HHS Public Access

Author manuscript

Biochem J. Author manuscript; available in PMC 2016 March 15.

Published in final edited form as:

Biochem J. 2015 March 15; 466(3): 525–536. doi:10.1042/BJ20140698.

$\alpha_v\beta_6$ Integrin Is Required for TGFβ1-Mediated Matrix Metalloproteinase2 Expression

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Abstract

TGFβ1 activity depends on a complex signaling cascade that controls expression of several genes. Among others, TGFβ1 regulates expression of matrix metalloproteinases (MMPs) through activation of Smads. Here, we demonstrate for the first time that the $\alpha_v\beta_6$ integrin interacts with TGFβ receptor II (TβRII) through the β_6 cytoplasmic domain and promotes Smad3 activation in prostate cancer cells. Another related α_v integrin, $\alpha_v\beta_5$, as well as the $\alpha_v\beta_{6/3}$ integrin, which contains a chimeric form of β_6 with a β_3 cytoplasmic domain, do not associate with TβRII and fail to show similar responses. We provide evidence that $\alpha_v\beta_6$ is required for upregulation of MMP2 by TGFβ1 through a Smad3-mediated transcriptional program in prostate cancer cells. The functional relevance of these results is underscored by the finding that $\alpha_v\beta_6$ modulates cell migration in a MMP2-dependent manner on an $\alpha_v\beta_6$ specific ligand, latency associated peptide (LAP)-TGFβ. Overall, these mechanistic studies establish that expression of a single integrin, $\alpha_v\beta_6$, is sufficient to promote activation of Smad3, regulation of MMP2 levels, and consequent

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[4]The abbreviations used are: ECM, Extracellular matrix; PrCa, Prostate cancer; TβR, TGFβ receptor; MMP, Matrix metalloproteinase; LAP, Latency associated peptide; type I Col, type I Collagen; SIS3, Specific inhibitor of Smad3; IB, Immunoblotting; IP, Immunoprecipitation; qRT-PCR, Quantitative Real Time PCR; Fn, Fibronectin; Zg, Zymography; TIMP2, Tissue Inhibitor of Metalloproteinase2; MT1-MMP, Membrane type-1 matrix metalloproteinase.

The other authors do not have any conflict of interests.

AUTHORS' CONTRIBUTION

Conception and design: Anindita Dutta, Jing Li and L.R. Languino.

Performed experiments: Anindita Dutta, Jing Li, Carmine Fedele, Aejaz Sayeed and Thomas Manes (who generated chimeric integrins).

Acquisition of data: Anindita Dutta, Jing Li, Carmine Fedele, Aejaz Sayeed.

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Administrative, technical, or material support: Shelia M. Violette for having provided antibodies to $\alpha_v\beta_6$ integrin.

Study supervision: LR. Languino.

catalytic activity, as well as cell migration. Our study describes a new TGFβ1/$\alpha_v\beta_6$/MMP2 signaling pathway that, given TGFβ1 pro-metastatic activity, may have profound implications for prostate cancer therapy.

Keywords

Integrins; prostate cancer; SMAD3; TGFβ1 receptor; MMP2

INTRODUCTION

The interactions between extracellular matrix (ECM) proteins and integrins, transmembrane receptors which comprise an α-subunit and a β-subunit [1, 2], support cancer cell functions including cell adhesion, proliferation, gene expression and modulation of migratory/invasive phenotypes [3–5]. Among others, the $\alpha_v\beta_6$ integrin is largely expressed in a wide variety of cancer types [6–10]. Its function is likely to be crucial for cancer progression [10–12], including prostate cancer (PrCa) where this molecule is largely undetectable in normal tissues, but abundantly expressed in metastatic tumors as evaluated in a mouse model of PrCa [13].

TGFβ1 has both tumor suppressor and tumor promoter activities [14, 15]. However, tumor cells often acquire a resistance to the growth-suppressive activities of TGFβ1 during tumor progression and hence, TGFβ1 shows growth stimulatory effects on these cells [16]. There are three types of TGFβ receptors (TβR)s: type I (TβRI), type II (TβRII) and type III (TβRIII) receptors. Type I and type II receptors comprise a single transmembrane segment and a cytoplasmic segment with a Ser/Thr kinase domain and form a heterodimeric complex upon ligand binding to initiate intracellular signaling [17]. Phosphorylation of TβRII at Ser213 is required for TGFβ1-mediated downstream signaling [18]. TβRIII, known as betaglycan, does not have an intrinsic signaling function, and has been shown to increase TGFβ1 binding to the signaling receptor TβRII [19].

