PTTG induces EMT through integrin AlphaV Beta3-focal adhesion kinase signaling in lung cancer cells

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Abstract

Pituitary tumor transforming gene (PTTG) is a well-studied oncogene for its role in tumorigenesis and serves as a marker of malignancy in several cancer types including lung. In the present study, we defined the role of PTTG in actin cytoskeleton remodeling, cell migration and induction of epithelial mesenchymal transition (EMT) through the regulation of integrin $\alpha_V\beta_3$-FAK signaling pathway. Overexpression of PTTG through an adenovirus vector resulted in a significant increase in the expression of integrins α_V and β_3, a process that was reversed with the down-regulation of PTTG expression through the use of an adenovirus expressing PTTG-specific siRNA. Western blot analysis of cells infected with adenovirus PTTG cDNA resulted in increased FAK and enhanced expression of adhesion complex molecules paxillin, metavincullin, and talin. Furthermore, downstream signaling genes Rac1, RhoA, Cdc42, and DOCK180 showed up-regulation upon PTTG overexpression. This process was dependent on integrin α_V as blockage by antagonist echistatin (RGD peptide) or α_V-specific siRNA resulted in a decrease in FAK and subsequent adhesion molecules. Actin cytoskeleton disruption was detected as a result of integrin-FAK signaling by PTTG as well as enhanced cell motility. Taken together our results suggest for the first time an important role of PTTG in regulation of integrins α_V and β_3 and adhesion complex proteins leading to induction of EMT.

Introduction

Integrins are a super family of heterodimeric transmembrane receptors responsible for cellular adhesion to extracellular matrix (ECM) proteins. A total of 18 α and 8 β subunits of integrins have been identified, which non-covalently bind to form 24 distinct transmembrane heterodimers, each with a specific, non-redundant function (Hynes, 2002). Specificity of an integrin in interacting with an extracellular ligand is determined by heterodimer composition of α and β subunits. The integrin $\alpha_V\beta_3$ binds to arginine-glycine-aspartic acid (RGD) containing compounds of the ECM such as vitronectin and fibronectin (Orlando and Cheresh, 1991), as well as blood and cell surface proteins (Ruoslahti, 1996). Integrins not only can trigger cytoskeletal rearrangements within the ECM but also connects to the cellular cytoskeleton through the actin-based microfilament system to mediate signals for the control of diverse cellular functions including survival, proliferation, differentiation, adhesion, and migration leading to changes in gene expression through outside-in signal

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Conflict of interest
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transduction (Giancotti and Tarone, 2003; Hynes, 2002). This is accomplished with the aid of scaffolding proteins such as talin, vinculin, paxillin, and α-actinin as well as kinases (Berrier and Yamada, 2007). At least three kinases are activated through integrin-mediated cell attachment: focal adhesion kinase (FAK), protein kinase C (PKC), and Src (Berrier and Yamada, 2007; Ruoslahti, 1994), which modifies downstream signaling. FAK is a non-receptor protein tyrosine kinase (Parsons, 2003) that binds to the cytoplasmic tail of the integrin β-subunit via its SH3 domain located on the N-terminal tail (Huveneers et al., 2008; Schaller et al., 1995). The C-terminal tail of FAK contains protein-protein interaction sites that span ~100 amino acids (Martin et al., 2002). NMR and x-ray crystallography analysis show that this region contains a four-helix bundle that can be found on other adhesion proteins (Arold et al., 2002; Hayashi et al., 2002; Liu et al., 2002). Paxillin is activated by tyrosine phosphorylation and has been shown to be associated with the regulation of cytoskeletal organization, focal adhesion formation, cell migration, and cell motility (Petit et al., 2000; Turner, 2000). In addition to binding to integrins, vinculin also binds to the N-terminus of paxillin as well as actin. Binding of vinculin to actin is believed to contribute to the formation of structural links between integrin proteins and the actin cytoskeleton (Ezzell et al., 1997).

Metastasis is a physiological process in which tumor cells detach from the primary tumor, travel via the bloodstream to distance sites, invade, and form secondary tumors. This is accomplished through changes in cell polarity, which causes the tumor cells to lose cell-to-cell contact and exchange their epithelial markers for mesenchymal (Thiery, 2003), a process known as the epithelial to mesenchymal transition (EMT),which is a key program during the embryonic development but has more recently been implicated in cancer invasion and metastasis (Firrincieli et al., 2010; Thiery, 2003). Changes in cell polarity is accomplished through the loss of E-cadherin expression (Christiansen and Rajasekaran, 2006; Schmalhofer et al., 2009) and cell junction proteins, and coupled to the gain of mesenchymal markers such as vimentin and N-cadherin (Agiostratidou et al., 2007; Blanco et al., 2004). Changes in the expression and function of certain integrins, such as β_1, has been implicated in cancer (Zuk and Hay, 1994). These polarity changes and resulting cellular focal adhesion changes enhance the ability of cells to migrate and invade the ECM, which is considered a functional hallmark of the EMT process (Christiansen and Rajasekaran, 2006; Danen et al., 2002).

Pituitary tumor transforming gene (PTTG) also known as securin regulates chromosomal segregation under normal physiological conditions (Kumada et al., 1998; Zou et al., 1999). PTTG was initially cloned from rat pituitary tumor cells as a 974 bp mRNA that encodes a 199 amino acid protein, which is not expressed in the normal pituitary (Pei and Melmed, 1997). Subsequently, PTTG was cloned from the adult testes and embryonic liver (Kakar and Jennes, 1999; Lee et al., 1999). PTTG is overexpressed in a variety of solid tumors including lung, ovary, uterine, pituitary, thyroid, liver, brain, and renal clear cell carcinoma (Chamaon et al., 2005; Cho-Rok et al., 2006; El-Naggar et al., 2007; Heaney and Melmed, 1999; Honda et al., 2003; Tang et al.; Tsai et al., 2005; Zhang et al., 1999). Furthermore, cloning and sequencing of PTTG isolated from tumors shares sequence homology with that isolated from human testes cDNA, suggesting that it is overexpression and not mutation that contributes to its oncogenic properties (Zou et al., 1999). Overexpression of PTTG is capable of stimulating cell proliferation in HEK293 and inducing cellular transformation in vitro using NIH3T3 and HEK293 cells as well as promotes tumor development in nude mice showing its tumorigenic potential without necessitating a partner oncogene (Hamid et al., 2005; Kakar and Jennes, 1999; Pei and Melmed, 1997). A 60% reduction of PTTG protein using siRNA in the lung cancer cell line H1299 showed inhibited colony formation using a soft agar assay and reduced xenograft tumor formation in nude mice (Kakar and Malik, 2006), furthermore demonstrating the role of PTTG in tumor growth and progression. In

addition, a relationship between PTTG levels and cancer metastasis have been reported in several cancer types (Liang *et al.*; Shibata *et al.*, 2002; Solbach *et al.*, 2004; Yan *et al.*, 2009). PTTG overexpression has also been shown to increase matrix-metalloproteinase 2 (MMP-2) expression and secretion resulting in increased cell migration and invasion to promote metastasis (Malik and Kakar, 2006). However, the relationship between PTTG and integrins α_V and β_3 in the promotion of actin cytoskeleton alternations through focal adhesion complex formation has not been explored. In the present study, we investigated the role of PTTG in regulation of integrins α_V and β_3 expression and the adhesion complex assembly to trigger alteration of the actin cytoskeleton and promote EMT in lung cancer cells.

Results

Overexpression of PTTG results in increased expression of integrins $\alpha_V\beta_3$, FAK, p-FAK and associated adhesion complex proteins paxillin, metavincullin, talin, Rac1, RhoA, Cdc42, and DOCK180

Adhesion complex formation is essential for rearrangement of the actin cytoskeleton for enhanced cell motility. In the present study, we used *in vitro* experiments to understand the molecular mechanisms involved in the formation of the focal adhesion complex by PTTG through the activation of integrins $\alpha_V\beta_3$ and subsequent activation of the FAK signaling pathway. For this purpose we generated an adenovirus expression system to over express PTTG cDNA (Ad-PTTG cDNA) and an adenovirus expressing PTTG siRNA (Ad-PTTG siRNA) to down-regulate the expression of PTTG. Human non-small cell lung carcinoma cell line H1299 and adenocarcinomic human alveolar basal epithelial cancer cell line A549 were selected to determine if these changes in expression were localized to a particular cell type or represented lung cancer in a broader sense. Quantitative real-time PCR (qPCR) analysis of PTTG mRNA showed a significant increase in expression upon infection of both A549 (Fig. 1A) and H1299 (Fig. 1C) cell lines with Ad-PTTG cDNA as compared to uninfected cells or cells infected with control Ad-GFP. Overexpression of PTTG was further confirmed by performing immunofluorescence analysis of both A549 and H1299 cells, which showed a significant increase in immunoreactive protein in Ad-PTTG cDNA infected cells compared to uninfected or cells infected with the control vector Ad-GFP (Fig. 1B, D).

