity assay. In this assay, ³²P-phosphorylated casein, which was generated by ³²P-phosphate-labeling (³²P-labeling) of casein (dephosphorylated) with protein kinase A in the presence of γ -³²P-ATP, was incubated with cell lysates of Mv1Lu cells treated with or without IGFBP-3 or TGF- β 1 (TGF- β). After incubation, ³²P-phosphate released from ³²P-casein via the action of stimulated PPase in cell lysates and nucleus extracts were separated from remaining ³²P-casein by 10% trichloroacetic acid (TCA) precipitation in the presence of a carrier protein (BSA). The ³²P-phosphate released was recovered in the supernate of the 10% TCA solution. The IGFBP-3- and TGF- β -stimulated PPase activities were estimated by subtracting the radioactivity of ³²P-phosphate released by cell lysates or nucleus extracts of cells treated without IGFBP-3 or TGF- β from that released by cell lysates or nucleus extracts of cells treated with IGFBP-3 or TGF- β . Using this assay, we characterized the kinetics, IGFBP-3 or TGF- β concentration dependence and OA sensitivity of the IGFBP-3- or TGF- β -stimulated PPase activity in Mv1Lu cells. As shown in Figure 1, IGFBP-3 and TGF- β stimulated the PPase activities in a time- and concentration-dependent manner. The IGFBP-3-stimulated PPase activity in the cell lysates appeared to be linear with time up to 3 hr treatment in these cells treated with 1 μ g/ml of IGFBP-3 (Figure 1A). The TGF- β -stimulated PPase activity in the cell lysates also exhibited a linear relationship with the treatment time for 3 hr in Mv1Lu cells treated with 40 pM TGF- β (data not shown). IGFBP-3 and TGF- β stimulated the PPase activities in a concentration-dependent manner (Figure 1B and Figure 1C, respectively). The half-maximum concentration of IGFBP-3 to stimulate the PPase activity was estimated to be ~10 nM (0.3 μ g/ml) (Figure 1B) which is close to the half-maximum concentration of IGFBP-3 for binding to the IGFBP-3 receptor (T β R-V) and for inhibiting cell growth in Mv1Lu cells.^{4,5} TGF- β also stimulated a PPase activity in Mv1Lu cells in a concentration-dependent manner with a half-maximum concentration of ~40 pM which is close to the K_d (50 pM) of TGF- β binding to T β R-V^{2,3} in these cells (Figure 1C). TGF- β at 10 pM stimulated a significant level of PPase activity (0.8×10^3 cpm; 200 cpm/pmol phosphate) (Figure 1C). However, the IGFBP-3-stimulated PPase activity is distinct from the TGF- β -stimulated PPase activity in its greater sensitivity to OA inhibition. OA at 0.5 nM completely inhibited the IGFBP-3-stimulated PPase activity (Figure 1D). OA at 1 nM did not significantly affect the TGF- β -stimulated PPase activity (Figure 1D). These results suggest that IGFBP-3 and TGF- β stimulate PPase activities by interaction with T β R-V in Mv1Lu cells and that IGFBP-3- and TGF- β -stimulated PPases are different enzymes with distinct sensitivity to OA inhibition in these cells.

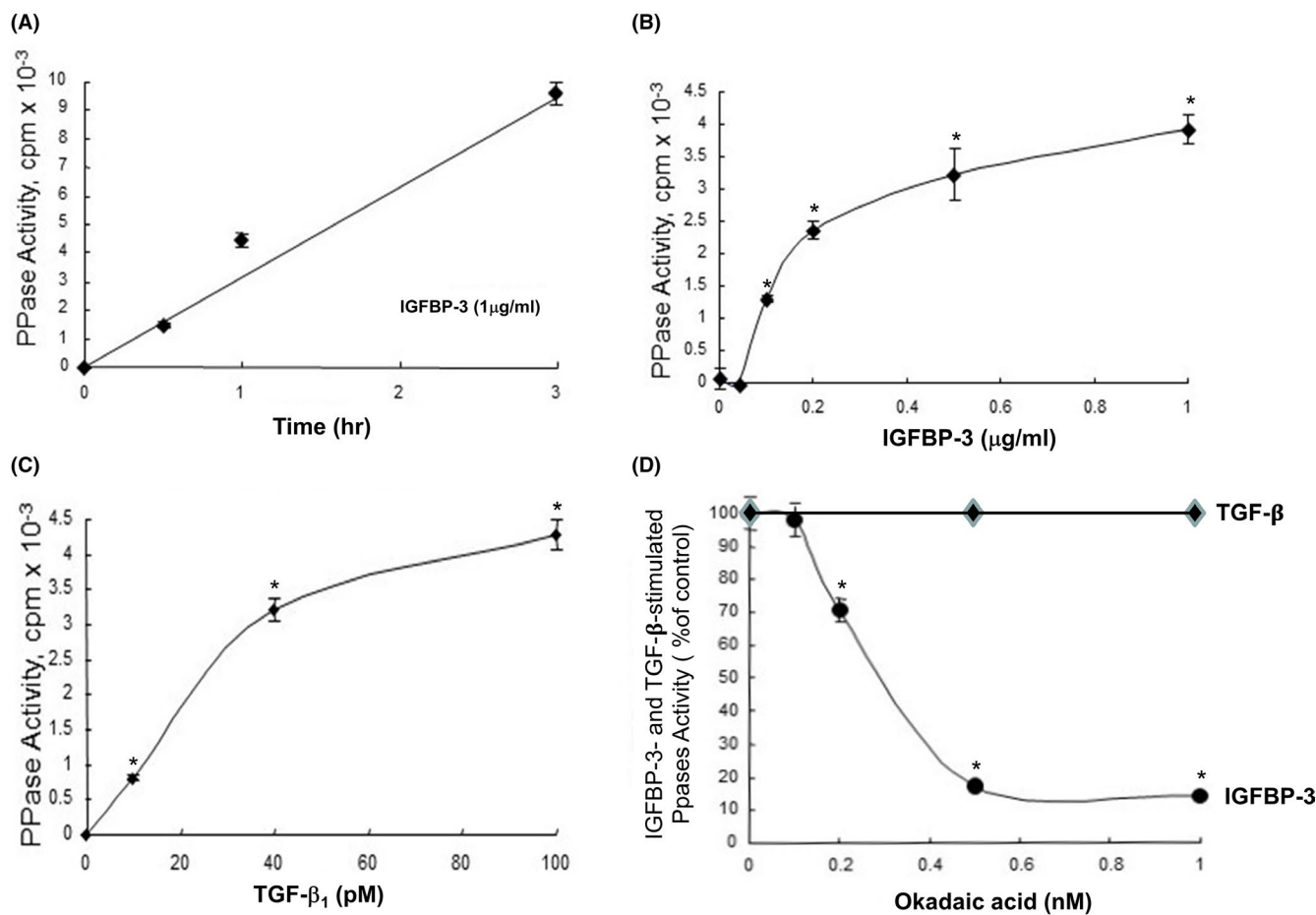


FIGURE 1 IGFBP-3 and TGF- β stimulate PPase activities in a time (A)- and concentration (B, C)-dependent manner but with distinct sensitivity to OA inhibition (D) in Mv1Lu cells. Cells were treated with vehicle only, 1 μ g/ml of IGFBP-3 (A), different concentrations of IGFBP-3 (B) and TGF- β (C) or treated with 0.6 μ g/ml of IGFBP-3 or 40 pM TGF- β in the presence of several concentrations of OA (D). After different time periods (A) or 2 hr incubation (B,C,D), cell lysates were assayed using ³²P-casein as substrate for assaying IGFBP-3- or TGF- β -stimulated PPase activity. The IGFBP-3- or TGF- β -stimulated PPase activity (200 cpm/pmol phosphate) was estimated by subtracting the ³²P radioactivity (cpm) released from ³²P-casein by cell lysates of cells treated with vehicle only from that released by cell lysates of cells treated with IGFBP-3 or TGF- β . The assays were performed in triplicates. The data are mean \pm SD *Significantly higher (B,C) or lower (D) than that of cells treated with vehicle only (control) or with IGFBP-3 or TGF- β only: $p < 0.001$

3.2 | IGFBP-3 and TGF- β stimulate PPase activities in a T β R-V-dependent manner in Mv1Lu and CHO-K1 cells

The T β R-V has been identified as the IGFBP-3 receptor which mediates its IGF-independent growth inhibitory activity.²⁻⁵ It has also been identified as an important TGF- β receptor required for mediating TGF- β growth inhibitory activity when canonical signaling mediated by T β R-I and T β R-II potentiates TGF- β growth inhibitory activity (~100% at 1–5 pM TGF- β) in wild-type Mv1Lu cells (7.9.10). To define the role of T β R-V in mediating IGFBP-3- and TGF- β -stimulated PPase activities, we determined the effects of T β R-V antagonists such as a dual TGF- β /IGFBP-3 peptide antagonist (β_1^{25})^{4,11} and RAP, a LRP-1 (T β R-V) antagonist,²⁵ and 10 nM insulin^{22,23} on IGFBP-3- and TGF- β -stimulated PPase activities in Mv1Lu cells, and

wild-type and LRP-1 (T β R-V)-deficient Chinese hamster ovary (CHO) epithelial cells (CHO-K1 and CHO-LRP-1^{-/-} cells, respectively). As shown in Figure 2, IGFBP-3- and TGF- β -stimulated PPase activities in Mv1Lu cells (Figure 2A,B,E) and wild-type CHO cells (CHO-K1 cells) (Figure 2C). Treatment of these cells with RAP or β_1^{25} alone stimulated non-specific (IGFBP-3- or TGF- β -independent) PPase activity (Figure 2A,B). Co-treatment of these cells with RAP or β_1^{25} and IGFBP-3 or TGF- β stimulated non-specific (IGFBP-3- or TGF- β -independent) PPase activity which was statistically indifferent from that treated with RAP or β_1^{25} alone, suggesting that RAP or β_1^{25} completely abolished the IGFBP-3- and TGF- β -stimulated PPase activities (Figure 2A,B, respectively). The important role of T β R-V in mediating the IGFBP-3-stimulated PPase activity was further supported by experiments using CHO-K1 and CHO-LRP-1^{-/-} cells (Figure 2C,D, respectively). IGFBP-3