As downstream effectors of TGFβ1 signaling, Smad molecules [20], whose genes have been reported to be mutated in various cancers [21], are often exploited in malignancy. While Smads 1, 5 and 8 are downstream of bone morphogenetic protein, rather than of TGFβ1, and Smad6 is an inhibitor of this growth factor [17, 22], Smads 2, 3 and 4 are downstream of TGFβ1. Phosphorylated Smad2/3 form dimers or trimers with Smad4, translocate to the nucleus and interact with both DNA-binding cofactors as well as co-activators or co-repressors, to modulate transcription of TGFβ-target genes [20]. Previous studies reported that Smad2 and Smad3 expression are highly relevant to PrCa progression as upregulation of these molecules correlates with higher Gleason scores in patients. Higher nuclear p-Smad2 and Smad4 are found in PrCa Gleason grade 5 as compared to PrCa with Gleason grade 3 or 4, whereas the levels of Smad7, which prevents phosphorylation of Smad2 or Smad3, are not changed with different Gleason grades [23]. Overexpression of Smad3 has been shown to correlate with Gleason score in human PrCa, and may contribute to disease progression in humans [24]. Both nuclear and cytoplasmic overexpression of Smad4 protein correlates with tumor grade, stage and DNA ploidy in PrCa patients [25], although nuclear Smad4 is

decreased in high Gleason score cancer [26] and Smad4 downregulation enhances the metastatic potential of PrCa cells [27].

Critical downstream effectors of TGFβ1 in cancer progression are integrins [28] and matrix metalloproteinases (MMPs) [29, 30]. Among others, TGFβ1 upregulates $\alpha_v\beta_6$ [28] and MMP2 protein expression [31], MMP2 mRNA stability [32] as well as secretion [31], and this response has been functionally implicated in osteolytic lesions in murine models of PrCa metastasis [33, 34]. Another study by Miralles et al. shows that TGFβ1 is a key activator of MMP2 in the pancreatic islets [35]. A different study by Sehgal et al. shows that TGFβ1 induces secreted protein activity for MMP2 although it has no effect on new MMP2 RNA synthesis [36]. Specifically, Ahmed et al. report expression of $\alpha_v\beta_6$ in ovarian cancer tissues and show that $\alpha_v\beta_6$ causes enhanced levels of two pro-MMPs, MMP2 and 9, in several cancer cell lines [7, 37]. Smad 2, 3 and Smad4 are involved in regulating MMP2 [38] and other MMPs [39, 40]. In addition, there are reports showing that MMP2 and MMP9 are increased in response to integrin expression [7, 37, 41, 42] and that TGFβ in cooperation with $\alpha_3\beta_1$ promotes MMP9 mRNA stability in normal keratinocytes [42]. Although clinical data have implicated MMP2 and MMP9 as independent predictors of PrCa metastasis [43] and associated MMP2 with reduced disease-free survival [44], a potential role of integrins in their regulation by TGFβ1 has not been investigated.

Our data show that the $\alpha_v\beta_6$ integrin interacts with TβRII and promotes TGFβ1-induced phosphorylation of Smad3. This causes upregulation of MMP2 and as a result, $\alpha_v\beta_6$ – expressing cells show increased cell migration in a MMP2-dependent manner.

MATERIALS AND METHODS

Reagents and Antibodies

BSA was from Sigma-Aldrich, type I Collagen (type I Col) was from Invitrogen, TGFβ1 and LAP-TGFβ1 were from R&D Systems. Specific inhibitor of Smad3 (SIS3) was from Santa Cruz Biotechnology [45]. SIS3 was dissolved in DMSO. We used the following rabbit antibodies (Abs) against: TβRII, ERK1/2, fibronectin (FN), focal adhesion kinase (FAK) and AKT from Santa Cruz Biotechnology; phospho-Smad3 (ser423/425) from Cell Signaling Technology; Smad3 from Zymed; β_3 cytoplasmic domain [46]; β_5 cytoplasmic domain and membrane type-1 matrix metalloproteinase (MT1-MMP) from Millipore; and MMP2 from Cell Signaling Technology. This Ab against MMP2 is specific for MMP2 and reacts with pro- and active human MMP2.

C19 goat Ab against β_6 cytoplasmic domain and goat IgG was from Santa Cruz Biotechnology.

We used the following mouse (m)Abs against: Smad2 from Invitrogen, tissue inhibitor of metalloproteinases2 (TIMP2) from Abeam, β_6 from Chemicon, 10D5, for FACS and immunoprecipitation (IP) and 2A1 for immunoblotting (IB) [12, 47]. In addition, the following mouse (m)Abs against: α_v, L230; β_1, TS2/16; β_3, AP3, all from Life Technologies; β_1, C-18 and Smad4 from Santa Cruz Biotechnology were used.

Non-immune mouse and rabbit IgG (from Sigma-Aldrich) were used as negative controls.