Integrins are the family of heterodimeric transmembrane adhesion receptors shown to be overexpressed in different tumors and tumor cell lines including lung cancer (Chen *et al.*, 2005). To determine if PTTG regulates the expression of commonly expressed integrins α_V and β_3 in cancer, we overexpressed PTTG in A549 and H1299 cells by infecting the cells with Ad-PTTG cDNA and analyzed the expression of α_V and β_3 integrins using qPCR, which showed a significant increase in mRNA expression of both integrins α_V and β_3 (Fig. 2A, B). In addition, FACS analysis using the specific antibody for $\alpha_V\beta_3$ showed a significant increase in $\alpha_V\beta_3$ in cells infected with Ad-PTTG cDNA compared to uninfected cells or cells infected with Ad-GFP (Fig. 2C, D). In contrast, down regulation of PTTG by using Ad-PTTG siRNA resulted in a significant decrease in the levels of expression of integrins α_V and β_3 (Fig. 2A, B), suggesting regulation of expression of α_V and β_3 integrins by PTTG. Consistent with these findings, immunofluorescence analysis of A549 and H1299 cells for integrin $\alpha_V\beta_3$ revealed a significantly higher level of protein expression for $\alpha_V\beta_3$ in both cell lines infected with Ad-PTTG cDNA compared to uninfected cells or cells infected with Ad-GFP (Fig. 2E, F).

Tumor cell adhesion and migration, mediated by members of the integrin family, are linked to tumor cell growth and malignancy. Once ligated, integrins have several adhesion complex associated signaling molecules that mediate their biological function, such as FAK, leading to anchorage-independent survival and proliferation (Reddig and Juliano, 2005; Westhoff *et*

al., 2004). As PTTG influences expression of integrins $\alpha_V\beta_3$, we investigated the effect of overexpression of PTTG on the phosphorylation and activation of FAK. Interestingly, we found that overexpression of PTTG in both A549 and H1299 cells resulted in an increase in the mRNA expression of total FAK compared to uninfected cells or cells infected with Ad-GFP (Fig. 3A, B). Increased expression of FAK was again confirmed by Western blotting. Both A549 (Fig. 3C,) and H1299 cells (Fig. 3D) showed a significantly higher level of FAK protein expression when infected with Ad-PTTG cDNA compared to uninfected cells and cells infected with Ad-GFP. As FAK is a non-receptor protein tyrosine kinase that becomes tyrosine phosphorylated and subsequently activated when integrins adhere to various matrix proteins, leading to increased cell migration (Zheng *et al.*, 1999),we therefore investigated if PTTG-induced $\alpha_V\beta_3$ resulted in increased phosphorylation of FAK in lung cancer. Western blot analysis of phosphorylated FAK (p-FAK) showed an increase in level of p-FAK in response to increased total FAK in both A549 and H1299 cell lines upon Ad-PTTG cDNA infection compared to uninfected cells or cells infected with Ad-GFP (Fig. 3C, D). In response to phosphorylation, immunofluorescence of p-FAK showed localization to the cellular membrane upon treatment with Ad-PTTG cDNA (Fig. 3Eii), indicative of increased focal adhesion complex assembly.

Additional adhesion complex molecules include talin, paxillin, and metavincullin (Ross, 2004). These signaling molecules interact with each other to produce adhesion dependent responses (Nishiya *et al.*, 2001). In the case of lung cancer using A549 cells, PTTG overexpression resulted in a significantly higher level of expression of paxillin, metavincullin, and talin (Fig. 4A). Paxillin itself has several downstream signal transduction molecules including Rac1, RhoA, Cdc42, and DOCK180 (Valles *et al.*, 2004). These molecules play important roles in migration and cell matrix adhesion (Danen *et al.*, 2002). To fully understand their roles in mediating the function of integrins $\alpha_V\beta_3$ in response to overexpression by PTTG, we studied the expression of these molecules by qPCR. A549 cells infected with Ad-PTTG cDNA showed a higher level of mRNA expression for each Rac1, RhoA, Cdc42, and DOCK180, compared to uninfected cells or cells infected with control Ad-GFP (Fig. 4B).

Knockdown of integrin α_V by α_V siRNA results in decreased mRNA expression of its adhesion associated complex molecules paxillin, metavincullin, talin, Rac1, DOCK180, RhoA, and Cdc42

To confirm the role of PTTG in the induction of EMT through the activation of integrin α_V, we knocked-out integrin α_V mRNA by using siRNA (α_V siRNA). Down regulation of integrin α_V was confirmed by using immunohistochemical analysis (Fig. 5A). Infection of A549 cells with Ad-PTTG cDNA along with co-transfection using α_V siRNA showed a significant decrease in mRNA expression of paxillin and metavincullin (Fig. 4A). Additionally, the downstream targets of paxillin, Rac1, RhoA, Cdc42, and DOCK180 were also down regulated (Fig. 4B), verifying the role of integrin α_V in the assembly of the adhesion complex. Furthermore, down regulation of DOCK180 upon treatment with α_V siRNA was confirmed by immunofluorescence (Fig. 5B).

Effect of knockdown of integrins $\alpha_V\beta_3$ by RGD peptides on expression of p-FAK

Activation and phosphorylation of FAK by ECM-integrins is an important phenomena for the cells to achieve migratory phenotype (McLean *et al.*, 2005). In the present study, to investigate the potential role of PTTG in the induction of EMT through the activation of integrins and FAK pathway, we infected A549 and H1299 cells with Ad-PTTG cDNA or Ad-GFP and treated with echistatin, a specific RGD peptide, to block the activation of integrins. After 48 hr of total treatment, cells were harvested and protein extracts were analyzed for p-FAK and total FAK by western blot analysis. Both A549 and H1299 cell

lines when infected with Ad-PTTG cDNA and RGD peptide showed a similar level of p-FAK to untreated cells (Fig.3C, D), indicating that RDG was able to block the effect of PTTG-induced FAK expression.

Effect of knockdown of integrin α_V by siRNA on cell migration

A549 cells were infected with Ad-PTTG cDNA or Ad-GFP control vector and co-transfected with α_V siRNA or control siRNA. A wound was formed by scrapping away the cells and the cells were examined after 24 hr of wound formation. Overexpression of PTTG with and without control siRNA showed nearly complete healing of the wound after 24 hr compared to uninfected and Ad-GFP infected cells, which had fewer cells migrated into the gap (Fig. 6A). However, addition of α_V siRNA significantly impaired the cells ability to migrate (Fig. 6A), suggesting that integrin α_V is crucial to mediate the function of PTTG in tumor cell migration.

Actin cytoskeletal organization

In A549 cells, it has been shown that EMT leads to increased migratory and invasive abilities, which are characterized by re-organization of actin cytoskeleton through destruction and cellular protrusion formation (Keshamouni *et al.*, 2006). To determine the role of PTTG in actin cytoskeletal reorganization, we infected A549 cells with Ad-PTTG cDNA and after 48 hr, cells were stained for F-actin and vinculin. Cells infected with Ad-PTTG cDNA showed morphological changes including formation of protrusions and destruction of actin filaments compared to uninfected cells (Fig. 6B), indicating a potential role of PTTG in the induction of EMT.

Expression of EMT markers E-cadherin and vimentin

To verify the induction of EMT, we tested the expression of epithelial marker E-cadherin and mesenchymal marker vimentin in A549 and H1299 cells on infection of cells with Ad-PTTG cDNA for 48 hr. In both cell lines, overexpression of PTTG resulted in a significant decrease in E-cadherin and increase in vimentin compared to Ad-GFP or untreated cells (Fig. 7), demonstrating the role of PTTG in the induction of EMT.