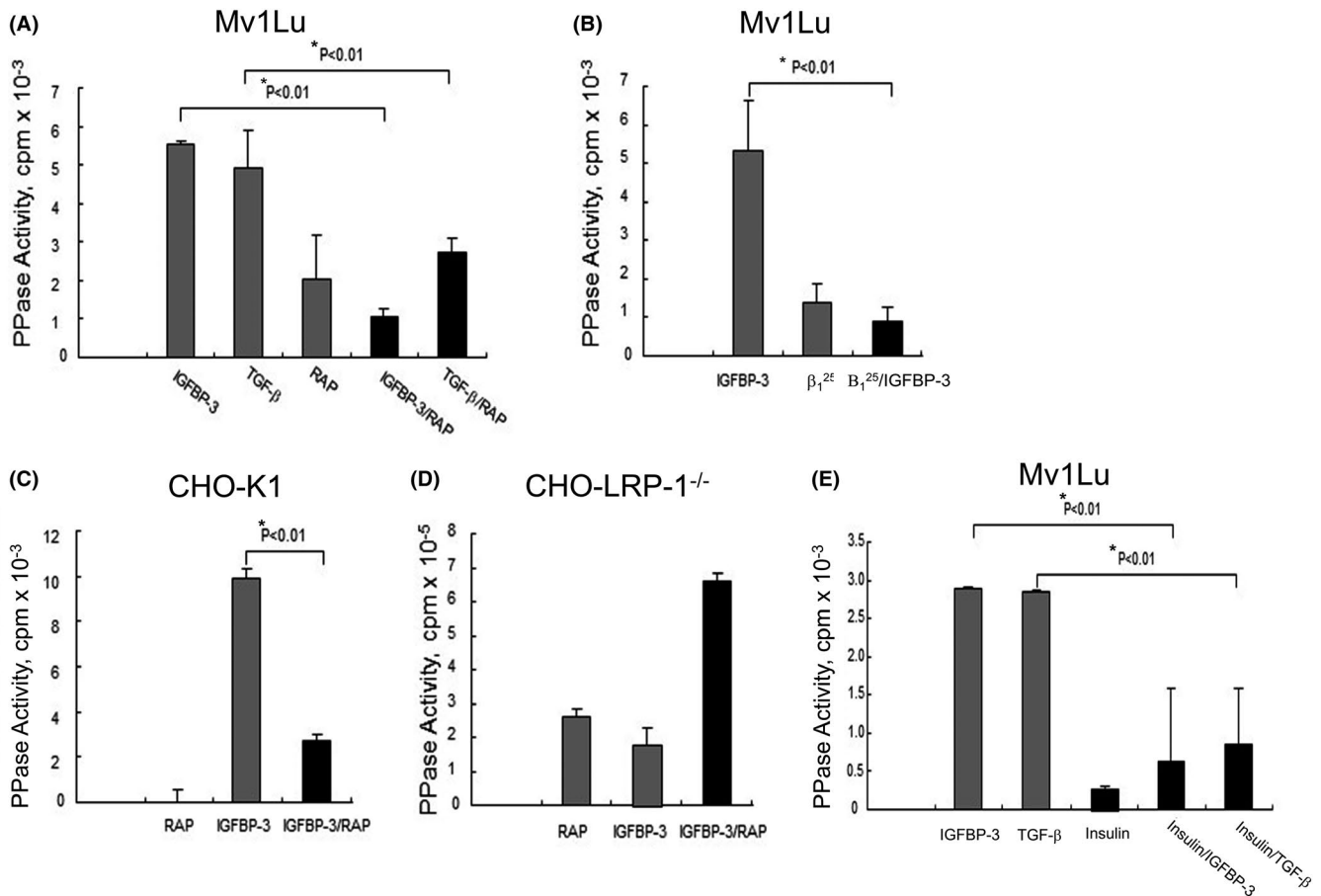


FIGURE 2 IGFBP-3- and TGF-β-stimulated PPase activities are inhibited by co-treating cells with LRP-1 (TβR-V) antagonist (RAP), TGF-β peptide antagonist (β₁²⁵), or insulin in Mv1Lu (A, B, E) and CHO-K1 (C) cells but not in CHO-LRP-1^{-/-} (D) cells. (A, B, E) Mv1Lu cells were treated with vehicle only, 1 μg/ml of IGFBP-3 or 40 pM TGF-β in the presence or absence of RAP (60 μg/ml), β₁²⁵ (10 μg/ml), or insulin (10 nM). After 2 hr at 37°C, cell lysates were assayed for IGFBP-3- and TGF-β-stimulated PPase activities as described above. (C, D) Wild-type CHO (CHO-K1) (C) and CHO-LRP-1-null (CHO-LRP-1^{-/-}) (D) cells were treated with vehicle only or 1 μg/ml of IGFBP-3 in the presence and absence of RAP (60 μg/ml). After 2 hr at 37°C, cell lysates were assayed for the IGFBP-3- or TGF-β-stimulated PPase activity. The assays were performed in triplicates. The data are mean ± SD *Significantly lower than that of cells treated with IGFBP-3 or TGF-β only: *p* < 0.01 (A, B, C, E). Both IGFBP-3- and RAP-stimulated non-specific (IGFBP-3-independent) PPase activity in CHO-LRP-1^{-/-} cells which lacked the expression of TβR-V (LRP-1) and did not exhibit TβR-V-mediated IGFBP-3-stimulated PPase activity (D). IGFBP-3 and RAP exhibited the additive effects on stimulating non-specific (IGFBP-3-independent) PPase activity (which could be mediated by different non-specific PPases in cell lysates) in CHO-LRP-1^{-/-} cells. Combination of insulin with IGFBP-3 or TGF-β also exhibited additive effects on stimulating non-specific (IGFBP-3- or TGF-β-independent) PPase activity in Mv1Lu cells (E)

stimulated a PPase activity which was blocked in the presence of RAP in CHO-K1 cells (Figure 2C). In Figure 2C, the RAP alone value was taken as 0 cpm. It means that RAP alone, like vehicle only, exhibited non-specific (IGFBP-3-independent) PPase activity which was taken as a mean of 0 cpm (±SD, *n* = 3). In Figure 2D, both IGFBP-3- and RAP-stimulated non-specific (IGFBP-3-independent) PPase activity in CHO-LRP-1^{-/-} cells which lacked the expression of LRP-1 (TβR-V) and did not exhibit IGFBP-3-stimulated PPase activity. IGFBP-3 and RAP appeared to exert additive effects on stimulating non-specific (IGFBP-3-independent) PPase activity in CHO-LRP-1^{-/-} cells (Figure 2D). However, deficiency of LRP-1 appeared to greatly increase non-specific (IGFBP-3-independent) PPase activity up to ~10⁵ cpm activity baseline levels in these CHO-LRP-1^{-/-} cells

as compared to those (10³ cpm activity baseline levels) seen in wild-type CHO-K1 cells (Figure 2D vs. Figure 2C). LRP-1 (TβR-V) acts as a tumor suppressor for epithelial cells.⁷ Loss of LRP-1 in CHO-K1 cells leads to transformation into carcinoma cells (CHO-LRP-1^{-/-} cells) which exhibited a spindle-shaped fibroblastoid morphology, frequently observed in invasive carcinoma cells.¹⁵ Transformation of wild-type CHO epithelial cells into CHO-LRP-1^{-/-} cells (carcinoma cells) appeared to greatly upregulate non-specific (IGFBP-3-independent) PPase activity in these CHO-LRP-1^{-/-} cells.

We previously demonstrated that insulin at 10 nM blocks IGFBP-3-induced growth inhibition in Mv1Lu cells²² and partially blocks TGF-β-induced growth inhibition in the presence of anti-α5β1 integrin in these cells.²³ We also

demonstrated that insulin (10 nM)-activated IGF-1R catalyzes tyrosine-phosphorylation of IRS-1/2, conferring resistance of tyrosine-phosphorylated IRS-1/2 to IGFBP-3-stimulated dephosphorylation and IGFBP-3-induced growth inhibition in these cells.²² We hypothesize that IGFBP-3- and TGF- β -stimulated PPases are involved in IGFBP-3- and TGF- β -induced growth inhibition, respectively, in target cells.⁷ We examined the effects of insulin on IGFBP-3- and TGF- β -stimulated PPase activities. Mv1Lu cells were treated with insulin (10 nM) and IGFBP-3 (0.3 μ g/ml) or TGF- β (50 pM) simultaneously for 2 hr. It is important to note that IGF-1R-catalyzed tyrosine phosphorylation of IRS-1/2 occurs much faster than PPase-catalyzed dephosphorylation of these proteins.^{22,23} The IGFBP-3- or TGF- β -stimulated PPase activity in the cell lysates of stimulated cells was determined using ³²P-phosphorylated casein as a substrate. As shown in Figure 2E, insulin (10 nM), effectively attenuated IGFBP-3- and TGF- β -stimulated PPase activities in Mv1Lu cells. This is consistent with the notion that insulin-stimulated IGF-1R-catalyzed tyrosine phosphorylation of IRS-1/2 results in the inability of the tyrosine-phosphorylated IRS-1/2 to mediate activation of either IGFBP-3-stimulated PPase or TGF- β -stimulated PPase in these cells treated with IGFBP-3 or TGF- β .^{22,23} Combination of insulin with IGFBP-3 or TGF- β appeared to have additive effects on stimulating non-specific (IGFBP-3-independent) PPase activity (in Figure 2E). These results support the notion that non-tyrosine-phosphorylated but Ser/Thr-phosphorylated IRS-1/2 are involved in T β R-V-mediated activation of IGFBP-3- and TGF- β -stimulated PPases, and in T β R-V-mediated cell growth inhibition by IGFBP-3 and TGF- β .^{10,22,23}

3.3 | IGFBP-3 and TGF- β stimulate colocalization of T β R-V and IRS-1/2 at the plasma membrane and cytoplasm-to-nucleus translocation of IRS-2 and IGFBP-3-stimulated PPase complexes in Mv1Lu cells

We previously hypothesized that IGFBP-3 and TGF- β induce growth inhibition by interaction with T β R-V, which recruits IRS-1/2 and IGFBP-3-stimulated or TGF- β -stimulated PPase to form ternary complexes at the cytoplasmic tail of T β R-V, resulting in the activation of IGFBP-3-stimulated PPase or TGF- β -stimulated PPase, dephosphorylation of IRS-1/2 by activated IGFBP-3- or TGF- β -stimulated PPase in the ternary complexes at the cytoplasmic tail of T β R-V, dissociation of dephosphorylated IRS-1/2-IGFBP-3-stimulated PPase or dephosphorylated IRS-1/2-TGF- β -stimulated PPase binary complexes from the cytoplasmic tail of T β R-V, and subsequent translocation of dephosphorylated IRS-1/2-IGFBP-3-stimulated PPase or dephosphorylated IRS-1/2-TGF- β -stimulated PPase binary complexes from cytoplasm to the nucleus where it induces cell cycle

arrest by dephosphorylating retinoblastoma-family proteins.⁷ To test this hypothesis, we performed immunofluorescence microscopy of Mv1Lu cells treated with and without (0.3 μ g/ml) IGFBP-3 and TGF- β using specific antibodies to T β R-V/LRP-1 and IRS-1/2. As shown in Figure 3A,B, IGFBP-3 and TGF- β -stimulated colocalization of T β R-V and IRS-1/2 at the plasma membrane (Figure 3A*f,i* and Figure 3B*f,i*, inset) and cytoplasm-to-nucleus translocation of IRS-2 but not IRS-1 (Figure 3B*e,h* vs. Figure 3A*e,h*). These results suggest that IGFBP-3 and TGF- β stimulate complex formation of T β R-V, IRS-1/2, and possibly IGFBP-3- or TGF- β -stimulated PPase at the plasma membrane and cytoplasm-to-nucleus translocation of IRS-2 likely as PPase complexes in Mv1Lu cells. To analyze the presence of IGFBP-3-stimulated PPase-IRS-2 complexes in nucleus extracts of Mv1Lu cells, the IGFBP-3-stimulated PPase activity associated with IRS-2 in nucleus extracts was then determined. As shown in Figure 3C, IGFBP-3 stimulated a PPase activity in cell lysates of treated Mv1Lu cells. Approximately 50% of it was present in nucleus extracts and could be immunoprecipitated by antibodies to IRS-2 (Figure 3D vs. Figure 3C). Insulin completely abolished the IGFBP-3-stimulated PPase activity in cell lysates (Figure 3C). These results suggest that IGFBP-3 stimulates complex formation and cytoplasm-to-nucleus translocation of IGFBP-3-stimulated PPase and IRS-2 in Mv1Lu cells.