Cells and Culture Conditions

PC3 cells were from ATCC. Two PC3 sublines: PC3-high and PC3-zero, previously designated PC3-H and PC3-L respectively [48], were used: PC3-high (PC3-H) which are positive for β_6 expression and PC3-zero (PC3-L) which are negative for β_6 expression; these cells were cultured in RPMI medium containing 10% FBS, 2 mmol/L glutamine, 100 μg/ml streptomycin and 100 U/ml penicillin. Another prostate cancer cell line, RWPE (from ATCC), was maintained in Defined Keratinocyte Serum Free Medium (K-SFM) (from Invitrogen) containing Defined K-SFM growth supplement. Cells were starved in serum-free culture medium for 24 h before being incubated with TGFβ1 (10, 20, 40 ng/ml) [49] for 48 h. After TGFβ1 stimulation, cells were analyzed by IB, zymography or quantitative real-time PCR (qRT-PCR). BPH1 cells were provided by Dr. Simon W. Hayward (Vanderbilt University, TN) and authenticated as previously described [50]. These cells were maintained in RPMI supplemented with 5% FBS, 2 mmol/L glutamine, 100 μg/ml streptomycin and 100 U/ml penicillin and the serum-free culture medium was used as a control for MMP activity by zymography [51].

Viral Constructs and Cell Transfection

PC3-zero cells were stably transfected using human β_6 integrin cDNA subcloned in pBABE retroviral vector as described before [52]. The β_6 integrin cDNA was generously provided by Dr. Ian Hart, Cancer Research UK Clinical Centre [53]. Human β_5 integrin cDNA in pBluescript ATCC (NO. 95496) was subcloned into pBABE retroviral vector by EcoRI sites. The pBABE-β_5 construct was confirmed by DNA sequencing. PC3-zero cells were stably transfected using human β_6 or β_5 integrin cDNA in pBABE retroviral vector, selected and maintained in culture medium containing 10% FBS and 0.5 μg/ml puromycin.

PC3-zero cells expressing chimeric β_6/β_3 or β_3/β_6 integrin were generated in our laboratory by subcloning in pcDNA-3 (pcDNA) mammalian expression vector (Invitrogen). Detailed construct information is included in the section of construction of integrin chimeras. PC3-zero cells expressing pcDNA vector were also generated. All these transfectants were selected and maintained in culture medium containing 10% FBS and 100 μg/ml G418. $\alpha_v\beta_6$ -PC3-zero cells expressing either a Ctrl.-shRNA or the MMP2-shRNA were generated in our laboratory as previously published [52].

All lentiviral constructs were obtained from Open Biosystems. PC3-high cells were stably transfected with lentivirus pLKO.1 β_5-shRNA (Number RHS3979-9624928) or lentivirus pLKO.1 β_6-shRNA (Number RHS3979-9624888). Stable transfectants of PC3-high and PC3-zero cells were screened using puromycin and maintained in culture medium containing 10% FBS and 0.5 μg/ml puromycin. Two different clones for each stable transfectants were used for each experiment.

Cells were transiently transfected using oligofectamine with 100 nM of the following siRNAs against: Smad2 (Dharmacon) [5′-GUCCCAUGAAAAGACUUAA(TT)-3′], Smad3 (Dharmacon) [5′-AAUGGUGCGAGAAGGCGGUCAdTdT-3′], Smad4 (Dharmacon) [5′-GUGUGCAGUUGGAAUGUAAUU-3′(UU overhang)], β_{1C} (Dharmacon) [5′-

CCUCUGACUUCCAGAUUCC-3′], a mixture of two different siRNAs against TβRII (Dharmacon) [5′-CAACAACGGUGCAGUCAAG-3′ and 5′-GACGAGAACAUAACACUAG-3′], β_6 integrin (siRNA duplex, D2, IDT Inc.) [(sense) 5′-ACCACGGGAACGGCUCUUUCCAGTG-3′ and (antisense) 5′-CACUGGAAAGAGCCGUUCCCGUGGUGA-3′]. After two rounds of transfection with siRNA, cells were serum starved for 24 h and stimulated with 20 ng/ml TGFβ1 for 48 h. Cells were then analyzed for β_6 integrin protein levels and MMP2 protein or mRNA levels.

Construction of Integrin Chimeras

Two chimeric integrins were expressed in PC3-zero cells: one referred to as β_3/β_6, contains the β_3 extracellular and transmembrane domains (residues 1 – 741 [54]) and a β_6 cytoplasmic tail (corresponding to residues 731 to 788 of β6); another, called β_6/β_3, consists of β_6 extracellular and transmembrane domains (residues 1 – 730 [55]) and a β_3 cytoplasmic tail (corresponding to residues 742 to 788 of β3). The amino acids spanning the putative transmembrane domain/cytoplasmic domain transitions are identical: IWKLL, corresponding to residues 740 - 744 in β_3 and 729 - 733 in β_6. The putative transmembrane domain ends at the residue W 741 in β_3, 730 in β_6, and the cytoplasmic domain starts at the residue K 742 in β_3, 731 in β_6. Therefore the sequences surrounding the junctions in the newly generated constructs are: β_3/β_6 integrin chimera …*AALLIW*KLLVSFH… and β_6/β_3 integrin chimera … VLLCIW*KLLITIH*….