Discussion

Epithelial to mesenchymal transition (EMT) is a physiologic process that allows morphological and genetic changes of carcinoma cells from an epithelial to a mesenchymal phenotype. During EMT process, epithelial cell surface proteins that mediate cell-to cell contact, such as E-cadherin, are replaced by mesenchymal markers N-cadherin and vimentin, which provides more transient adhesive properties (Micalizzi *et al.*, 2010). Numerous inducers of EMT in cancer cells have been identified including transforming growth factor-β (TGF-β), Wnt/β-catenin, Snail/Slug, Twist, and talin (Becam *et al.*, 2005; Huber *et al.*, 2005; Kim *et al.*, 2002; Savagner *et al.*, 1997; Yang *et al.*, 2004; Zavadil and Bottinger, 2005). These factors have been reported to be critical as repressor of E-cadherin resulting in induction of EMT during developmental (Mercado-Pimentel and Runyan, 2007; Taneyhill *et al.*, 2007). PTTG itself has been shown to increase cell proliferation and tumorigenesis in HEK293 cells, while mutation of PTTG decreased tumorigenesis (Hamid *et al.*, 2005). In addition, down regulation of PTTG in lung cancer cells resulted in reduced colony formation and tumor formation in nude mice (Kakar and Malik, 2006). While PTTG can alter expression of MMP-2 (Malik and Kakar, 2006), co-expression of integrin $\alpha_V\beta_3$ with MMP-2 resulted in MMP-2 activation and correlated with progession of melanoma (Hofmann *et al.*, 2000). Our results show that PTTG can act as an inducer of EMT by altering expression of E-cadherin and vimentin as well as integrin-mediated focal adhesion complex formation to alter the actin cytoskeleton. Adherin junctions incorporate the actin

cytoskeleton allows the cells to "hold hands" and link cell to cell across the entire tissue, enabling a sheet of epithelial cells to move and function as a single cell (Vaezi *et al.*, 2002). Therefore, changes in the actin cytoskeleton are a prerequisite for cell migration and invasion (Yilmaz and Christofori, 2009), and thus transition between an early stage neoplasm that is localized to the primary site to a late stage where metastasis has occurred. In addition to E-cadherin expression, alterations in the expression of integrins has been implicated in malignant transformation, tumor progression, and metastasis (Mizejewski, 1999). Integrins $\alpha_V\beta_3$ has been shown to be frequently overexpressed in tumor cells, including lung cancer, melanoma, glioblastoma, and breast cancer (Chen *et al.*, 2005; Gladson and Cheresh, 1991; Rolli *et al.*, 2003; Seftor *et al.*, 1992). High level of expression of many integrins has been correlated with tumor progression and metastasis including $\alpha_5\beta_1$, α_6, and $\alpha_V\beta_3$ (Gong *et al.*, 1997; Hofmann *et al.*, 2000). In addition, mice treated with intetumumab, a monoclonal antibody to integrin α_V, significantly reduced lung metastasis in A549 non-small cell lung cancer xenograft model (Ning *et al.*, 2010). In the present study we demonstrated the potential role of PTTG in induction of EMT through the promotion of integrins $\alpha_V\beta_3$. Overexpression of PTTG resulted in a significant increase in level of integrins α_V and β_3 mRNA and protein (Fig. 2A–B, 3). In contrast down regulation of PTTG resulted in a reversal of the effects at both the mRNA and protein level (Fig. 2A–B), clearly demonstrating the involvement of PTTG in the induction of EMT through increased expression of integrin $\alpha_V\beta_3$. Thus this regulation occurs at the transcriptional level. No significant change in expression of other integrins such as α_5, α_6 or $\beta1$ was observed (Supplement Figure 1), indicating that α_V and β_3 probably are the main integrins in mediating the function of PTTG.

Integrin signaling is depending on the formation of adhesion complexes that includes both adaptor/scaffolding proteins as well as kinases that provide enzymatic activity (Berrier and Yamada, 2007). Proteins involved in adhesion complex formation include talin, paxillin, vinculin, α-actinin, FAK, and Src that interact either directly or indirectly to affect adhesion dependent responses (Playford and Schaller, 2004). Src interacts via its SH3 domain with integrin β_3 to activate FAK signaling to promote tumor growth leading to cytoskeleton driven morphological changes (Huveneers *et al.*, 2008). In addition to integrins, soluble growth factors also promote FAK activation, and thereby, FAK integrates growth factor receptor- and matrix-derived signals to elicit biological responses, including the induction of a migratory phenotype (McLean *et al.*, 2005). FAK–Src complex has been shown to promote the phosphorylation of many FAK-associated Src substrates including CAS, paxillin, and p190RhoGAP, which have a central role in the reorganization of the actin cytoskeleton and migration (Playford and Schaller, 2004). So far several studies have reported the role of FAK signaling in the induction of EMT (Deng *et al.*, 2010; Ponnusamy *et al.*, 2010; Wendt *et al.*, 2010). In our present study, we observed that overexpression of PTTG resulted in a significant increase in the total level of FAK and p-FAK (Fig. 3). Interestingly, knockout of integrins $\alpha_V\beta_3$ by using RGD peptide resulted in a significant decrease in the total level of FAK protein (Fig. 3) As a result of increased integrin and FAK expression by PTTG, we found a significant increase in the expression of adhesion complex proteins paxillin, metavincullin, and talin (Fig. 4A). Subsequent downstream molecules Rac1, RhoA, Cdc42, and DOCK180 were also overexpressed an effect which was reversed with the down regulation of integrin α_V (Fig. 4B). The small GTPases Rac1, RhoA, and Cdc42 have been implicated in regulating the assembly and disassembly of the actin cytoskeleton (Altun-Gultekin *et al.*, 1998; Berrier and Yamada, 2007; Klemke *et al.*, 1998; Nakashima *et al.*, 1999) while Cdc42-induced filopodia act as precursors for Rac-induced lamellipodia, establishing an integrin-dependent Rho GTPase activation in the regulation of cell spreading and migration (Guillou *et al.*, 2008). DOCK180 has been shown to function as a guanine nucleotide exchange (GEF) factor for Rac to increase cell motility and enhance invasion (Jarzynka *et al.*, 2007; Wang *et al.*, 2010), a crucial step in the EMT process.

Studies have shown that integrins $\alpha_V\beta_3$ and $\alpha_5\beta_1$ differentially regulate RhoA activity in response to fibronectin binding (Danen *et al.*, 2002). Consistent with our results, overexpression of integrins $\alpha_V\beta_3$ and subsequent overexpression of Rac1, RhoA, and Cdc42 as a result of PTTG overexpression leading to enhanced cell migration (Fig. 6A) and actin cytoskeleton destruction (Fig. 6B) as a step in induction of EMT.

Conclusion

In the present study we have demonstrated for the first time the potential role of PTTG in the induction of EMT through the activation of integrin $\alpha_V\beta_3$ and its associated focal adhesion kinase pathway (Fig. 8).

Materials and methods

Generation of plasmid and adenovirus constructs

The full length PTTG cDNA, PTTG siRNA, and control siRNA were sub-cloned into adenovirus shuttle vector (pShuttle). Positive clones were sequenced to confirm the orientation and authenticity of sequence as described previously (Panguluri and Kakar, 2009). The adenovirus expression systems were generated and purified in association with the Virus Vector Core Facility, Gene Therapy Center, University of North Carolina at Chapel Hill.

Cell culture and infection of cells with adenovirus expressing PTTG cDNA, GFP or PTTG-siRNA

Human non-small cell lung carcinoma cell line H1299 and adenocarcinomic human alveolar basal epithelial cancer cell line A549 were purchased from American Type Culture Collection (ATCC, Rockville, MD, USA) and cultured in DMEM supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA) and 1% antibiotic/antimycotic (Sigma, St. Louis, MO, USA). The cell lines were routinely sub cultured every 3–4 days.

For infection of cells with adenovirus, cells were seeded in 6-well plates. After 24 hr, cells were infected with adenovirus expressing PTTG cDNA (Ad-PTTG cDNA), adenovirus expressing GFP (Ad-GFP), control siRNA (Ad-control siRNA) or PTTG-specific siRNA (AdPTTG siRNA) as described previously (Panguluri and Kakar, 2009).