3.4 | IGFBP-3-induced growth inhibition, but not TGF- β -induced growth inhibition, is reversed by OA and PP2A_c siRNA in Mv1Lu and 32D cells

Since OA blocked the activity of the IGFBP-3-stimulated PPase and the TGF- β -stimulated PPase activity was relatively resistant to inhibition by 0.2–1 nM OA (Figure 1D), OA should be able to reverse IGFBP-3-induced growth inhibition, but not TGF- β -induced growth inhibition, in Mv1Lu cells. To test this, we determined the effects of OA on growth inhibition (as measured by [Methy-³H] thymidine incorporation) induced by IGFBP-3 and TGF- β . As shown in Figure 4A, OA reversed the growth inhibition induced by different concentrations of IGFBP-3. OA reversed IGFBP-3-induced growth inhibition in a concentration-dependent manner (Figure 4A). At 5 nM, OA reversed IGFBP-3 (0.1 μ g/ml)-induced growth inhibition by ~80% (Figure 4A) but did not significantly affect TGF- β -induced growth inhibition in these cells (Figure 4B). IGFBP-3-stimulated PPase is sensitive to OA inhibition, suggesting that IGFBP-3-stimulated PPase is likely to be identical to PP2A which is known to be highly sensitive to OA.^{26,27} To test this, we used murine myeloid cells which stably expressed human IR and IRS-2 (32D cells) and responded to IGFBP-3-induced growth inhibition.²² PP2A is a heterotrimer composed of a 36-kDa catalytic C subunit (PP2A_c), a 65-kDa

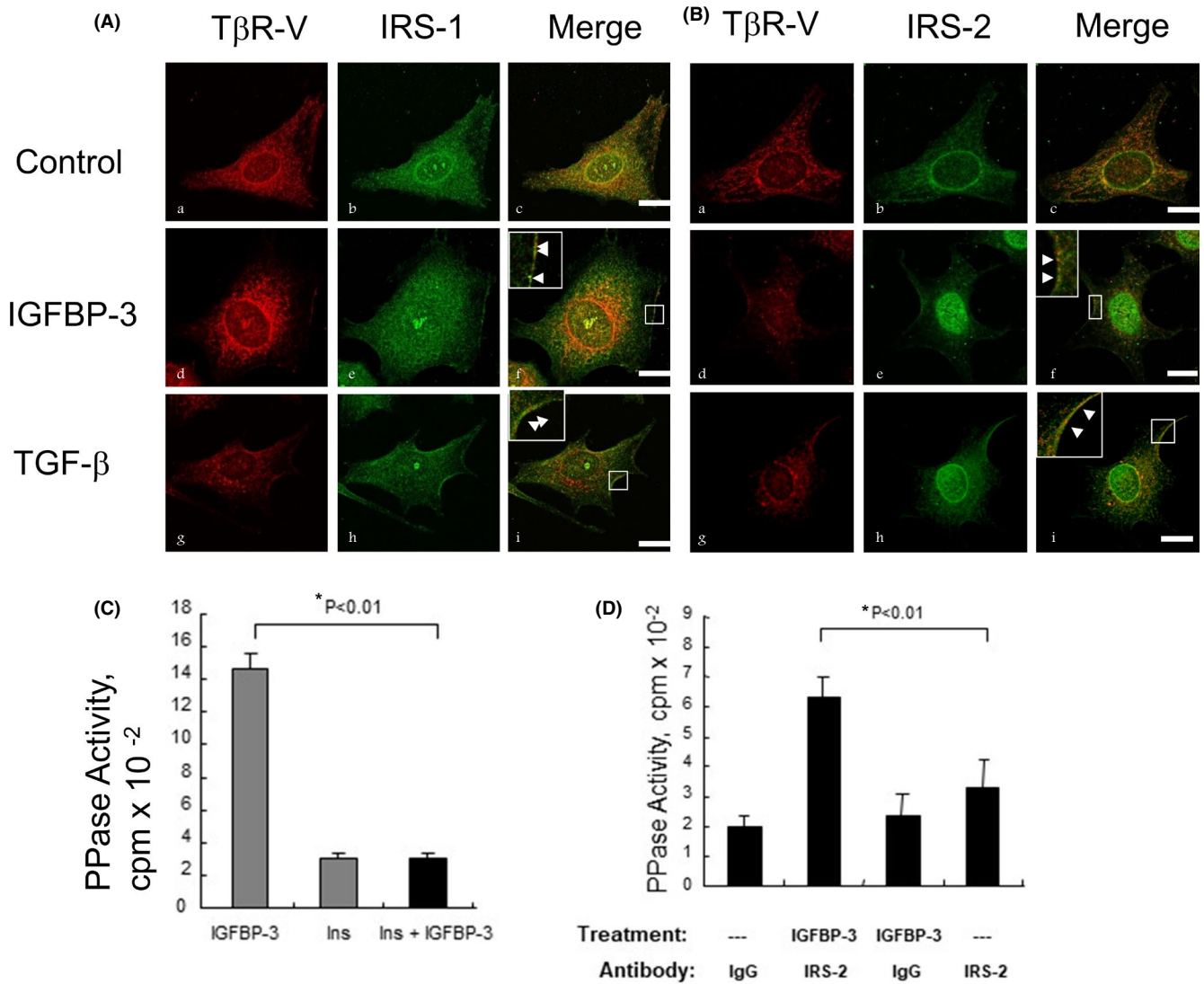


FIGURE 3 IGFBP-3 or TGF- β stimulates co-localization of T β R-V and IRS-1 (A) or IRS-2 (B) at the plasma membrane and cytoplasm-to-nucleus translocation of IRS-2 (B), but not IRS-1 (A), and cytoplasm (C)-to-nucleus (D) translocation of IRS-2-associated IGFBP-3-stimulated PPase activity in Mv1Lu cells. (A, B) Cells grown on coverslips in p35 culture dishes were treated with control (vehicle only), IGFBP-3 (1 μ g/ml) or TGF- β (40 pM). K_d for IGFBP-3 and TGF- β binding to T β R-V were estimated to be 10 nM (0.3 μ g/ml) and 50 pM, respectively.^{3,4} After 2 hr at 37°C, cells were fixed and stained by immunofluorescence using antibodies to T β R-V and IRS-1/2. Arrowheads indicate the co-localization of T β R-V and IRS-1/2 at the plasma membrane (Af,i and Bf,i, inset); scale bar = 10 microns. Both IGFBP-3- and TGF- β -stimulated cytoplasm-to-nucleus translocation of IRS-2 but not IRS-1 (Be,h vs. Ae,h). (C,D) Cells were treated with IGFBP-3 (1 μ g/ml), insulin (10 nM), or insulin (10 nM) + IGFBP-3 (1 μ g/ml) for cell lysate assay, and with IGFBP-3 (1 μ g/ml) and vehicle only (---) for nucleus extract assay. After 2 hr at 37°C, cell lysates (C) were assayed for IGFBP-3-stimulated PPase activity and nucleus extracts (D) were immunoprecipitated with control IgG and IgG to IRS-2. The immunoprecipitates were then assayed for the IGFBP-3-stimulated PPase activity. The assays were performed in triplicates. The data are mean \pm SD *Significantly lower (C) or higher (D) than that of cells treated with IGFBP-3 only or vehicle only: $p < 0.01$

scaffolding A subunit, and a 56-kDa substrate-recognizing B subunit (PP2A-B56).²⁷ We examined the effect of PP2A_c siRNA transfection on IGFBP-3-induced growth inhibition in these murine 32D cells. This PP2A_c siRNA was developed based on the murine sequence. 32D cells were transfected with control siRNA, 2 and 4 nM PP2A_c siRNA by electroporation and treated with IGFBP-3. As shown in Figure 4C,D, PP2A_c siRNA (2 and 4 nM) reversed the growth inhibition induced by IGFBP-3 (Figure 4C), but not by TGF- β (Figure 4D), in a

dose-dependent manner in murine 32D cells. Four nM PP2A_c siRNA reversed IGFBP-3-induced growth inhibition at 0.1 μ g/ml by ~78% in these murine cells (Figure 4C). This degree of inhibition is comparable to the ~70% downregulation of PP2A_c protein by murine PP2A_c siRNA transfection (vs. control siRNA transfection) (Figure 4E) as determined by 7.5% SDS-PAGE followed by quantitative Western blot analysis of cell lysates from 32D cells transfected with control siRNA (-) and 4 nM murine PP2A_c siRNA (+) (Figure 4E, top panel, lane 2

vs. lane 1 and bottom panel, quantitative analysis in three independent experiments). Murine PP2A_c siRNA was unable to reverse IGFBP-3-induced growth inhibition in mink Mv1Lu cells (data not shown). This is consistent with the inability of murine PP2A_c siRNA to significantly downregulate mink PP2A_c (Figure 4E, top panel, lane 4 vs. lane 3). These results suggest that IGFBP-3-induced growth inhibition is reversed by OA in Mv1Lu cells and by murine PP2A_c siRNA in murine 32D cells.

3.5 | IGFBP-3 stimulates cytoplasm-to-nucleus translocation of PP2A in Mv1Lu cells

As described above, IGFBP-3-stimulated PPase activity and IGFBP-3-induced growth inhibition are blocked or reversed by co-treatment with very low concentrations of OA

in Mv1Lu and 32D cells and by transfection with PP2A_c siRNA in murine 32D cells. These suggest that the IGFBP-3-stimulated PPase is identical to PP2A. We hypothesized that IGFBP-3 induces growth inhibition by stimulating IRS-2-dependent activation and cytoplasm-to-nucleus translocation of PP2A_c in Mv1Lu cells. To test this, Mv1Lu cells were treated with 0, 2, and 10 nM (or 0.06 and 0.3 μg/ml, respectively), IGFBP-3 for 2 hr. The cytoplasm and nucleus fractions in treated cells were then isolated and subjected to 7.5% SDS-PAGE followed by Western blot analysis. As shown in Figure 5A, IGFBP-3 at 2 and 10 nM increased accumulation of PP2A_c in the nucleus fraction by 1.5- to 1.7-fold (n = 3) as compared to that in cells treated with vehicle only (0 nM IGFBP-3). These results suggest that IGFBP-3 promotes cytoplasm-to-nucleus translocation of PP2A_c (likely as the IRS-2 complex) in Mv1Lu cells.