β_3/β_6 integrin chimera: A recombinant cDNA encoding the β_3/β_6 chimera was constructed in our laboratory by directional ligation of cDNA and PCR-generated fragments. Identical sequences of β_3 and β_6 at the transmembrane domain - cytoplasmic domain interface allowed for the generation of cohesive termini between β_3 and β_6 fragments at this juncture. This was effected by incorporating a restriction enzyme site (Bsa I) that cuts at a distance away from its recognition sequence (GGTCTCN′NNNN′) into oligos used in PCR; the cohesive termini are generated upon digestion with BsaI. Two PCR products, one containing the transmembrane domain of β_3 integrin (fragment 1) and one containing the cytoplasmic domain of β_6 (fragment 2), were generated by using the following oligos and templates: fragment 1. (β_3 forward, nucleotide (nt) 2001-2022) GTGACGAGATTGAGTCAGTGAA and (β_3 reverse, nt 2218-2239, containing the Bsa-restriction enzyme site) GGTCTCCCAGATGAGCAGGGCGGCAAGG using β_3 cDNA in pRc/CMV as template; fragment 2. (β_6 forward nt 2410-2431, containing the Bsa-restriction enzyme site) GGTCTCATCTGGAAGCTACTGGTGTCA and (SP6 in vector) ATTTAGGTGACACTATAG using β_6 in pcDNA-3 as template. The following gel-purified (QIAEX II) fragments (cDNAs or fragments) were assembled in a ligation reaction with pcDNA-3 digested with EcoRI and XbaI to create a recombinant cDNA encoding the β_3/β_6 chimera: β_3 cDNA digested with EcoRI and AflII + fragment 1 digested with AflII and BsaI + fragment 2 digested with BsaI and XbaI.

β_6/β_3 integrin chimera: Similarly, a recombinant cDNA encoding the β_6/β_3 chimera was constructed in our laboratory by directional ligation of cDNA and PCR-generated fragments. The two PCR products, one containing the transmembrane domain of β_6 integrin (fragment 3) and one containing the cytoplasmic domain of β_3 (fragment 4), were generated by using

the following oligos and templates: fragment 3. (β_6 forward nt 2060-2081) CCAACCTGTGAACGATGTCCTA and (β_6 reverse nt 2395-2416, containing the Bsa-restriction enzyme site) GGTCTCCCAGATGCACAGTAGGACAACC using β_6 in pcDNA-3 as template; fragment 4. (β_3 forward nt 2231-2253, containing the Bsa-restriction enzyme site) GGTCTCATCTGGAAACTCCTCATCAC and (SP6 in vector) ATTTAGGTGACACTATAG using β_3 cDNA in pRc/CMV as template. The following gel-purified (QIAEX II) fragments were assembled in a ligation reaction with pcDNA-3 digested with EcoRI and XbaI to generate a recombinant cDNA encoding the β_6/β_3 chimera: β_6 cDNA digested with EcoRI and BstEII + fragment 3 digested with BstEII and BsaI + fragment 4 digested with BsaI and XbaI. The absence of mutations in all PCR-amplified fragments was verified by sequencing.

Flow Cytometric analysis

FACS analysis was performed to determine integrin expression using mAbs: TS2/16 to β_1 or 10D5 to β_6-Non-immune mouse IgG was used as negative control.

Immunoblotting and Immunoprecipitation

PC3 or RWPE cells were resting or stimulated with 20 ng/ml TGFβ1 for 48 h. Cell lysates were prepared, separated by SDS-PAGE and analyzed by IB as described before [56].

IP experiments were performed as described before [56] with the following modification: cells were lysed in the following lysis buffer [20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1% NP-40, 1 mM Na_3VO_4, 1 mM PMSF, 10 μg/ml Aprotinin, 10 μg/ml Leupeptin, 4 μg/ml Pepstatin and 1 μM Calpain inhibitor] and pre-clearing was performed by two consecutive incubations with protein G-Sepharose at 4°C for 45 min. Binding to the specific Abs was achieved by incubation at 4°C overnight, followed by incubation with protein G-Sepharose for 2 h at 4°C. After five washes with lysis buffer, immunocomplexes were eluted and separated under reducing or non-reducing condition (reducing for β_3, β_5 or TβRII and non-reducing for β_6). The immunocomplexes were separated by SDS-PAGE and analyzed by IB. In order to detect the association between TβRII and β_6, either β_6 or TβRII was immunoprecipitated from the cell lysates using 10D5 (this mAb reacts with mouse, rat or human β_6) to β_6 and sc-400 (designated as 1, a rabbit polyclonal affinity purified Ab raised against a peptide mapping within a cytoplasmic domain of TβRII of human origin; reacts with mouse, rat or human TβRII) or sc-1700 (designated as 2, a rabbit polyclonal Ab raised against amino acids 1-567 representing full length TβRII of human origin; reacts with mouse, rat or human TβRII) to TβRII. To perform the IP experiment with other integrin subunits, β_3 or β_5 was immunoprecipitated from the cell lysate using mAb AP3 to β_3 or a rabbit Ab against the cytoplasmic domain of β_5.