Western blot analysis

Cells were plated in 6-well plates and infected with Ad-GFP or Ad-PTTG cDNA and treated with 100 nm echistatin (RGD peptide) after 24 hr of infection to block integrin $\alpha_V\beta_3$. After 48 hr of infection, cells were washed with PBS and lysed in chilled lysis buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% NP-40, 1 mM Na_3VO_4, and 1 mM NaF] supplemented with Complete Mini Protease Inhibitor tablets (Roche Molecular Biochemical, Indianapolis, IN, USA). Protein was quantitated using BSA as a standard. Equal amount of protein (40 μg) from each sample was resolved on a 12% SDS-PAGE gel and transferred onto a nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ, USA). Blots were probed with anti-FAK or anti-phospho-FAK (Tyr397) from Cell Signaling Technology (Danvers, MA, USA) at a dilution of 1:1,000 in Tris-buffered saline (pH 7.6) containing 0.1% Tween-20 (TBST). Immunoreactive proteins were visualized using the Enhanced Chemiluminescent Detection system (ECL) kit from GE Health System (Piscataway, NJ, USA) according to instructions provided by the supplier. The membrane was stripped by using western blot stripping reagent (BioRad, Hercules, CA, USA) and reprobed with GAPDH antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) to normalize the variation in loading of samples.

Immunofluorescence

Cells were cultured in chamber slides and infected with adenovirus as described above. After 48 hr of infection, cells were fixed with 4.0% paraformaldehyde in PBS for 30 min and then permeabilized with 0.2% Triton X-100 for 15 min at room temperature. Cells were rinsed twice with PBS then incubated for 1 hr with anti-PTTG polyclonal antibody (El-Naggar *et al.*, 2007) at a dilution of 1:1,000 or anti-integrin α_V (at a dilution of 1:1,000). After successive washes, cells were then incubated with Alexa Fluor 488 anti-rabbit or Alexa Fluor 548 anti-mouse secondary antibody. After incubation with secondary antibody for 45 min, cells were rinsed with PBS and incubated with DAPI for 20 min to label nuclei. Cells were then mounted with aquapolymount antifade solution (Sigma) and examined under a fluorescence microscope or with a MRC 600 confocal laser scanning microscope (Bio-Rad).

FACS analysis

FACS analysis was performed by using FACS Caliber Cytometer (BD Biosciences, San Jose, CA, USA). Briefly, after 48 hr of infection of A549 and H1299 cells with Ad-GFP or Ad-PTTG cDNA, cells were trypsinized and washed with wash buffer (PBS containing 0.1% BSA). After blocking for 1 hr, cells were incubated with anti-integrin $\alpha_V\beta_3$-Alexa Fluor 488 (Santa Cruz Biotechnology). The cells were washed with wash buffer followed by fixation for 1 hr. Cells were resuspended for analysis on a FACS Caliber cytometer and analyzed with FlowJo software.

Preparation of total RNA, synthesis of first strand cDNA, and quantitative real-time PCR

After 48 hr of infection with appropriate adenovirus, cells were harvested and total RNA was purified using trizol reagent (Sigma) according to the manufacturer's protocol and as described previously (El-Naggar *et al.*, 2007). First strand cDNA was synthesized using the iScript™ cDNA synthesis kit (BioRad) using 1 μg total RNA according to manufacturer's protocol.

The real-time PCR reaction mixture was prepared in a Light Cycler® 480 (Roche Diagnostics) Multiwell 96 wells plate containing 1 μM of each primer, 10 μl of 2X master mix and 1μl of cDNA template, in a final reaction volume of 20 μl. The real-time PCR amplification was performed using the specific primers for PTTG, integrin α_V, integrin β_3, FAK, paxillin, metavincullin, talin, RhoA, Rac1, Cdc42, and DOCK180 (Table 1) using the following cycle parameters: enzyme activation at 95°C for 10 min; 45 cycles of 95°C for 10 s, 63°C for 10 s, and 72°C for 10 s. Following the amplification phase, a cooling step was performed at 40°C for 10 s (ramp rate of 1.5°C/s). Acquisition of the fluorescence signal was performed using the Mono Hydrolysis Probe setting (483–523 nm) following the 72°C extension phase of each cycle. GAPDH primers were included to normalize variation from sample to sample. All experiments were repeated three times using three independent preparations of cDNA.

Wound migration assay

Cells were treated with Ad-GFP or Ad-PTTG cDNA for 48 hr prior to wound formation. Wounds were formed by scraping away the cells and replacing the media. Cells were examined after 24 hr of wound formation.

Cytoskeleton organization analysis

Actin cytoskeleton staining was carried out by using Actin Cytoskeleton and Focal Adhesion staining kit purchased from Millipore (Temecula, CA, USA). Briefly, cells were cultured in chamber slides and infected with Ad-GFP or Ad-PTTG cDNA. After 48 hr of

infection, cells were fixed with 4.0% paraformaldehyde in PBS for 30 min and then permeabilized with 0.2% Triton X-100 for 15 min at room temperature. After washing twice (5–10 min each) with washing buffer (PBS containing 0.05% Tween-20), followed by incubation with blocking solution (PBS containing 1% BSA) for 30 min. Cells were then incubated for 1 hr at room temperature with anti-vinculin at a dilution of 1:300. After two successive washes, cells were then exposed to corresponding FITC-conjugated secondary antibody at a dilution of 1:500 for 60 min at room temperature. For double labeling, cells were simultaneously incubated with TRITC-conjugated Phalloidin at the dilution of 1:1 000 to map the local orientation of actin filaments. After washing of cells with washing buffer, nuclei were counterstained with DAPI (diluted 1:500) for 20 min at room temperature followed by three washes (5–10 min each) with washing buffer. The cells were then mounted with aquapolymount antifade solution (Sigma) and observed under an Olympus IX50 fluorescence microscope or using a MRC 600 laser scanning microscope (Bio-Rad).