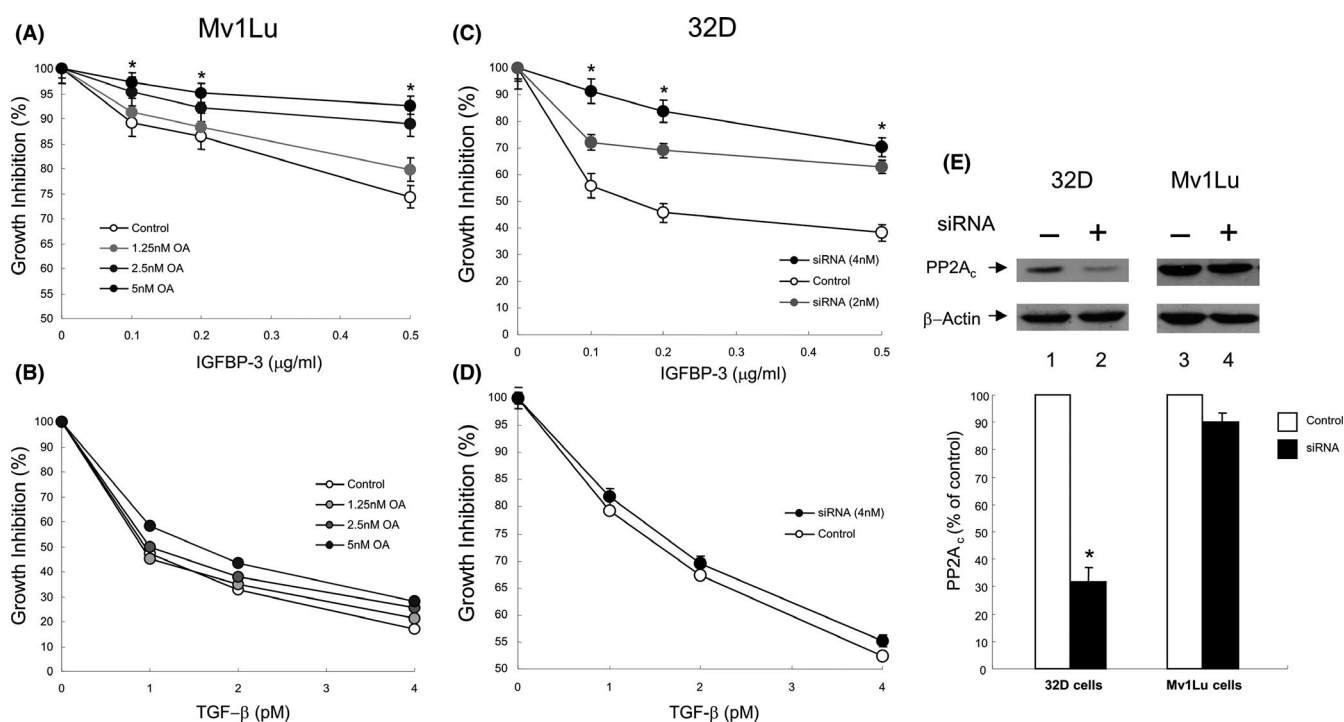


FIGURE 4 IGFBP-3 but not TGF-β-induced growth inhibition is reversed by OA in Mv1Lu cells (A, B) and by PP2A_c siRNA in 32D cells (C, D) via attenuating PP2A_c expression in these cells (E). (A,B) Mv1Lu cells were treated with several concentrations of OA for 1 hr at 37°C and then treated with several concentrations IGFBP-3 (A) or TGF-β (B). After 18 hr at 37°C, the cell growth was determined by measurement of [methyl-³H] thymidine incorporation into cellular DNA. The [methyl-³H] thymidine incorporation in cells incubated without OA was taken as 100% cell growth. The reversibility (%) by OA of IGFBP-3- or TGF-β-induced growth inhibition was estimated by $100 \times (\text{IGFBP-3- or TGF-}\beta\text{-induced growth inhibition in the absence of OA} - \text{IGFBP-3- or TGF-}\beta\text{-induced growth inhibition in the presence of OA}) / \text{IGFBP-3- or TGF-}\beta\text{-induced growth inhibition in the absence of OA}$. The assays were performed in triplicates. The data are mean \pm SD *Significantly lower than that of cells treated with control (vehicle only) (A): $p < 0.01$. (C,D) 32D cells were transfected with 0, 2, and 4 nM (C) or 0 and 4 nM (D) murine PP2A_c siRNA by electroporation. Transfected cells were treated with several concentrations of IGFBP-3 (C) or TGF-β (D). After 18 hr at 37°C, the cell growth was determined by measurement of [methyl-³H] thymidine incorporation into cellular DNA. The cell growth in cells treated with control siRNA (control) was taken as 100% (C, D). The data are mean \pm SD *Significantly lower than that of cells treated with control siRNA (control) (C): $p < 0.01$. (E) Murine PP2A_c siRNA attenuates the expression of PP2A_c protein in murine 32D cells but not in mink Mv1Lu cells. 32D and Mv1Lu cells were transfected with control siRNA (-) and 4 nM murine PP2A_c siRNA (+) by electroporation. Transfected cells were analyzed by Western blot analysis using antibody to PP2A_c (top panel), which were representatives of a total of three experiments, and quantified by densitometry (bottom panel). Murine PP2A_c siRNA was effective in attenuating PP2A_c expression in 32D cells (murine cells) (top panel, lane 2 vs. lane 1) but not in Mv1Lu cells (mink lung cells) (top panel, lane 4 vs. lane 3). The analysis was performed in triplicates. The data are mean \pm SD *Significantly lower than that of cells treated with control siRNA (-): $p < 0.01$

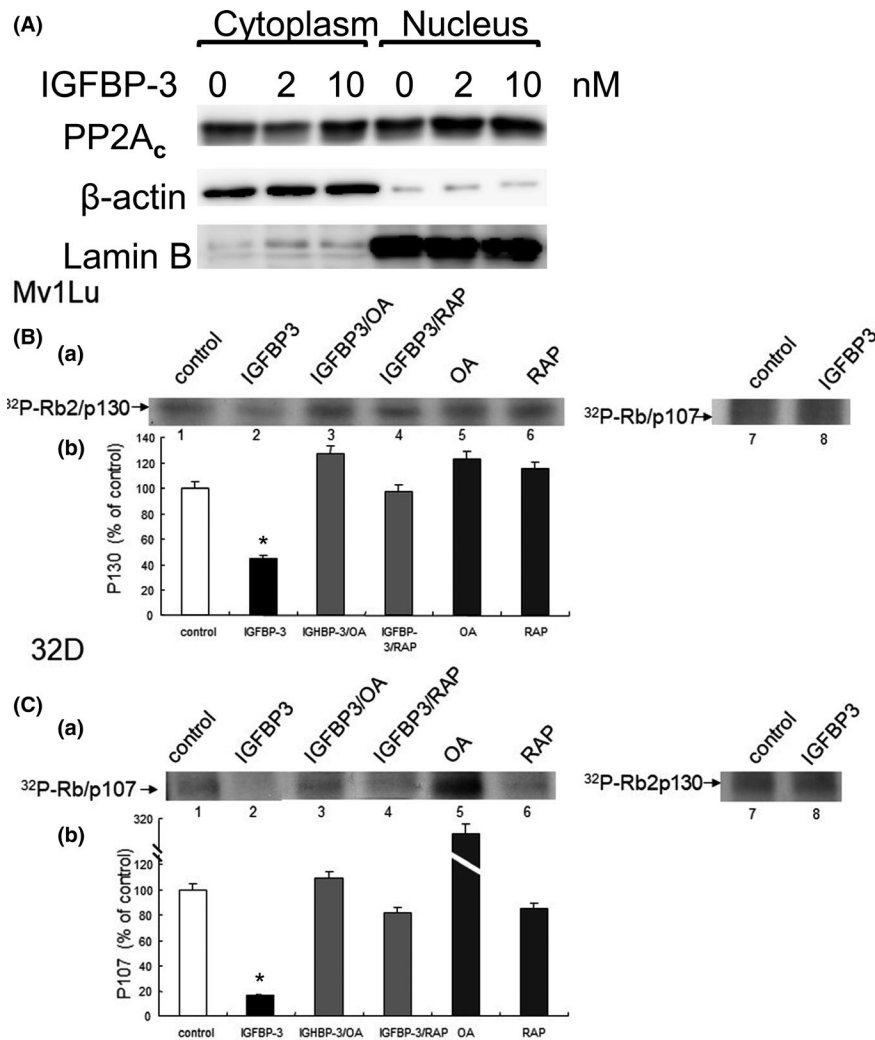


FIGURE 5 IGFBP-3 stimulates cytoplasm-to-nucleus translocation of PP2A_c in Mv1Lu cells (A) and inhibits growth by inducing dephosphorylation of pRb-related proteins, p130 and p107, in Mv1Lu (B) and 32D (C) cells, respectively. Mv1Lu cells were treated with 0, 2, and 10 nM (or 0, 0.06 and 0.3 μg/ml, respectively), IGFBP-3 for 2 hr. The cytoplasm and nucleus fractions were separated by centrifugation and analyzed by 7.5% SDS-PAGE followed by Western blot analysis using antibodies to PP2A_c, β-actin, and lamin B. The final volume of the total cytoplasm fraction was 10 times higher than that of the total nucleus fraction. However, an equal volume of cytoplasm and nucleus fractions was analyzed by 7.5% SDS-PAGE followed by Western blot analysis. At 2 and 10 nM, IGFBP-3 appeared to increase cytoplasm-to-nucleus translocation of PP2A_c by ~2 fold. Western blot analysis was the representative of a total of three experiments. Lamin B and β-actin served as nuclear and cytoplasmic internal standards, respectively. (B and C) Mv1Lu (B) and 32D (C) cells were pre-incubated with [³²P]-orthophosphate for 2 hr, washed and incubated in the culture medium with excess phosphate. ³²P-labeled cells were treated with vehicle only or 0.3 μg/ml of IGFBP-3 in the presence and absence of OA (5 nM) and RAP (60 μg/ml). After 2 hr at 37°C, cell lysates were immunoprecipitated with antibodies to p130 and p107. The immunoprecipitates were analyzed by 7.5% SDS-PAGE and quantified by a Perkin Elmer phosphorimager (B and C, panels a and b). Phosphorimager analysis was the representative of a total of three experiments. IGFBP-3 appeared to stimulate dephosphorylation of p130 and p107 in Mv1Lu and 32D cells, respectively (B and C, panels a, lane 2 vs. lane 1 and panel b, quantitative data). OA and RAP inhibited IGFBP-3-stimulated dephosphorylation of p130 and p107 (B and C, panel a, lanes 3 and 4 vs. lane 2 and panel b, quantitative data) in Mv1Lu and 32D cells, respectively. The quantitative data from three independent analyses were shown. The data are mean ±SD *Significantly lower than that of cells treated with vehicle only (control): *p* < 0.01

3.6 | IGFBP-3 inhibits growth by inducing dephosphorylation of pRb-related proteins, p130 and p107, in Mv1Lu and 32D cells, respectively

PP2A plays a critical multi-faceted role in the regulation of the cell cycle. It has been implicated in dephosphorylation of

two retinoblastoma protein (pRb)-related proteins, p130 and p107, which interact primarily with E2F4 and E2F5 and are most active in G0-the quiescent phase of the cell cycle.^{26,27} Moreover, pRb (p105) interacts primarily with E2F1–3 and is most active at the G1-to-S phase transition.^{26,27} These suggest that IRS-2-PP2A complexes may dephosphorylate pRb-related proteins (p130 and p107) in the nucleus of target

cells. To test this possibility, Mv1Lu and 32D cells were pre-labeled with ^{32}P -orthophosphate at 37°C for 1 hr, washed, and incubated with $0.3\ \mu\text{g/ml}$ ($10\ \text{nM}$) IGFBP-3 in the presence of excess unlabeled orthophosphate in the medium. After 2 hr at 37°C , ^{32}P -labeled cell lysates were immunoprecipitated with specific antibodies to p130 and p107 and analyzed by 7.5% SDS-PAGE and quantified by a phosphorimager (panels a and b, respectively). As shown in Figure 5B and Figure 5C, IGFBP-3-induced dephosphorylation of p130 and p107 in Mv1Lu and 32D cells, respectively (panel a, lane 2 vs. lane 1 and panel b, quantitative analysis from three experiments). The IGFBP-3-induced dephosphorylation of p130 and p107 was blocked in these cells co-treated with OA and RAP (Figure 5B,C, panel a, lanes 3 and 4 vs. lane 2 and panel b, quantitative analysis from three experiments).