Gelatin Zymography

Serum-free culture media collected from PC3-high, PC3-zero cells and their stable transfectants, were concentrated using Centricon filters (Millipore, MA) by centrifuging at 3000 rpm for 30 min at 4°C. Gel loading buffer [0.5 M Tris–HCl, pH 6.8, 10% SDS, 50% glycerol and 0.1% bromophenol blue] was added to 7 μg (PC3-high) or 10 μg (PC3-zero) of concentrated culture supernatant. Proteins were loaded and electrophoresis was performed

using SDS-PAGE (8%) containing 0.1% gelatin (Sigma-Aldrich) at 12 mA for 2 h at room temperature. After electrophoresis, the gels were incubated in 2.5% Triton X-100 solution for 1 h at room temperature and then in substrate buffer containing 50 mM Tris–HCl, pH 7.4, 10 mM $CaCl_2$ for 24 h at 37°C. The gels were stained with Coomassie brilliant blue, and destained in methanol: acetic acid: water (50: 10: 40) [57].

Quantitative Real-Time PCR analysis

qRT-PCR was performed as described before using Parental, shβ_5- and shβ_6-PC3-high cells [58]. Before RNA isolation and PCR analysis, cells were treated as follows: Parental, shβ_5- and shβ_6-PC3-high were stimulated with TGFβ1 or resting for 48 h. PC3-high cells were transiently transfected with β_{1C}, Smad2, Smad3, Smad4 siRNA or not transfected before being stimulated with or without TGFβ1 for 48 h. PC3-high and $\alpha_v\beta_6$-PC3-zero cells were pre-treated with 3, 10 or 30 μM SIS3 for 1 hr and then stimulated with TGFβ1 or unstimulated. The oligo sequences used are as follows: MMP2 (Forward) 5′-GCAACCCAGATGTGGCCAAC-3′, (Reverse) 5′-CGCTCCAGACTTGGAAGGCA-3′; and MMP9 (Forward) 5′-ATAGACTACTACAGGCT-3′, (Reverse) 5′-TAGCACGGATAGACCA-3′; GAPDH (Forward) 5′-GGGAAGGTGAAGGTCGGAGT-3′, (Reverse) 5′-GTTCTCAGCCTTGACGGTGC-3′.

Statistical analysis

Statistical significance between datasets was calculated using t-test and all graphs were generated using Microsoft Excel. The error bars were calculated and represented in terms of mean ± SD. A two-sided *P*-value of less than 0.05 was considered statistically significant.

Migration assay

Transwell chambers (12 μm pore diameter, Costar) were coated with BSA (1%), type I Collagen (50 μg/ml) or LAP-TGFβ1 (10 μg/ml) overnight at 4°C and migration assay were performed as described previously [59]. Briefly, cells were stimulated with TGFβ1 for 48 hr; after detachment and trypsin inactivation, the cells were seeded (300,000 cells/well) on transwell chambers at 37°C for 6 h in presence or absence of TGFβ1. After fixation with 3.7% paraformaldehyde (PFA), cells attached on both layers of the porous filter were stained with DAPI (1 μg/ml) and pictures of nuclei were acquired by fluorescence microscopy (Olympus IX71 or Nikon Eclipse TS-100 inverted microscopes equipped with fluorescence unit). Then, cells on the top layer were removed using a cotton swab, and pictures of nuclei from cells migrated to the bottom layer were acquired. Five and twenty random fields were acquired for quantification of attached and migrated cells, respectively. Cell Profiler [60] software (www.cellprofiler.org) was then used for quantification of nuclei number, using 10–30 pixel units as range for discrimination between single nuclei and potential aggregates. The ratio between number of cells migrated onto the bottom layer and total (top + bottom) number of cells attached on the filter was calculated for each group of transfectants. Chi-Square test was used for statistical analysis.