Statistical analysis

Data comparing differences between two groups were statistically analyzed using unpaired Student's t-test. Differences were considered significant when $p < 0.05$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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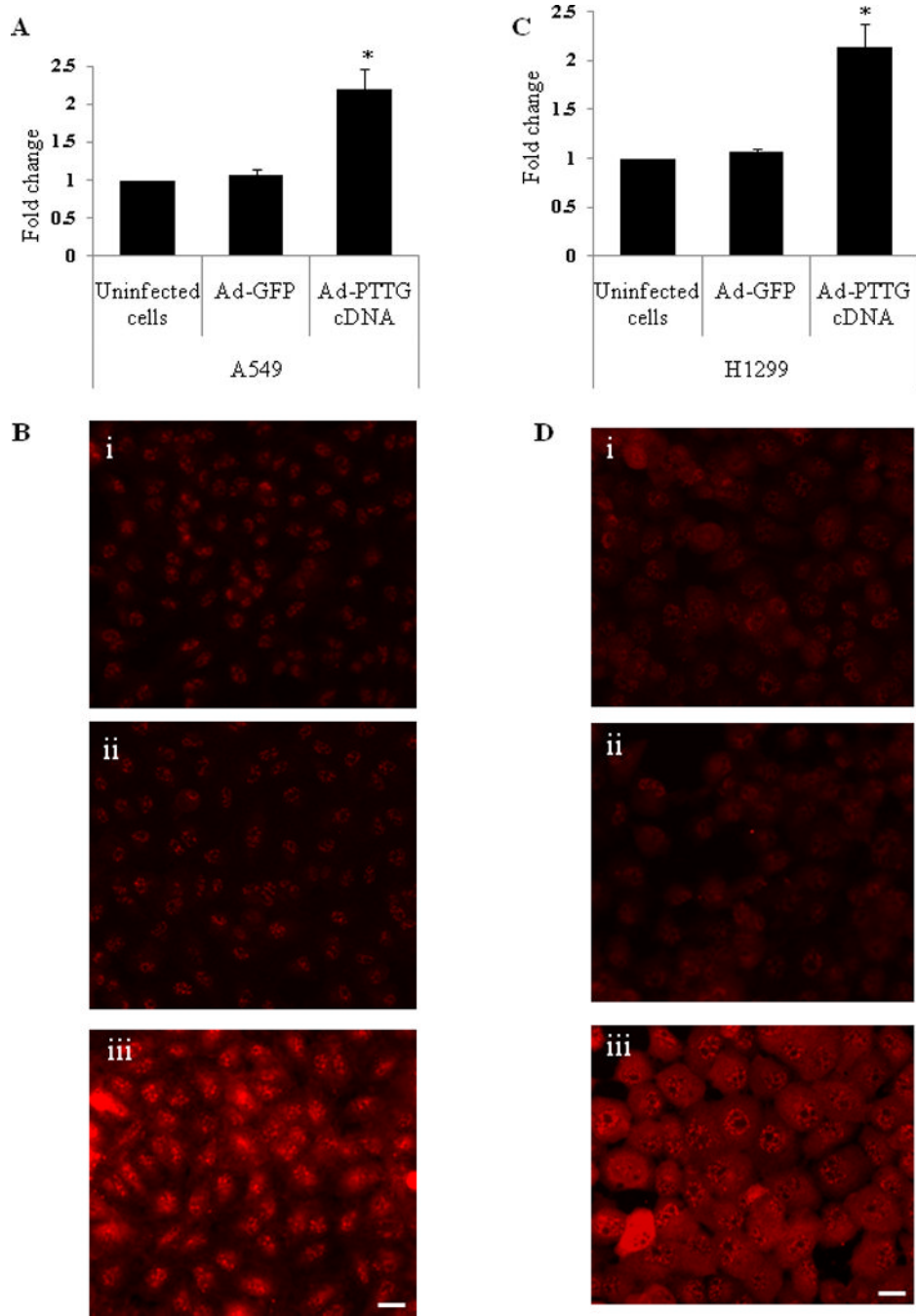
Figure 1.
mRNA and protein expression of PTTG in A549 and H1299 cells. (A) mRNA expression in A549 uninfected cells, cells infected with Ad-GFP, or infected Ad-PTTG cDNA using qPCR. (B) PTTG protein expression in A549, i: uninfected cells, ii: Ad-GFP infected cells, iii: Ad-PTTG cDNA infected cells. (C) mRNA expression of PTTG in H1299 uninfected cells, cells infected with Ad-GFP, or infected with Ad-PTTG cDNA using qPCR. (D) PTTG protein expression in H1299, i: uninfected cells, ii: cells infected with Ad-GFP vector, iii: cells infected with Ad-PTTG cDNA. White bar shown in the right panels is 20 μm. qPCR values were normalized with GAPDH used as an internal control. Columns indicated the mean (n = 3); error bars represent SEM. *p < 0.05.
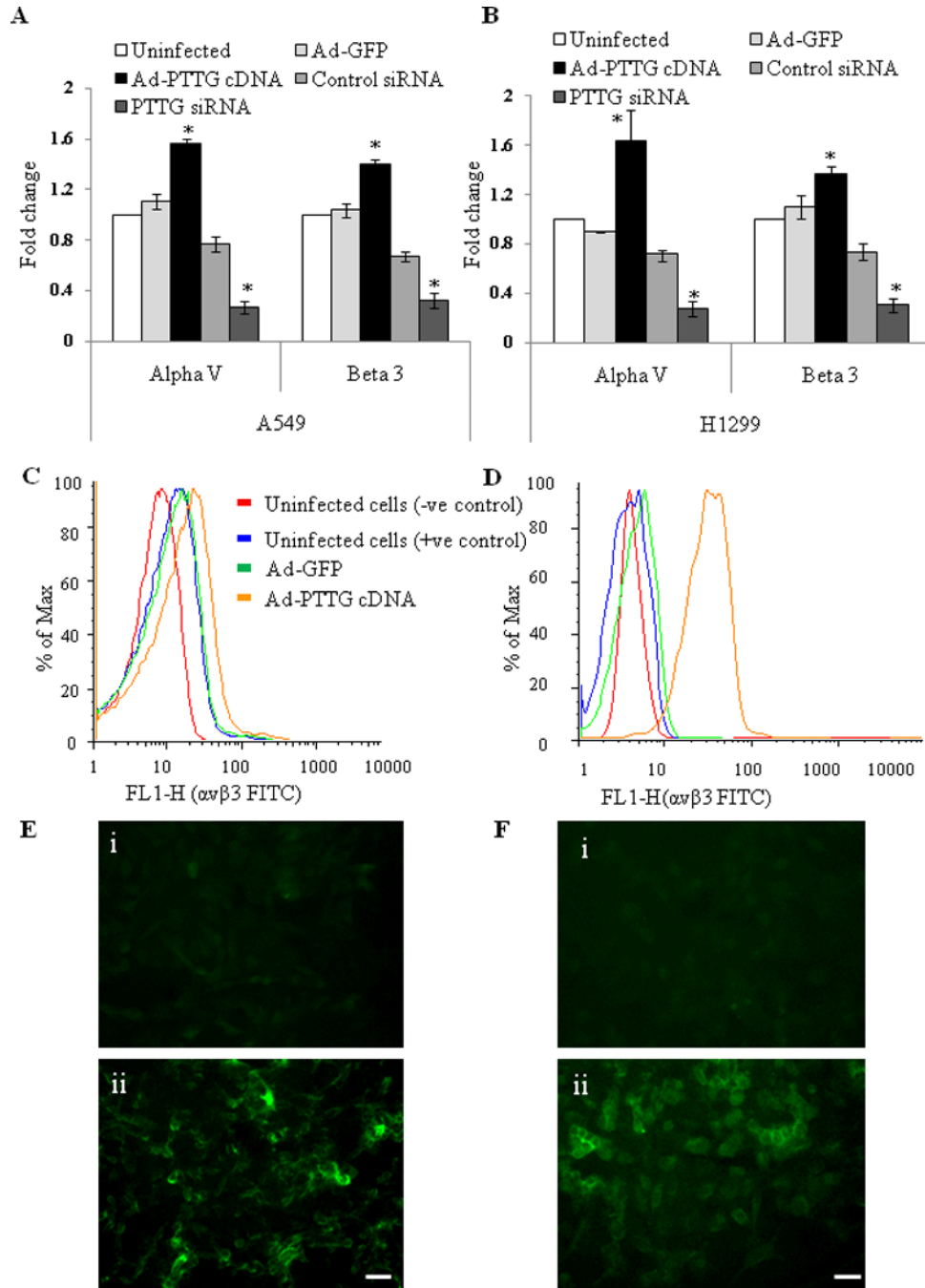
Figure 2.