3.7 | TGF- β induces colocalization of T β R-V and PP1_c at the plasma membrane and accumulation of PP1 and decreased levels of hyperphosphorylated pRb (P-Rb) in the nucleus in Mv1Lu cells

The TGF- β -stimulated PPase involved in TGF- β -induced growth inhibition has been identified as PP1 in human keratinocytes.²⁸ PP1 is responsible for dephosphorylating of pRb (p105) which is linked to TGF- β -induced growth inhibition in Mv1Lu cells.²⁹ The mechanism by which TGF- β stimulates PP1 activity is not clear. Since TGF- β induces growth inhibition by stimulating complex formation of T β R-V, IRS-1/2, and likely PP1 at the plasma membrane in Mv1Lu cells, we hypothesize that PP1 should be activated by its interaction with IRS-1/2 in the formation of the T β R-V-IRS-1/2-PP1 ternary complexes in TGF- β -treated cells. PP1 enzyme contains both a 37-kDa catalytic subunit (PP1_c) and at least one regulatory subunit which directs PP1_c to different substrates or sites. To test this, we performed immunofluorescence microscopy in Mv1Lu cells treated with $40\ \text{pM}$ TGF- β at 37°C for 0 and 1 hr using antibodies to T β R-V (LRP-1) and PP1_c (Figure 6A). TGF- β -stimulated colocalization of T β R-V and PP1_c at the plasma membrane, as indicated by arrowheads in Mv1Lu cells treated with TGF- β at 37°C for 1 hr (Figure 6Af). In contrast, Mv1Lu cells treated with $40\ \text{pM}$ TGF- β for 0 hr did not exhibit colocalization of T β R-V and PP1_c in these cells (Figure 6Ac).

Retinoblastoma protein (pRb) present in the cytoplasm and nucleus fractions are identified as hyperphosphorylated (as a slow-migrating form) and hypophosphorylated (as a fast-migrating form) forms of pRb, respectively, based on its mobility on 7.5% SDS-PAGE.^{29–31} Cytoplasmic pRb is known to be mainly the hyperphosphorylated form.³² These suggest that TGF- β stimulates cytoplasm-to-nucleus translocation of PP1_c and correspondingly increases the amount of hypophosphorylated pRb (as a fast-migrating form of pRb on 7.5% SDS-PAGE),

which is the PP1_c-dephosphorylated product of pRb in the nucleus. To demonstrate the subsequent cytoplasm-to-nucleus translocation of PP1_c and its effect on dephosphorylation of pRb in the nucleus, we performed immunofluorescence analysis in Mv1Lu cells treated with $40\ \text{pM}$ TGF- β at 37°C for 0, 1, and 2 hr using specific antibodies to PP1_c and hyperphosphorylated pRb (P-Rb). We reasoned that TGF- β promotes nucleus accumulation of PP1_c and should accordingly decrease the amount of P-Rb, its target substrate, in the nucleus. PP1 specifically dephosphorylates pRb in the nucleus of target cells.^{28,29} After treatment of cells with TGF- β and immunofluorescence staining, six images, which consist of 6–8 cells/image, were taken at different areas of cells grown on a coverslip. The image shown in the data was the representative of the six images. As shown in Figure 6B, after treatment of cells with TGF- β at 37°C for 1 or 2 hr, approximately 40%–50% cells on a coverslip exhibited significantly decreased yellow fluorescence (co-localization) in the nucleus, whereas ~90% cells (treated with TGF- β at 37°C for 0 hr) on a coverslip exhibited yellow fluorescence (colocalization) in the nucleus (Figure 6Bf,i and Figure 6Bc, respectively). TGF- β treatment of cells for 1 and 2 hr decreased the amount of P-Rb and colocalization of PP1_c and P-Rb in the nucleus (Figure 6Be,h and Figure 6Bf,i, respectively). These results support the suggestion that TGF- β promotes cytoplasm-to-nucleus translocation of PP1_c, resulting in dephosphorylation of pRb in the nucleus, which leads to cell growth arrest.

3.8 | TGF- β stimulates cytoplasm-to-nucleus translocation of PP1_c and increases formation of dephosphorylated pRb (Rb) in the nucleus in Mv1Lu (A) and A549 (B) cells

To further support the hypothesis that TGF- β stimulates cytoplasm-to-nucleus translocation of PP1, we determined the subcellular localization of PP1_c, PP2A_c, pRb (Rb), phosphorylated Smad2 (P-Smad2), phosphorylated IRS-1/2 (P-IRS-1/2), phosphorylation at Ser 270, lamin B, and β -actin using 7.5% SDS-PAGE and quantitative Western blot analysis with specific antibodies to PP1_c, PP2A_c, and others after subcellular cytoplasm/nucleus fractionation of Mv1Lu and A549 cells treated with $40\ \text{pM}$ TGF- β for 0, 1, and 2 hr. As shown in Figure 7, TGF- β increased the amounts of PP1_c (Figure 7A,B), dephosphorylated Rb (as a fast-migrating form of Rb on 7.5% SDS-PAGE) (Figure 7A,B), P-IRS-2 (Figure 7A), and P-Smad2 (Figure 7A,B) in the nucleus fraction in a time-dependent manner in these cells. Both P-IRS-1/2 contain Ser 270 but only P-IRS-2 entered the nucleus (Figure 3Be,h). After 2 hr, TGF- β increased the amounts of PP1_c, dephosphorylated Rb (as a fast migrating form on 7.5% SDS-PAGE), P-IRS-2 and P-Smad2 by 1.5- to 2-fold ($n = 3$) in the nucleus fraction in Mv1Lu and A549 cells. In contrast, TGF- β did not significantly increase the amount of PP2A_c in the nucleus fraction in these cells.

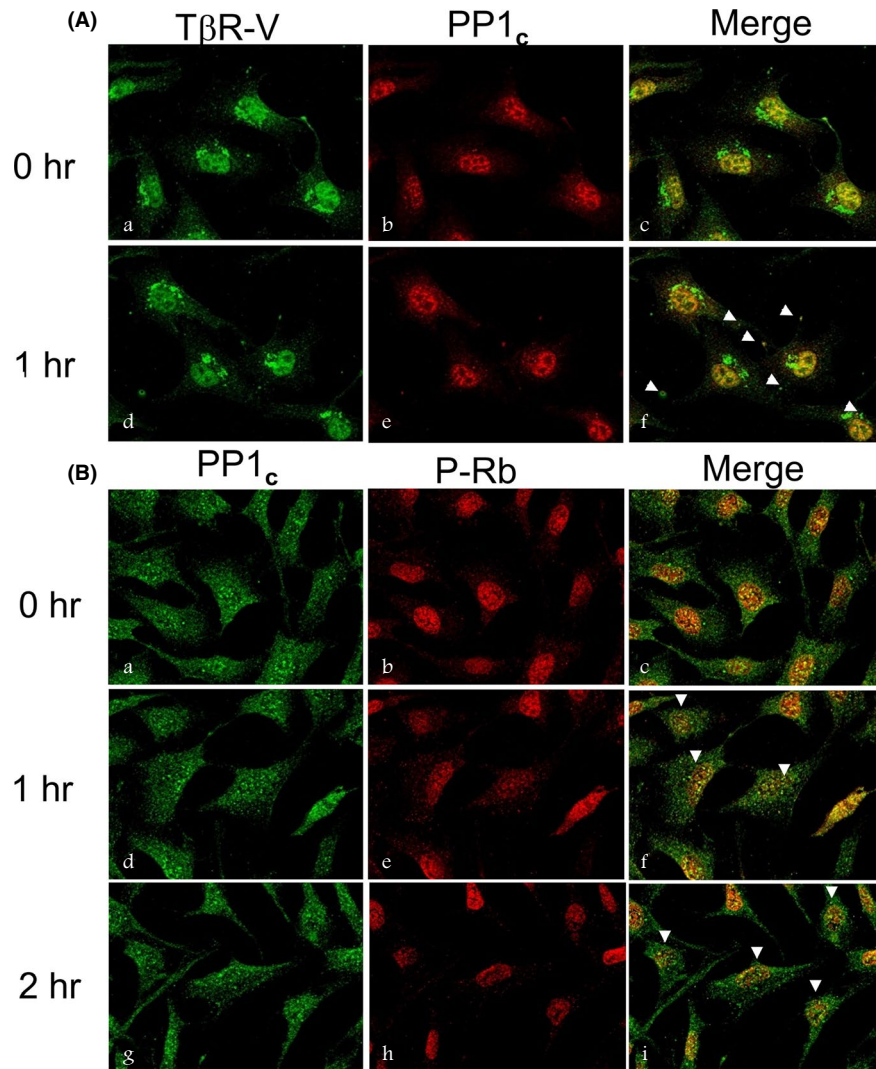


FIGURE 6 TGF- β induces co-localization of T β R-V and PP1_c at the plasma membrane (A) and accumulation of PP1_c and decreased levels of hyperphosphorylated pRb (P-Rb) in the nucleus (B) in Mv1Lu cells. Mv1Lu cells were grown to 50% confluence on coverslips in 35 mm culture dishes at 37°C for 24 hr. Mv1Lu cells were then treated with 40 pM TGF- β at 37°C. After 0 and 1 hr (A) or 0, 1, and 2 hr (B), cells were fixed and stained by immunofluorescence using antibodies to T β R-V and PP1_c (A) or using antibodies to PP1_c and P-Rb (B). (A) After immunofluorescence staining, cells on coverslips were counted. Cells treated with TGF- β at 37°C for 0 hr did not exhibit colocalization of T β R-V and PP1_c at the plasma membrane (Ac). However, cells treated with TGF- β at 37°C for 1 hr exhibited colocalization of T β R-V and PP1_c at the plasma membrane as indicated by arrowheads (Af). T β R-V (LRP-1) is known to undergo constitutive endocytosis and recycling in cells. Perinuclear labeling is likely to be endocytic vesicles which are often seen in juxtannuclear regions. This appearance of endocytic vesicles might be due to longer-time cell culture before the experiment. (B) After treatment of cells with TGF- β for 1 or 2 hr at 37°C, approximately 40%–50% cells on a coverslip exhibited significantly decreased yellow fluorescence (co-localization) in the nucleus, whereas ~90% cells (treated with TGF- β at 37°C for 0 hr) on a coverslip exhibited yellow fluorescence (colocalization) in the nucleus (Bf,i and Bc, respectively). Arrowheads indicate decreased colocalization (as marked by decreased yellow fluorescence) of PP1_c and P-Rb (Bf,i) due to decreased P-Rb (as marked by decreased red fluorescence) in the nucleus (Be,h)

Interestingly, Rb present in the cytoplasm and nucleus fractions were identified as phosphorylated (as a slow-migrating form) and dephosphorylated (as a fast-migrating form) forms of Rb, respectively, based on its mobility on 7.5% SDS-PAGE.^{29–31} Cytoplasmic Rb is known to be mainly the hyperphosphorylated form.³² These results suggest that TGF- β stimulates cytoplasm-to-nucleus translocation of PP1_c and P-IRS-2, and correspondingly increases the amount of dephosphorylated Rb (as a

fast-migrating form of Rb), which is the PP1-dephosphorylated product of Rb, in the nucleus.