RESULTS

$\alpha_v\beta_6$ is Required for TGFβ1 Upregulation of MMP2

Given the known ability of TGFβ1 to upregulate $\alpha_v\beta_6$ [28] and MMP2 [31, 32], we first asked whether $\alpha_v\beta_6$ is a downstream effector of TGFβ1 which mediates MMP2 induction (Figure 1 and Figure 2). For this study, we used two PrCa PC3 cell sublines, PC3-high ($\alpha_v\beta_6^+$) or PC3-zero ($\alpha_v\beta_6^-$) (described in the Material and Methods section) and another PrCa cell line, RWPE, which expresses high levels of $\alpha_v\beta_6$. In RWPE cells, TGFβ1 induces the expression of β_6 and MMP2 (Figure 1A, left panels). However, TGFβ1 stimulation fails to significantly increase β_6 and consequently MMP2 expression in β_6-silenced RWPE cells as compared to non-transfected or cells transfected with a non silencing (β_{1C}) siRNA (Figure 1A, left panels). Similarly, in RWPE cells, PC3-high cells stably transfected with β_5-shRNA (shβ_5) show increased levels of β_6 upon TGFβ1 stimulation. Densitometric analysis shows a 6.4 or 9.3 fold increase of β_6 expression in shβ_5-PC3-high cells upon 20 or 40 ng/ml TGFβ1 stimulation respectively, as compared to the unstimulated shβ_5-PC3-high cells (Figure 1A, right panels). However, downregulation of β_6 by shRNA (shβ_6) in PC3-high fails to induce MMP2 expression upon TGFβ1 stimulation (Figure 1A, right panels). Our FACS analysis confirms high levels of surface expression of $\alpha_v\beta_6$ integrin in RWPE and PC3-high cells (Figure 1B). This $\alpha_v\beta_6$-dependent TGFβ1 regulation is specific for MMP2 as another MMP family member, MT1-MMP, known to be upregulated by TGFβ1 in several cell types [61–63], is not affected upon abrogation of β_6 in PC3-high cells in presence or absence of TGFβ1 (Figure 1C). Densitometric analysis confirms that no significant changes occur in MT1-MMP expression upon these treatments. In parallel, we observed that TGFβ1 induces MMP2 activity in shβ_5-PC3-high cells as evaluated in culture supernatants by gelatin zymography, but it fails to induce MMP2 activity in shβ_6-PC3-high cells (Figure 1D). Similarly, MMP2 activity is induced by TGFβ1 in $\alpha_v\beta_6$ expressing PC3-zero transfectants, but not in $\alpha_v\beta_5$ expressing PC3-zero cells (Figure 1D).

As expected [28], we observed that TGFβ1 stimulation of PC3-high cells specifically increases β_6 and β_3 protein levels without affecting α_v or β_5 integrin subunits (Figure 2A and 2B). However, the cells that lack $\alpha_v\beta_6$ expression (PC3-zero), do not show a similar response as TGFβ1 stimulation of PC3-zero cells specifically increases β_3, but not β_5 or β_6 (Figure 2C). Since TGFβ1 is known to upregulate TIMP2 in gastric cancer [64], we investigated whether $\alpha_v\beta_6$ is required for TIMP2 regulation by TGFβ1 in PrCa. We observe that TIMP2 is increased upon TGFβ1 treatment in PC3-high cells and that abrogation of β_6 by shRNA (shβ_6) in these cells drastically downregulates the expression of TIMP2 under basal and induced conditions (Figure 2D). Furthermore, our data also show that TGFβ1 promotes MMP2 expression and consequently activity only in $\alpha_v\beta_6$ –expressing PC3-high cells (Figure 2E and not shown), but fails to show a similar response in $\alpha_v\beta_6^-$ PC3-zero cells (Figure 2E and not shown). Altogether, these data show that $\alpha_v\beta_6$ is required for TGFβ1-mediated increase in MMP2 expression and, consequently, activity.

$\alpha_v\beta_6$ Integrin Associates with TβRII

Given previous studies that have demonstrated that TβRII associates with integrins [65], we tested whether TβRII associates with $\alpha_v\beta_6$. TβRII co-immunoprecipitates with $\alpha_v\beta_6$ but not

with other integrins such as $\alpha_v\beta_3$ or $\alpha_v\beta_5$ in PC3-high cells (Figure 3A); a reverse co-IP experiment was also performed to confirm this association (Figure 3B). Additional immunoblotting analysis to confirm the specificity of the Ab to TβRII was also performed using a mixture of two different TβRII siRNAs in PC3-high, PC3-zero and RWPE cells (Figure 3C).

Association of $\alpha_v\beta_6$ Integrin with TβRII Induces MMP2 Expression

To confirm whether the association of $\alpha_v\beta_6$ with TβRII causes MMP2 expression, we transfected either a cDNA chimeric variant of the β_6 and β_3 integrin subunits containing the β_3 extracellular domain and β_6 cytoplasmic domain (β_3/β_6) or a chimeric form containing the β_6 extracellular domain and β_3 cytoplasmic domain (β_6/β_3) in PC3-zero cells (Figure 4A). IP using an Ab specific for the β_6 cytoplasmic domain shows association of TβRII with β_3/β_6 in PC3-zero cells, either in the absence or presence of TGFβ1 (Figure 4B, left panels). However, PC3-zero cells expressing β_6/β_3 do not show any association with TβRII when immunoprecipitated using an Ab directed against the extracellular domain of β_6 (Figure 4B, right panels). We next stimulated β_3/β_6-PC3-zero and β_6/β_3-PC3-zero cells with TGFβ1 and analyzed MMP2 expression. The results show MMP2 induction only in β_3/β_6-PC3-zero cells as compared to Parental, control cells transfected with a vector alone (pcDNA) or β_6/β_3-PC3-zero cells (Figure 4C) in the absence of TGFβ1. In addition, TGFβ1 stimulation to β_6/β_3-PC3-zero cells further increases MMP2 expression, but it fails to show a similar response in Parental, pcDNA or β_6/β_3-PC3-zero cells (Figure 4C). These results confirm that TβRII associates with the cytoplasmic domain of β_6 and the interaction between β_6 cytoplasmic domain and TβRII promotes TGFβ1-mediated induction of MMP2.