Expression of integrins α_V and β_3. (A) A549 and (B) H1299 cells infected with Ad-GFP or Ad-PTTG cDNA using qPCR. Values were normalized with GAPDH used as an internal control. Columns indicate the mean (n = 3); error bars represent SEM. *p < 0.05. (C–D) FACS analysis of cell stained with integrin $\alpha_V\beta_3$ Alexa Fluor 488 antibody: (C) FACS sorting in A549, (D) FACS sorting in H1299 cells. (E & F) Fluorescence staining for integrin $\alpha_V\beta_3$ in (E) A549 and (F) H1299 cells, i: uninfected cells, ii: Ad-PTTG cDNA infected. White bar shown in the right panels is 20 μm.
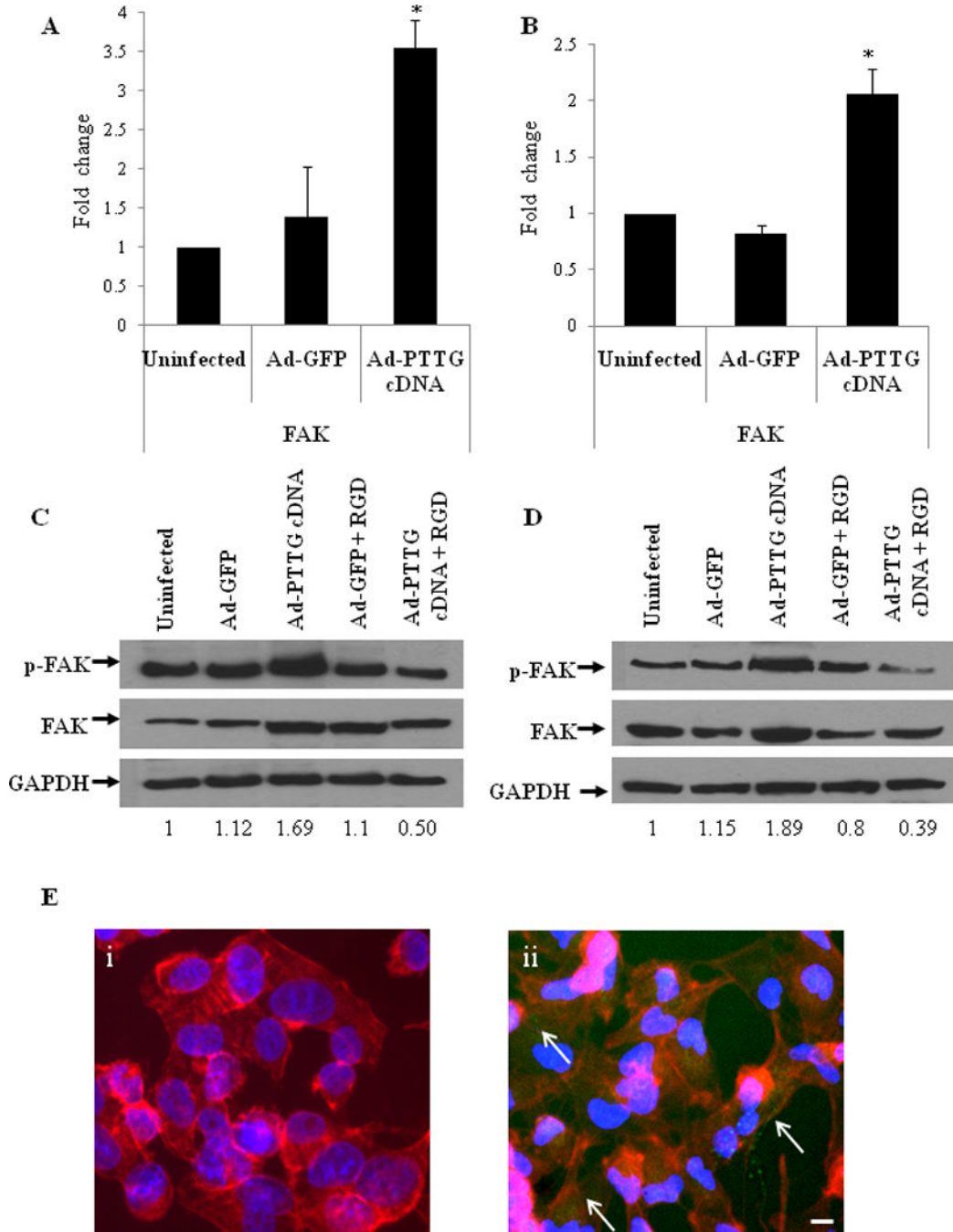
Figure 3.
mRNA expression of FAK and protein expression of p-FAK and FAK. (A, B) mRNA expression of FAK using qPCR in A549 and H1299, respectively. Values were normalized with GAPDH. Column indicate the mean (n = 3); error bars represent SEM. *$p < 0.05$. (C–D) Western blot analysis for p-FAK, FAK, and GAPDH in A549 and H1299 cells, respectively. Cells were infected with Ad-GFP or Ad-PTTG cDNA for 48 hr. (E, F) Immunofluoroscence staining for p-FAK in A549 cells. i: uninfected cells, ii: cells infected with Ad-PTTG cDNA. p-FAK was detected by using Alexa Fluor 548 anti-mouse secondary antibody (green). Actin filaments were stained by phalloidin (red) and DAPI (blue) for