4 | DISCUSSION

Here, we have provided evidence revealing that IGFBP-3 inhibits growth in epithelial cells by stimulating the

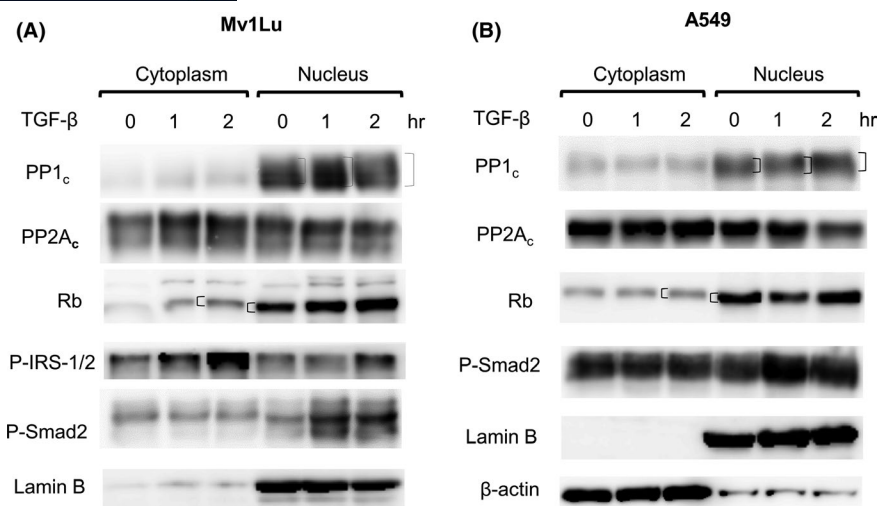


FIGURE 7 TGF- β stimulates cytoplasm-to-nucleus translocation of PP1_c in Mv1Lu (A) and A549 (B) cells and increases dephosphorylated pRb (a fast-migrating form of pRb) levels in the nucleus in these cells. Mv1Lu (A) and A549 (B) cells were treated with 40 pM TGF- β for 0, 1, and 2 hr. The cytoplasm and nucleus fractions were separated by centrifugation and analyzed by 7.5% SDS-PAGE followed by quantitative Western blot analysis using antibodies to PP1_c, PP2A_c, pRb (Rb), P-IRS-1/2 (phosphorylated IRS-1/2, Ser 270), P-Smad2 (phosphorylated Smad2), lamin B, and β -actin. pRb (Rb) (present in the nucleus) migrated as a fast-migrating form (dephosphorylated pRb) on 7.5% SDS-PAGE. pRb (Rb) (present in the cytoplasm) migrated as a slow form (phosphorylated pRb). The final volume of the total cytoplasm fraction was 10 times higher than that of the total nucleus fraction. An equal volume of cytoplasm and nucleus fractions was then analyzed by 7.5% SDS-PAGE followed by Western blot analysis. Western blots were representatives of a total of three experiments. Lamin B and β -actin served as nuclear and cytoplasmic internal standards, respectively

T β R-V-mediated growth inhibition signaling cascade which involves IRS-2, PP2A, and pRb-related proteins, p130 or p107. We propose a revised version of our previously published model⁷ to demonstrate the molecular mechanism of IGFBP-3-induced growth inhibition in epithelial cells and other cell types. In this model (Figure 8A), IGFBP-3 is a non-covalently linked homodimeric protein. It binds to T β R-V by interaction with its cell surface subdomains II and IV,^{4,5,7} resulting in dimerization of T β R-V and subsequent recruitment of Ser/Thr-phosphorylated IRS-1 or IRS-2 and then PP2A to the cytoplasmic tail of the dimeric T β R-V. Interacting proteins or specific protein substrates of PP2A are known to possess PP2A-B56 docking (or binding) motifs (L/MxxL/I/VxE) which contain contiguous six amino acid residues and are well conserved throughout the eukaryotic domain of life and in human viruses.³³ IRS-1 and IRS-2 possess PP2A-B56 docking motifs of LytrdE (residues 86–91) and LkeLfe (residues 294–299), respectively, which provide binding sites for PP2A-B56 (binding affinity: IRS-2 > IRS-1). PP2A in the T β R-V-IRS-1-PP2A or T β R-V-IRS-2-PP2A ternary complex becomes activated and dephosphorylates IRS-1 or IRS-2 in the complex, leading to dissociation of dephosphorylated IRS-1-PP2A or IRS-2-PP2A binary complexes from the cytoplasmic tail of T β R-V (at the plasma membrane). High-affinity-bound dephosphorylated IRS-2-PP2A complexes then undergo IRS-2-dependent translocation from cytoplasm to the nucleus where activated PP2A dephosphorylates pRb-related proteins, p130 and p107, resulting in growth arrest.

p130 and p107 possess high-affinity PP2A-B56 docking motifs of LsgIIE (residues 519–524) and LinIfE (residues 412–417), respectively. It is important to note that p130 and p107 do not possess specific PP1_c docking motifs (FxxR/KxR/K),³⁴ suggesting that p130 and p107 are the PP2A target substrates in the nucleus. In this communication, we also demonstrate that IGFBP-3 stimulates cytoplasm-to-nucleus translocation of IRS-2 but not IRS-1. IRS-2 has been shown to undergo nuclear translocation in normal and cancer cells.³⁵ It possesses a putative nuclear localization signal (NLS) motif of KKwRsK (residues 80–85). Moreover, after dissociation from the cytoplasmic tail of T β R-V, low-affinity-bound dephosphorylated IRS-1-PP2A complexes are mainly present in the cytoplasm and do not have known functions in cells treated with IGFBP-3.⁷ However, IGFBP-3 is known to inhibit phosphorylation of c-raf-MEK-ERK and p38 kinase in insulin-secreting cells.³⁶ PP2A is also known to inhibit the kinase activities of the kinases involved in T β R-I-activated non-Smad pathways, which include JNK,³⁷ TAK1-p38/JNK,³⁸ PI3K-AKT,³⁹ and RhoA-Rock⁴⁰ signaling, by dephosphorylating these kinases.^{41,42} It is likely that IRS-1-PP2A is responsible, at least in part, for IGFBP-3-induced inhibition of non-Smad signaling.³⁶

Several lines of evidence suggest that IGFBP-3 acts as a potential tumor suppressor gene. First, aberrant promoter methylation of IGFBP-3 gene, which silences its expression, is detected in human gastric cancer, colorectal cancer, breast cancer, and malignant mesothelioma cancer.²⁰ Second, low