Smad3 Mediates MMP2 Regulation by TGFβ1 in $\alpha_v\beta_6$–Expressing Cells

We performed a qRT-PCR analysis to investigate whether a TGFβ1 induction of MMP2 in Parental and shβ_5-PC3-high cells occurs at the mRNA levels. ShRNA mediated silencing of β_6 in PC3-high cells results in reduced MMP2 mRNA levels. On the other hand, MMP9 mRNA levels do not show any change upon β_6 downregulation in PC3-high cells (Figure 5A). Studies by different groups have shown that MMP2 is a Smad3 target gene [39], and since Smad3 is known to promote PrCa progression [24], we investigated the role of Smad3 in inducing MMP2 in TGFβ1-stimulated PC3-high cells. Silencing of Smad3 (Figure 5B and 5C) and Smad4 (data not shown) in PC3-high cells reduces TGFβ1-induced MMP2 expression; however, downregulation of Smad2 by siRNA or transfection of a non-relevant siRNA does not show a similar effect (Figure 5B and 5C). In addition, the differences in MMP9 transcript levels in the presence or absence of Smad3 in PC3-high cells are not statistically significant ($P>0.05$; data not shown). When we tested the activation of Smad3 by $\alpha_v\beta_6$ in the presence of TGFβ1 for 5, 10 or 30 min, we observe that TGFβ1 induces robust phosphorylation of Smad3 in Parental and shβ_5-PC3-high, but only a minor increase in shβ_6-PC3-high cells (Figure 5D, left panel). In support of this result, we confirm that exogenous expression of $\alpha_v\beta_6$ in PC3-zero cells sustains phosphorylation of Smad3 (Figure 5D, right panel). Furthermore, to evaluate whether activation of Smad3 is required for TGFβ1 induction of MMP2, we pre-treated PC3-high (Figure 6A and 6B) and $\alpha_v\beta_6$ expressing PC3-zero (Figure 6C) cells with different concentrations of SIS3, which specifically blocks the phosphorylation of Smad3 [45]. TGFβ1 stimulation of these cells

results into decreased phosphorylation of Smad3 as compared to vehicle treated cells (Figure 6A). As a consequence, TGFβ1 induction of MMP2 mRNA is reduced in SIS3 treated cells as compared to vehicle treated cells (Figure 6B and 6C). These data show that activation of Smad3, mediated by $\alpha_v\beta_6$, selectively regulates TGFβ1-induced MMP2 levels.

MMP2 mediates PrCa cell migration supported by $\alpha_v\beta_6$ integrin

Given our previous observations showing that MMP2 mediates $\alpha_v\beta_6$ downstream signaling in bone metastasis *in vivo* [52], here we investigated the contribution of $\alpha_v\beta_6$-dependent MMP2 on cell migration upon TGFβ1 stimulation of PrCa cells on an $\alpha_v\beta_6$ specific ligand LAP-TGFβ1[66]. TGFβ1 stimulation of Parental or shβ_5-PC3-high cells enhances migration on LAP-TGFβ1, whereas TGFβ1 stimulation of shβ_6-PC3-high cells has a minimal effect on cell migration on this ligand. On the other hand, migration of Parental, shβ_6-PC3-high cells and shβ_5-PC3-high cells on type I collagen is comparable (Figure 7A). On the basis of these results, we investigated whether downregulation of MMP2 in $\alpha_v\beta_6$ expressing cells contributes to this phenotype. We observe that TGFβ1 stimulation of $\alpha_v\beta_6$-PC3-zero or $\alpha_v\beta_6$-Ctr.shRNA-PC3-zero enhances migration on LAP-TGFβ1, whereas TGFβ1 stimulation of $\alpha_v\beta_6$-shMMP2-PC3-zero cells has a reduced effect on cell migration on this ligand. On the other hand, $\alpha_v\beta_6$-PC3-zero, $\alpha_v\beta_6$-Ctr.shRNA-PC3-zero and $\alpha_v\beta_6$-shMMP2-PC3-zero cells migrate equally well on type I Collagen (Figure 7B). Overall, our data indicate that MMP2 promotes TGFβ1-dependent PrCa cell migration in $\alpha_v\beta_6$-expressing PC3 cells.

$\alpha_v\beta_6$ Association with TβRII Increases MMP2 Levels in a Smad3-dependent Manner

Our working model shows that the $\alpha_v\beta_6$ integrin interacts with TβRII and promotes phosphorylation of Smad3. As a result, tumor cells produce MMP2, which is released in the ECM. On the other hand, either $\alpha_v\beta_{6/3}$ or other α_v- integrins ($\alpha_v\beta_x$) fail to associate with TβR and to phosphorylate Smad3, thus preventing TGFβ1 ability to induce MMP2 (Figure 8).

DISCUSSION

In this study, we show that the $\alpha_v\beta_6$ integrin is required for TGFβ1 signaling. We demonstrate that $\alpha_v\beta_6$ associates with TβRII and is required for TGFβ1-stimulated upregulation of MMP2, through Smad3 activation, and consequent MMP2-dependent cell migration.