nuclei. Bar shown in the right panels is 20 μm. Arrows indicate localization of p-FAK at the cellular membrane.
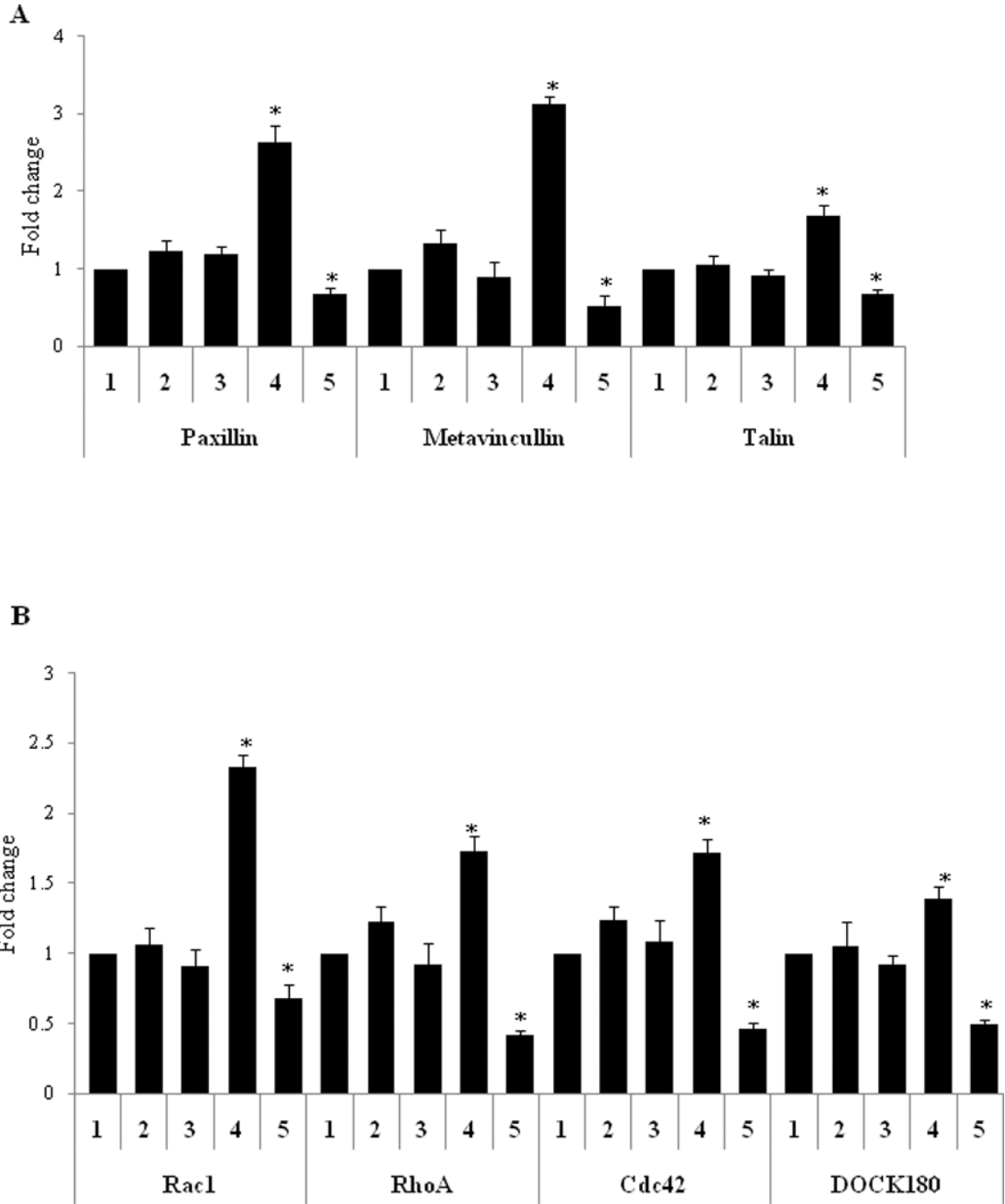
Figure 4.
mRNA expression in A549 cells using qPCR. (A) paxillin, metavincullin, and talin. 1: untreated cells, 2: cells infected with Ad-GFP, 3: cells infected with Ad-GFP + control siRNA, 4: cells infected with Ad-PTTG, and 5: cells infected with Ad-PTTG cDNA + α$_V$ siRNA. (B) Rac1, RhoA, Cdc42, and DOCK180. Values were normalized with GAPDH. Columns indicated the mean (n = 3); error bars represent SEM. *p < 0.05.
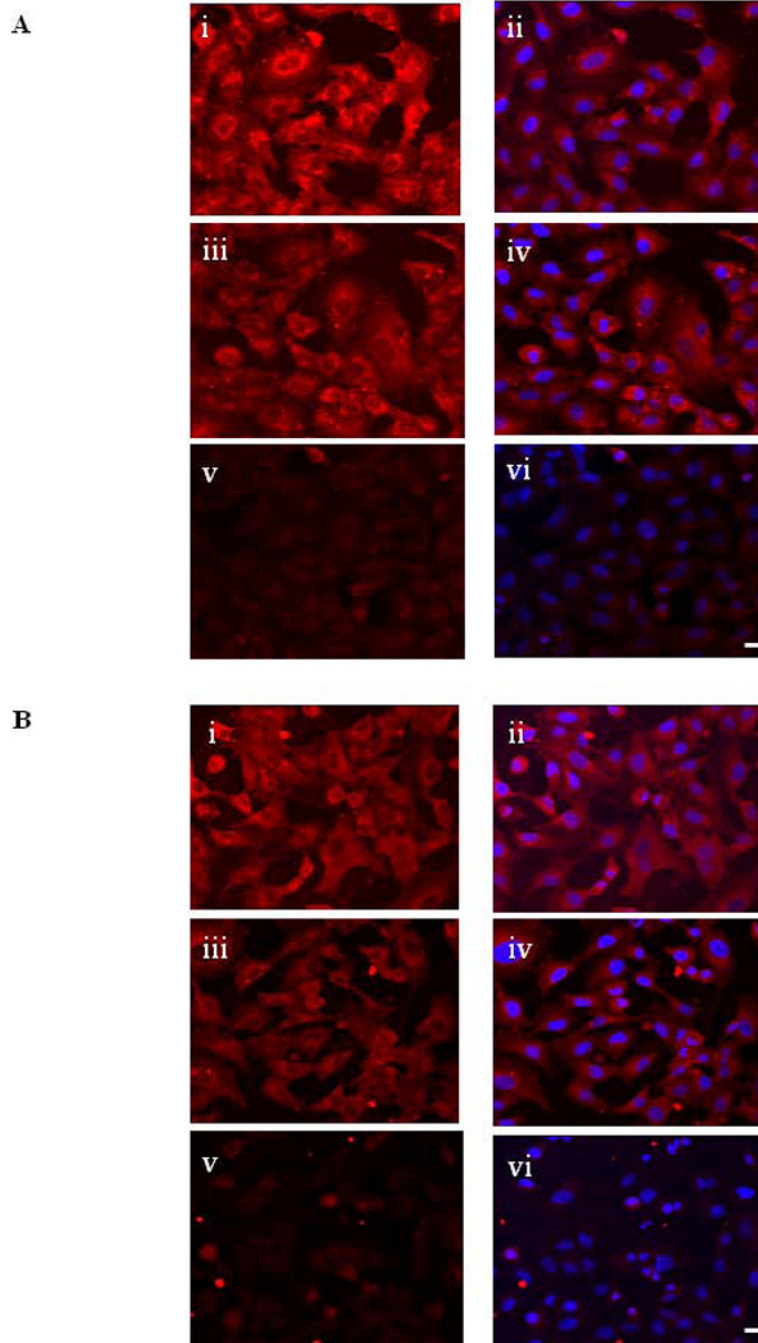
Figure 5.
(A) Fluorescence staining for α_V detected with Alexa Fluor 548 anti-mouse secondary antibody in A549 cells. i: un-transfected cells, ii: un-transfected cells counterstained with DAPI iii: cells transfected control siRNA, iv: control siRNA counterstained with DAPI, v: cells transfected with α_V siRNA, vi: α_V siRNA counterstained with DAPI. (B) Fluorescence staining for DOCK180 detected with Alexa Fluor 548 anti-mouse secondary antibody in A549 cells. i: un-transfected cells, ii: un-transfected cells counter stained with DAPI, iii: cells transfected with control siRNA, iv: control siRNA infected cells counter stained with DAPI, v: cells transfected with α_V siRNA, vi: α_V siRNA transfected cells countered stained with DAPI. White bar shown in the right panels is 20 μm.
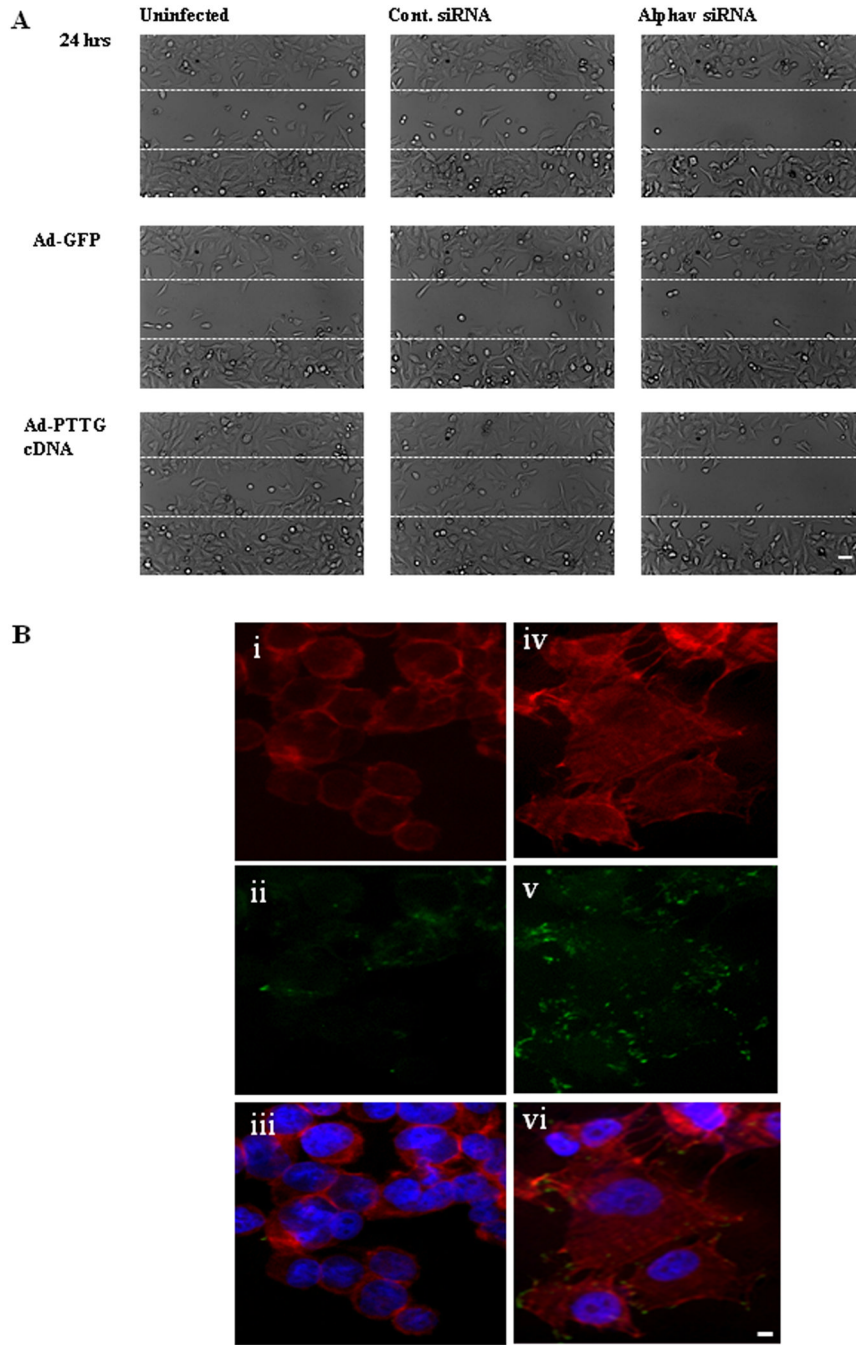
Figure 6.
(A) Wound migration assay in A549 cells. Cells were infected with Ad-GFP or Ad-PTTG cDNA and co-transfected with control siRNA or α_v siRNA. Cells were examined after 24 hr post wound formation and photographed. White bar shown in the right panels is 20 μm. (B) Fluorescence microscopy of focal adhesion and actin cytoskeleton in A549 cells. F-actin was detected using TRITC-conjugated phalloidin (red). Focal contacts were revealed using anti-vinculin (green). i: uninfected cells with phalloidin, ii: uninfected cells with anti-vinculin, iii: overlay of i and ii with DAPI counter stain, iv: Ad-PTTG cDNA infected cells with phalloidin, v: AdPTTG cDNA infected cells with anti-vinculin, vi: overlay of iv and v with DAPI counter stain.