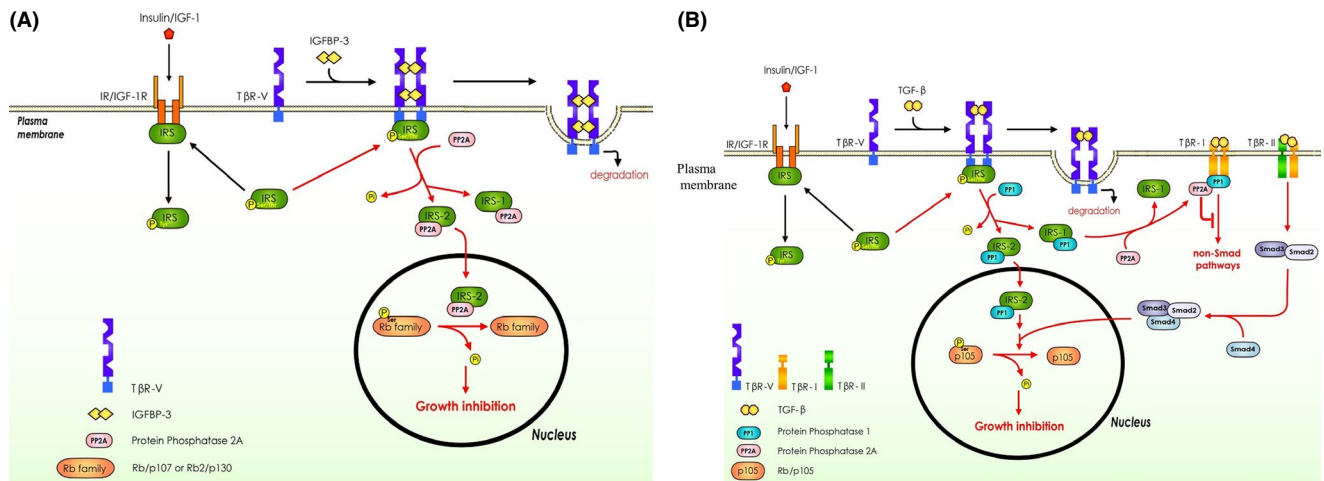


FIGURE 8 Models for the mechanisms by which IGFBP-3 (A) and TGF- β (B) induces cellular growth inhibition and the cross talk between T β R-V and IR/IGF-1R signaling (A, B). (A) IGFBP-3 induces growth inhibition by stimulating T β R-V-mediated IRS-2-dependent activation and cytoplasm-to-nucleus translocation of PP2A, resulting in dephosphorylation of pRb-related proteins (p130 and p107). In the IGFBP-3-stimulated tumor suppressor signaling cascade (T β R-V/IRS-2/PP2A/p107, p130), T β R-V is identical to LRP-1 which has a 515-kDa α chain that contains four cell surface ligand-binding subdomains (I, II, III, and IV) and an 85-kDa β chain comprising the transmembrane domain and cytoplasmic tail. IGFBP-3 binds to cell surface subdomains II and IV of T β R-V. Insulin and IGF-I antagonize IGFBP-3-induced cellular growth inhibition by stimulating tyrosine phosphorylation of IRS-1/2 via interaction with their cognate receptors. The signaling cascades potentiated in diabetic patients are indicated by red arrows. Under non-insulin-stimulating conditions, IRS-1/2 are mainly phosphorylated at serine residues. (B) In epithelial cells, TGF- β induces potent growth inhibition (100% growth inhibition at 1–5 pM) by stimulating the T β R-V-mediated IRS-2-dependent tumor suppressor signaling cascade (T β R-V/IRS-2/PP1/pRb) in concert with canonical TGF- β signaling mediated by T β R-I and T β R-II (T β R-I/T β R-II/Smad-2/3/4). The T β R-V-mediated tumor suppressor signaling cascade (T β R-V/IRS-2/PP1/pRb) is essential to dephosphorylate (activate) pRb (by PP1) for causing TGF- β growth inhibition. T β R-I–T β R-II-mediated canonical signaling is required for potentiating TGF- β growth inhibition (mediated by T β R-V) by transcriptional activation of CDK inhibitors¹⁴ which activate the T β R-V/IRS-2/PP1/pRb cascade via maintaining pRb unphosphorylated (active) in the nucleus.⁷ TGF- β also stimulates IRS-1-dependent activation and formation of PP1-PP2A complexes which target and suppress T β R-I-mediated non-Smad pathways. The T β R-I is present as T β R-V–T β R-I complexes⁹ mainly localized in plasma-membrane non-lipid raft microdomains. In normal epithelial cells, which express T β R-V, TGF- β , as a tumor suppressor, suppresses carcinogenesis by potently inhibiting cell growth in normal epithelial cells via stimulating T β R-V-mediated IRS-2-dependent tumor suppressor signaling (T β R-V/IRS-2/PP1/pRb) in concert with canonical TGF- β signaling (T β R-I/T β R-II/Smad2/3/4) and by suppressing T β R-I-mediated tumor progression via stimulating T β R-V-mediated IRS-1-dependent signaling (T β R-V/IRS-1/PP1-PP2A/T β R-I). Insulin and IGF-I antagonize TGF- β -stimulated T β R-V/IRS-1/2/PP1 signaling by stimulating tyrosine phosphorylation of IRS-1/2 via interaction with their cognate receptors, insulin receptor (IR), and IGF-1 receptor (IGF-1R). The tyrosine phosphorylation of IRS-1/2 catalyzed by IR and IGF-1 receptor, which occurs more rapidly than the Ser/Thr-specific dephosphorylation of IRS-1/2 by PP1 or PP2A, leads to multiple IR/IGF-1R downstream signaling pathways and prevents the formation of T β R-V-IRS-1/2-PP1 complexes. Tyrosine phosphorylation and Ser/Thr-specific dephosphorylation of IRS-1/2 are mutually exclusive.⁷ In diabetes, insulin or insulin signaling defects potentiate TGF- β -induced growth inhibition in target cells.⁷ In addition, high glucose in the plasma and tissues of diabetic patients may enhance T β R-V and T β R-I/T β R-II signaling via increasing TGF- β production and T β R-I/T β R-II expression.^{7,80} Increased ECM synthesis (which is mediated by T β R-I/T β R-II/Smad2/3/4 signaling) further attenuates insulin signaling and enhances TGF- β -induced growth inhibition,⁷ resulting in alopecia, impaired wound healing, accelerated glomerulopathy, and tissue fibrosis in diabetic patients. The signaling cascades potentiated in diabetic patients are indicated by red arrows

levels of IGFBP-3 expression in cancer tissues are correlated with poor prognosis for patients with esophageal squamous cell carcinoma⁴³ and hepatocellular carcinoma.⁴⁴ Third, low IGFBP-3 expression correlates clinically with higher tumor grade, advanced stage, and poor survival in ovarian endometrioid adenocarcinoma patients.⁴⁵ Here, we demonstrate that IGFBP-3 inhibits cell growth by stimulating the T β R-V-mediated tumor suppressor signaling pathway (T β R-V/IRS-1/2/PP2A/p130, p107). Among the components of this signaling cascade, T β R-V and PP2A have been proved to be tumor suppressor genes by that stable transfection of human

carcinoma cells and CHO-LRP-1^{-/-} cells with LRP-1 (T β R-V) cDNA restores the growth inhibitory response to IGFBP-3 and TGF- β , and normal epithelial morphology^{10,15} and by that loss of PP2A regulatory subunit B56 δ promotes spontaneous tumorigenesis in vivo.⁴⁶ In addition, knockout of the T β R-V (LRP-1) gene and both p130 and p107 genes in mice has been shown to cause embryonic and neonatal lethality, respectively.^{47,48} In normal epithelial cells, IGFBP-3 induces growth inhibition by stimulating T β R-V-mediated and IRS-1/2-dependent activation of PP2A. PP2A may serve as an important down-stream effector for mediating other known

cell biological activities of IGFBP-3 and other high-affinity IGFbps.^{1,36}

T β R-V is the only cell surface IGFBP-3 receptor identified by I¹²⁵-labeled IGFBP-3 affinity labeling (binding/cross-linking) followed by immunoprecipitation using antiserum to T β R-V in epithelial cells and other cell types.⁴⁻⁶ T β R-V also binds IGFBP-4 and IGFBP-5 but not IGFBP-1, IGFBP-2, and IGFBP-6, as determined by I¹²⁵-labeled IGFbps affinity labeling.^{5,6} It exhibits the highest binding affinity toward IGFBP-3 with a K_d of 10 nM.⁴⁻⁶ A TGF- β peptide antagonist β_1^{25} , which contains a minimal active site motif of WS/CXD in TGF- β and IGFBP-3 molecules, blocks TGF- β and IGFBP-3 binding to TGF- β receptors in epithelial cells and reverses growth inhibition induced by either TGF- β or IGFBP-3 in these cells.^{4,5,11,12} The transmembrane protein TMEM219 (25 kDa) was also identified as an IGFBP-3 receptor (termed IGFBP-3R) in 2010, using the yeast two-hybrid screening and a human breast cancer cell cDNA library.⁴⁹ In contrast to the IGFBP-3 receptor (T β R-V/LRP-1), IGFBP-3R/TMEM219 does not bind other high-affinity IGFbps⁴⁹ and have known function of a tumor suppressor gene. IGFBP-3R/TMEM219 was identified as a cell death receptor mediating IGFBP-3-induced anti-tumor effects in cancer cells^{49,50} and as an autophagy-activation receptor mediating IGFBP-3-activated autophagy in Vero cells.⁵¹ In addition, the K_d of IGFBP-3 binding to TMEM219/IGFBP-3R has been estimated to be 125 nM.⁵¹ IGFBP-3 (1 μ M) is utilized to stimulate TMEM219/IGFBP-3R-mediated autophagy activation in Vero cells (kidney epithelial cells).⁵¹ It is important to note that carcinoma cancer cells do not express the IGFBP-3 receptor (T β R-V) and loss of T β R-V confers cancer malignancy.⁷ Many lines of evidence suggest that the IGFBP-3 receptor (T β R-V) is the primary IGFBP-3 receptor in normal epithelial cells and other cell types.⁷

The transcriptional activation and growth inhibition activities of TGF- β have generally been thought to be mediated by the canonical T β R-I/T β R-II/Smad2/3/4 signaling cascade.¹³ However, these two activities appear to segregate in several cell types and under various conditions.⁵²⁻⁵⁴ This suggests that other signaling pathways must be involved in mediating the TGF- β activities. Although TGF- β -stimulated canonical T β R-I/T β R-II/Smad2/3/4 signaling can be modulated by other signaling pathways,^{55,56} it is mainly responsible for mediating the transcriptional activation of ECM synthesis-related genes. Smad2/3/4 responsive elements exist in the promoter regions of all responsive genes. In contrast, the signaling involved in TGF- β -induced growth inhibition in target cells is unknown. Other signaling pathways, in addition to the well-known canonical T β R-I/T β R-II/Smad2/3/4 signaling pathway, are suggested to be involved in the growth inhibitory response to TGF- β .^{57,58} The Ras/ERK signaling and PP2A are involved in mediating TGF- β -induced growth inhibition in certain cells.^{59,60} However, the main signaling

pathway, in concert with canonical T β R-I/T β R-II/Smad2/3/4 signaling,¹³ mediates the growth inhibitory response to TGF- β in epithelial cells remains unknown. T β R-I, T β R-II, T β R-III, and T β R-V co-express in all normal cell types studied. Since the T β R-III null mutation in mice does not affect the growth regulatory response to TGF- β in embryonic fibroblasts derived from these mice,⁶¹ the remaining candidate is T β R-V. Many carcinoma cells and primary tumors express no or very low levels of T β R-V expression.^{2,7,16-18} Growth of these cells is not inhibited by either TGF- β or IGFBP-3. In the absence of T β R-V in late-stage cancer, TGF- β induces EMT (epithelial-mesenchymal transition), autoinduction, and increased invasiveness by stimulating T β R-I-activated or T β R-I-mediated non-Smad signaling pathways³⁷⁻⁴⁰ as well as canonical Smad signaling (T β R-I/T β R-II/Smad2/3/4 signaling).¹³ T β R-II is apparently not involved in TGF- β -stimulated T β R-I-activated/mediated non-Smad signaling pathways³⁷⁻⁴² which are involved in cell survival, migration, proliferation, malignant transformation, and tumor growth. In fact, TGF- β stimulates tumor promoter signaling toward EMT is mediated by both non-Smad and Smad pathways. While no LRP-1 (T β R-V) is detected in hepatoma in human patients, it is present in the normal parenchymal tissue surrounding the hepatomas.¹⁶ These suggest that T β R-V is involved in mediating the growth inhibitory response to TGF- β in normal epithelial cells and that its loss contributes to the malignant phenotype in cancer cells.⁷ These are also consistent with the notion that T β R-V acts as a tumor suppressor gene and controls cell growth in normal epithelial cells by mediating TGF- β -induced growth inhibition in these cells. The loss or deficiency of T β R-V leads to the development of carcinoma cancer. Although no mutation in the LRP-1 (T β R-V) gene has been found related to cancer initiation or progression, the T allele of the C766 T polymorphism in the LRP-1 (T β R-V) gene is associated with an increased risk of breast cancer.⁶²

We previously proposed a model for the mechanism by which TGF- β inhibits growth in epithelial cells by binding to a site between cell surface subdomains I and II of T β R-V in target cells. In this model, TGF- β stimulates sequential association of IRS-1 or IRS-2 and a Ser/Thr-specific PPase with the cytoplasmic tail of T β R-V by inducing T β R-V dimerization via its covalently linked homodimeric structure. In the ternary complexes, the Ser/Thr-specific PPase becomes activated and dephosphorylate IRS-1/2. Dephosphorylated IRS-1-PPase or IRS-2-PPase binary complexes dissociate from the cytoplasmic tail of T β R-V and undergo IRS-1/2-dependent translocation from cytoplasm to the nucleus where the PPase dephosphorylates pRb (retinoblastoma protein) or pRb-related proteins, resulting in growth arrest. This model lacked the identity of PPase, IRS-1/2, and retinoblastoma family proteins which are involved in TGF- β -stimulated T β R-V-mediated tumor suppressor (growth inhibition) signaling.⁷ Here, we provide several lines of evidence to suggest that

PP1, IRS-1/2, and pRb (p105) are involved in the TGF- β -stimulated T β R-V-mediated tumor suppressor signaling cascade (T β R-V/IRS-2/PP1/pRb). These include: (1) TGF- β stimulates colocalization of T β R-V and IRS-1/2 at the plasma membrane in Mv1Lu cells, as demonstrated by immunofluorescence staining. (2) TGF- β stimulates cytoplasm-to-nucleus translocation of IRS-2 but not IRS-1 in Mv1Lu cells, as demonstrated by immunofluorescence staining. (3) PP1 is known to be responsible for mediating TGF- β -stimulated dephosphorylation of pRb in keratinocytes and Mv1Lu cells.^{28,29} (4) TGF- β -stimulated PPase (PP1) activity is abolished in cells co-treated with RAP (LRP-1/T β R-V antagonist) or insulin^{7,22,23} in Mv1Lu cells. (5) TGF- β -stimulated PPase (PP1) activity is distinct from IGFBP-3-stimulated PPase (PP2A) activity in its relative insensitivity to OA inhibition, which appears to be the biochemical character of PP1 activity.^{26,27} PP2A is completely inhibited at 1 nM OA, compared to greater than 1 μ M OA for PP1.²⁶ (6) OA at 0.5 nM completely inhibits IGFBP-3-stimulated PPase (PP2A) activity, but not TGF- β -stimulated PPase (PP1) activity, in Mv1Lu cells treated with IGFBP-3 and TGF- β . (7) TGF- β stimulates cytoplasm-to-nucleus translocation of PP1_c, resulting in dephosphorylation of pRb (p105) in the nucleus, as demonstrated by Western blot analysis following subcellular fractionation (to yield cytoplasm and nucleus fractions) and immunofluorescence analysis. (8) PP1 as well as PP2A are the master regulators of the eukaryotic cell cycle.²⁷ The above evidence supports an updated model (Figure 8B) in which TGF- β induces growth inhibition in target cells by stimulating T β R-V-mediated signaling (T β R-V/IRS-2/PP1) which leads to dephosphorylation of pRb (p105) in the nucleus, resulting in cell growth arrest.

In this model (Figure 8B), TGF- β , a covalently associated homodimeric cytokine, interacts with T β R-V at a site between subdomains I and II,^{7,10,13} resulting in dimerization of T β R-V and sequential recruitment of IRS-1 or IRS-2 and PP1 to the cytoplasmic tail of dimeric T β R-V to form T β R-V-IRS-1-PP1 or T β R-V-IRS-2-PP1 ternary complexes. IRS-1 and IRS-2 possess PP1_c docking motifs of FrssfR (residues 438–443) and FefRpR (residues 298–303), respectively (PP1_c docking affinity: IRS-2 > IRS-1). IRS-2 appears to comprise of overlapping high-affinity PP1 and PP2A docking motifs of FefRpR (residues 298–303) and LkeLfe (residues 294–299), respectively, suggesting that PP1 and PP2A docking to IRS-2 are mutually exclusive. After dephosphorylation of IRS-2 by activated PP1 in the ternary complex, dephosphorylated IRS-2-PP1 binary complexes dissociate from the cytoplasmic tail of T β R-V and enter the nucleus via the nucleus-targeting function of IRS-2. In the nucleus, PP1 dephosphorylates pRb (p105), resulting in cell growth arrest. In normal epithelial cells, TGF- β potently inhibits cell growth (~100% growth inhibition at 1–5 pM) by stimulating the T β R-V-mediated signaling

cascade (T β R-V/IRS-2/PP1/pRb) to dephosphorylate (activate) pRb by PP1 in concert with canonical TGF- β signaling (T β R-I/T β R-II/Smad2/3/4).⁷ TGF- β -stimulated canonical signaling potentiates T β R-V-mediated growth inhibition in these epithelial cells, at least in part, by transcriptional activation of cyclin-dependent kinase (CDK) inhibitors which maintain pRb unphosphorylated (active) in the nucleus.^{7,13} Moreover, upon TGF- β stimulation in cells, dephosphorylated IRS-1-PP1 complexes also dissociate from T β R-V and bind (or anchor) to a high-affinity PP1 docking motif FesfKR (residues 393–398) in the cytoplasmic domain of T β R-I in the T β R-V–T β R-I complex.⁹ Since PP1_c itself possesses a high-affinity PP2A-B56 docking motif of LrlLfe (residues 82–87), on the way to bind to T β R-I (Figure 8B), PP1_c also recruits PP2A to form the high-affinity-bound T β R-I-PP1-PP2A complex. PP2A recruited by T β R-I as the high-affinity-bound PP1-PP2A complex^{63,64} effectively suppresses or silences TGF- β -stimulated T β R-I-mediated non-Smad pathways by dephosphorylation of the kinases involved in non-Smad pathways in normal epithelial cells. However, in cancer cells lacking T β R-V expression,⁷ T β R-I is transiently localized in lipid rafts due to its specific interaction with caveolin-1, a structural component of lipid rafts.⁶⁵ Lipid rafts serve as major platforms for non-Smad signaling regulation in cell migration and proliferation.⁶⁶ In these cancer cells, T β R-I-mediated non-Smad signaling pathways are activated by TGF- β due to defective recruitment of PP1_c-PP2A by T β R-I to suppress non-Smad signaling.⁴² Furthermore, in carcinoma cells, loss or very low levels of T β R-V expression do not affect TGF- β -stimulated canonical signaling (T β R-I/T β R-II/Smad-2/3/4), as evidenced by TGF- β -stimulated expression of PAI-1 in these cells.¹⁰ Thus, as a tumor promoter, TGF- β stimulates both Smad and non-Smad signaling (termed tumor promoter signaling), leading to epithelial mesenchymal transition (EMT), autoinduction, invasiveness, and chemoresistance in these cancer cells. PP2A plays a pivotal role in suppressing the development of cancer malignancy via TGF- β -induced T β R-I recruitment of PP1-PP2A complexes^{41,42} to suppress non-Smad signaling pathways.^{37–40} The expression and activity of PP2A are commonly reduced in cancer tissues. Small molecule PP2A activators have been developed to treat cancer.⁶⁷

TGF- β is known to act as a tumor suppressor and a tumor promoter during tumorigenesis. The mechanism of switching TGF- β from a tumor suppressor to a tumor promoter in the process of tumorigenesis remains unclear.⁶⁸ We hypothesize that the presence and absence of T β R-V expression in target cells appear to be critical in determining whether TGF- β is a tumor suppressor or a tumor promoter.^{7,69} In normal epithelial cells which express T β R-V, TGF- β suppresses carcinogenesis by potently inhibiting cell growth via stimulating T β R-V-mediated IRS-2-dependent tumor suppressor

signaling cascade (T β R-V/IRS-2/PP1/pRb) in concert with T β R-I-T β R-II-mediated canonical TGF- β signaling (T β R-I/T β R-II/Smad2/3/4) and by suppressing tumor progression via stimulating T β R-V-mediated IRS-1-dependent activation and formation of PP1-PP2A complexes (T β R-V/IRS-1/PP1-PP2A) which targets and suppresses T β R-I-mediated non-Smad tumor progression signaling (Figure 8B). In cancer cells, loss or very low levels of T β R-V expression cause the inability of TGF- β to inhibit cell growth but promote tumor growth. In the absence of T β R-V and PP1-PP2A complexes in cancer cells, T β R-I becomes active as a homodimeric protein. In these cells, TGF- β is able to stimulate T β R-I-mediated non-Smad signaling pathways. Together with canonical Smad signaling,¹³ non-Smad signaling contributes to TGF- β -induced EMT, autoinduction, invasiveness, chemoresistance, and immunomodulation in late-stage cancer. This raises an interesting question: how much minimum expression threshold of T β R-V in epithelial cells is required for switching TGF- β from a tumor suppressor to a tumor promoter. To test this, we used pseudomonas exotoxin treatment of Mv1Lu cells to select mutant cells with reduced expression of T β R-V (LRP-1).¹⁰ We found that PEA-C11 cells, a representative clone, possess ~15% as much cell surface T β R-V as parent cells and that TGF- β , at 40 pM, inhibits cell growth in PEA-C11 cells by 70% as compared with ~100% inhibition in wild-type Mv1Lu cells.¹⁰ These results suggest that relatively low levels of T β R-V at the cell surface are enough to maintain the status of TGF- β as a growth inhibitor or T β R-V as a tumor suppressor in target cells. They also suggest that T β R-V (LRP-1)-knockdown approach (e.g., siRNA) may not be perfect for examining the role of T β R-V in the cellular function of interest because of possibly enough presence of remaining T β R-V (~15%) in LRP-1 knockdown cells.⁷⁰

Activation of tumor suppressors or their signaling for the treatment of human cancers has been a long sought, yet elusive, strategy for an effective therapy.⁷¹ T β R-V is the only known membrane receptor which acts as a tumor suppressor required for epithelial cells.^{7,69} More than 80% of human cancers are carcinomas. The T β R-V-mediated tumor suppressor signaling cascade should be an ideal target for developing a strategy to prevent and treat carcinoma cancer. As described above, TGF- β -stimulated T β R-I-T β R-II-mediated canonical signaling is responsible for potentiating TGF- β growth inhibition mediated by T β R-V by transcriptional activation of CDK inhibitors which activates the T β R-V-mediated tumor suppressor signaling cascade (T β R-V/IRS-2/PP1/pRb) to maintain pRb unphosphorylated (active). Small molecule TGF- β enhancers (statins, vitamin D2, vitamin D3, cyanidin, apocyanin, dynasore, resveratrol, aspirin, ethanol, and DMSO), which enhance TGF- β activity in epithelial cells, have been identified using a TGF- β -stimulated luciferase reporter gene assay in MLE cells-Clone 32.^{69,72-76} These small molecule

enhancers enhance TGF- β -stimulated T β R-I-T β R-II-mediated canonical signaling (T β R-I/T β R-II/Smad2/3/4) by recruiting T β R-I-T β R-II hetero-oligomeric complexes from lipid rafts to non-lipid raft microdomains^{69,72,73,75,76} and facilitating TGF- β -induced signaling at coated-pit stages during clathrin-mediated endocytosis.⁷⁴ In epithelial cells, T β R-V is mainly localized in plasma-membrane non-lipid raft microdomains which serve as signaling platforms for TGF- β receptor (T β R-I/T β R-II/T β R-V)-mediated growth inhibition signaling. These TGF- β enhancers could be used to prevent and treat the majority (carcinoma) of human cancers,⁶⁹ and other chronic inflammatory diseases such as atherosclerotic cardiovascular disease (ASCVD).^{77,78} Diets containing natural TGF- β enhancers (fruits/vegetables/nuts rich in triterpenoids, polyphenols and antioxidants), synthetic TGF- β enhancers (such as statins, resveratrol, cyanidin and aspirin), moderate ingestion of red wine, and exercise (which increases plasma HDL levels) are known to be associated with low risk of developing chronic inflammatory diseases such as cancer and ASCVD.^{69,73,77} HDL (high-density lipoproteins), and ethanol enhance TGF- β activity by recruiting T β R-I and T β R-II from cytoplasmic vesicles (intracellular pool) and lipid rafts/caveolae to non-lipid raft microdomains.^{73,75,78} TGF- β has been known to be a protective cytokine against carcinoma and ASCVD.^{19,79}

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CONFLICT OF INTEREST

The authors declare that there are no competing financial interest associated with this work.

AUTHORS' CONTRIBUTIONS

C.L.C. performed most of the experiments in the Department of Biochemistry and Molecular Biology, Saint Louis University School of Medicine. F.W.H. was involved in identifying the negative regulation of non-Smad pathways by T β R-V. S.S.H. and J.S.H. were involved in research designing and finalizing the manuscript.

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