A

B
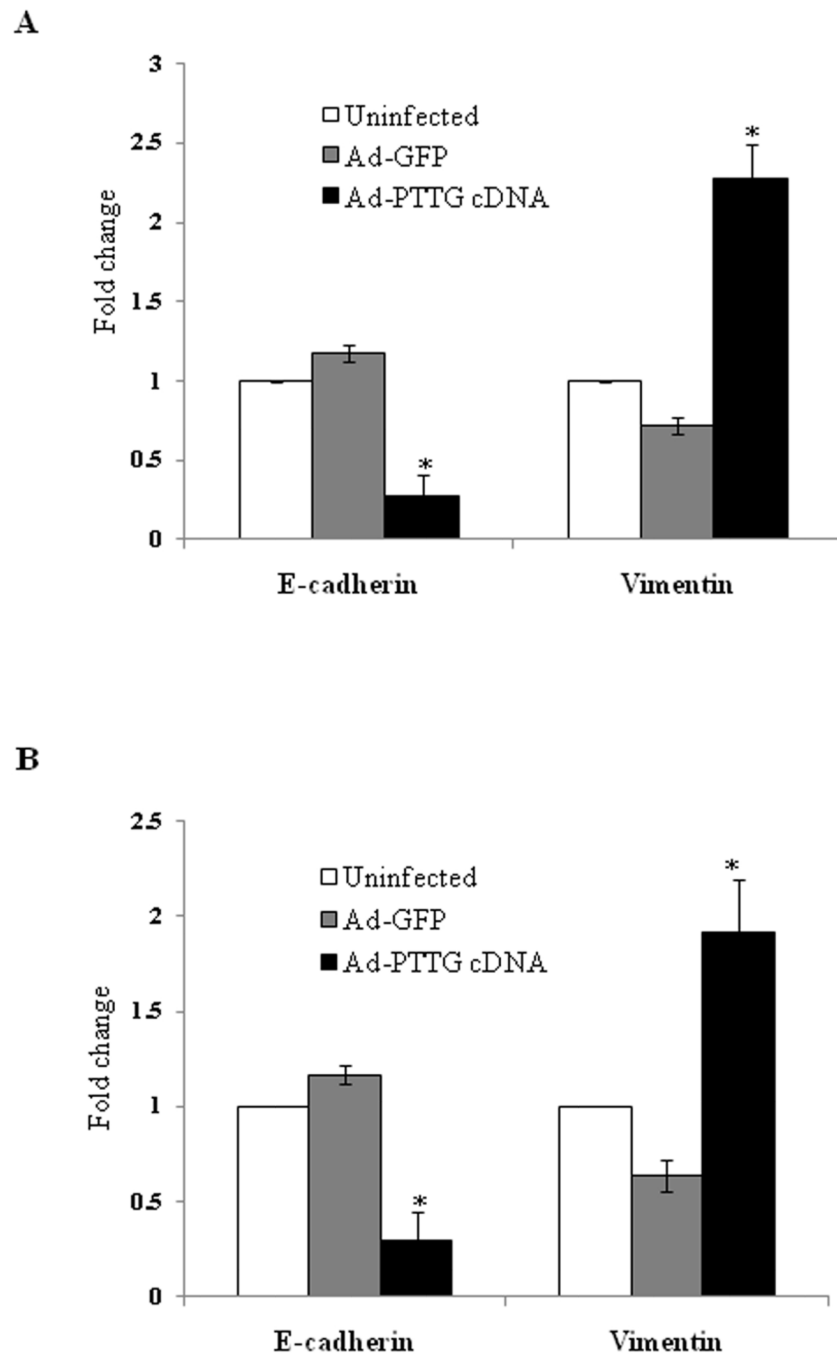
Figure 7.
E-cadherin and vimentin mRNA expression in (A) A549 and (B) H1299 using qPCR.
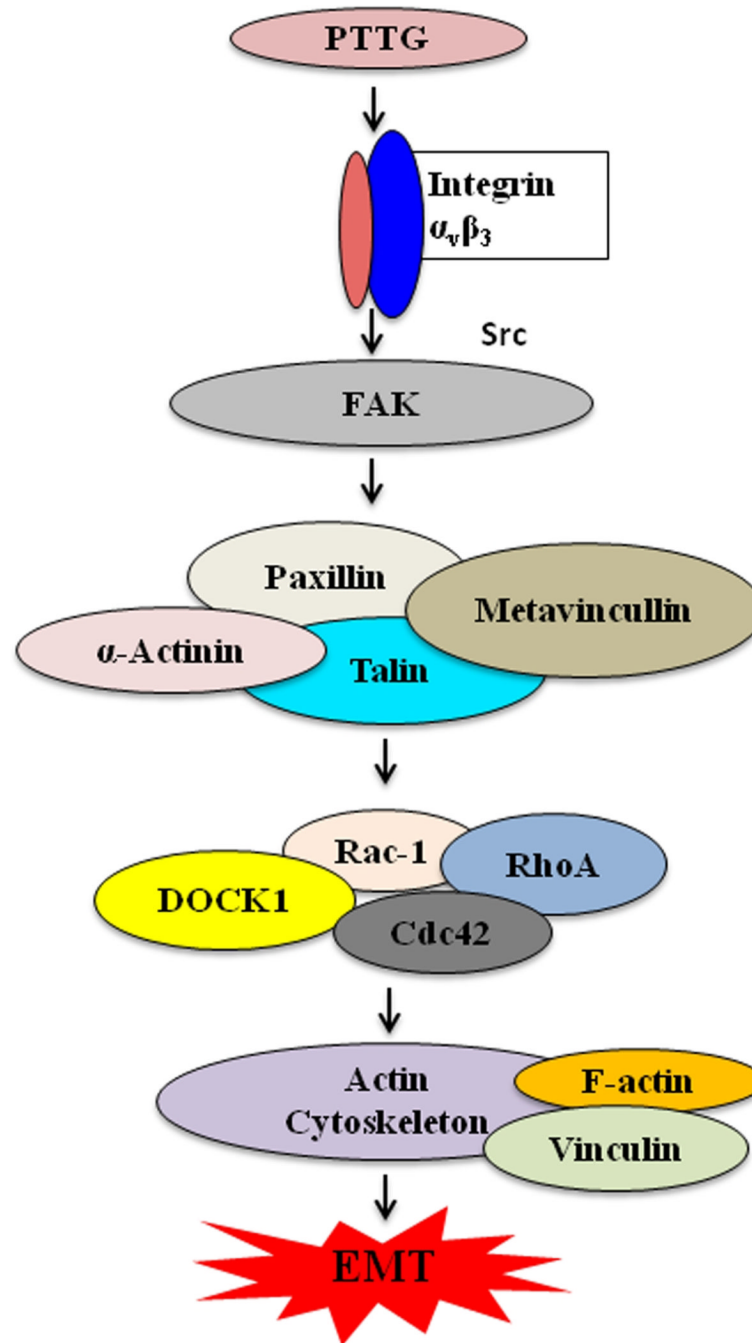
Figure 8.
Schematic presentation of regulation of EMT by PTTG.

Table I

Primers Sequences for various genes

Gene	Sequences
GAPDH	Sense 5'-TGA TGA CAT CAA GAA GGT GGT-3'
	Antisense 5'-TCC TTG GAG GCC ATG TGG GCC-3'
Integrin α_V	Sense 5'-AATCTTCCAATTGAGGATATCAC-3'
	Antisense 5'-AAAACAGCCAGTAGCAACAAT-3'
Integrin β_3	Sense 5'-CCGTGACGAGATTGAGTCA-3'
	Antisense 5'-AGGATGGACTTTCCACTAGAA-3'
FAK	Sense 5'-GAA GTC TTC AGG GTC CGA TTG-3'
	Antisense 5'-CAT TCT CGT ACA CCT TAT CAT TCG-3'
Paxillin	Sense 5'-TGG CTT CGC TGT CGG ATT TC-3'
	Antisense 5'-GTC AAG GGC TGT CAC CAC TTT ATC-3'
Metavincullin	Sense 5'-CTT TCC CCT CTG ACA TGG AA-3'
	Antisense 5'-GAA TAA GTG CCC GCT TGG TA-3'
Talin	Sense 5'-ACC AGG TGA AGG TGA AGG TG-3'
	Antisense 5'-TTT TAG CGG CCT GAG TGA GT-3'
RhoA	Sense 5'-CGG AAT GAT GAG CAC ACA AGG-3'
	Antisense 5'-ATG TAC CCA AAA GCG CCA ATC-3'
Rac1	Sense 5'-GCC GAT TGC CGA TGT GTT-3'
	Antisense 5'-CTC GGA TCG CTT CGT CAA A-3'
DOCK180	Sense 5'-GGA AAT GAA AGG CGC TTC T-3'
	Antisense 5'-TCC ATT CCC ATC ATG CCG TTC-3'
Cdc42	Sense 5'-AAG ACC CCA ATT TAC CTG AAA GC-3'
	Antisense 5'-TGG CGA AAG TCT CCA AGC G-3'
PTTG	Sense 5'-GCC TTA GAT GGG AGA TCT CA-3'
	Antisense 5'-GCT TTA ACA GTC TTC TCA GT-3'
E-Cadherin	Sense 5'-TGA CAC CCG GGA CAA CGT TTA TTA-3'
	Antisense 5'-CTA GTC TAG ACC CCT AGT GGT CCT CG-3'
Vimentin	Sense 5'-GAC AAT GCG TCT CTG GCA CGT CTT-3'
	Antisense 5'-TCC TCC GCC TCC TGC AGG TTC TT-3'