The ability of $\alpha_v\beta_6$ to associate with TβRII and consequently activate the TGFβ1 pathway is novel. Although a direct association between $\alpha_v\beta_6$ and TβRII is not confirmed by our findings, specific molecular requirements appear to be necessary. We present evidence that the β_6 cytoplasmic domain mediates this association and provides a high degree of specificity to the system since it cannot be replaced by the β_3 cytoplasmic domain. The functional implication of this interaction mediated by the β_6 cytoplasmic domain is that MMP2 upregulation is observed only if this domain is expressed and does not require ligand binding to $\alpha_v\beta_6$. Specifically, increased MMP2 levels are evident as shown by using a chimeric integrin in our assays, only if an association of an integrin containing the β_6 cytoplasmic domain and TβRII occurs. Thus, minute changes in cellular integrin repertoires,

such as downregulation of $\alpha_v\beta_6$ or expression of $\alpha_v\beta_3$ or $\alpha_v\beta_5$, which fail to cause the described signaling cascade, may cause pathological events where TGFβ1 signaling becomes aberrant. In this context, it is worth stressing that this mechanism appears to occur in the absence of the specific cytokine-ligand; $\alpha_v\beta_6$-TβRII interaction is observed in either presence or absence of TGFβ1, as previously described for TβRII interaction with $\alpha_5\beta_1$ or $\alpha_v\beta_5$ in normal cells [67, 68]. In contrast, TGFβ1 stimulation is needed for the association of $\alpha_v\beta_3$ integrin with TβRII in human normal lung fibroblasts [65].

$\alpha_v\beta_6$ activates Smads whereas an $\alpha_v\beta_3$-TβRII-mediated pathway activates p38 MAPK, thus suggesting that the difference in the status of TβRII upon association with two different integrins which share the same α partner, α_v, may cause significant variations in the downstream signaling [69].

In our study, the relevance of $\alpha_v\beta_6$-TβRII association lies on the effect of TGFβ1 on MMP2 production and consequent MMP2-dependent cell migration. Our results highlight a specific function of $\alpha_v\beta_6$ integrin in upregulating MMPs, and for the first time the requirement of integrins in the TGFβ1/MMP pathway. Other studies have shown that *in vitro* integrins upregulate MMPs [7, 70] with apparent discrepancies attributed to differences in cancer cell types, and that stimulation of TGFβ1 induces secretion and activation of MMP2 [71, 72] as well as increased half-life of MMP2 mRNA [32]. However, a selective upregulation of MMP2 mediated by integrins upon TGFβ1 stimulation had not been previously shown. Noteworthy is the evidence that *ex vivo* primary cultures of breast cancer cells produce mature form of MMP9 when expressing activated $\alpha_v\beta_3$ integrin [73]. We conclude that the increased levels of MMP2 facilitate cell migration regulated by $\alpha_v\beta_6$ expressing cells and are likely to recapitulate previous effects observed *in vivo* where cancer cells were shown to cause osteolytic lesions [52] or metastasize to a higher extent when expressing $\alpha_v\beta_6$ [11]. We speculate that this pathway may be shared by other integrins, such as $\alpha_v\beta_3$, whose interaction with TβRII results in enhanced EMT, invasion [69] and proliferation [65] in a TGFβ1-dependent manner. Overall, these studies and our analysis suggest that MMP regulation by integrins is likely to be relevant to human cancer progression to a metastatic phenotype.

Our results show a direct correlation between MMP2 and TIMP2 expression which appears to be regulated by $\alpha_v\beta_6$ upon TGFβ1 stimulation. These data are in agreement with a previous study that showed MMP2 and TIMP2 co-expression in prostate adenocarcinoma [74], although increased TIMP2 expression is usually associated with decreased tumor growth, invasiveness and metastasis in PrCa [75]. A direct or inverse correlation between MMP2 and TIMP2 expression appears to be organ-site specific; indeed, the correlation has been shown to be direct and to predict poor prognosis in studies related to renal cell carcinomas and bladder cancer [76, 77], but to be inverse in endometrial carcinoma [78]. It should be stressed that MMP2 is known to be activated on the cell surface by forming a complex with TIMP2, which functions as inhibitor of MMPs but is also required for pro-MMP2 activation [79], and with MT1-MMP. We speculate that this migration promoting activity occurs through specific induction of MMP2 and TIMP2 without any change in MT1-MMP levels, as seen in Figure 1C. Moreover, MMP2 enzymatic activity is known to be controlled by its binding to the cell surface $\alpha_v\beta_3$ integrin [80]. This previous observation

explains why this membrane-bound active MMP2 is detected in whole cell lysates as also described by another study showing its accumulation in the intracellular vesicles of endothelial cells [81].

The relevance of our results is also shown by the signaling pathway activated by $\alpha_v\beta_6$, which requires activation of Smad3 for the observed increase in MMP2 levels as evaluated using SIS3, an inhibitor of Smad3 phosphorylation [45]. Smad3 has been shown to be overexpressed in human PrCa, and may contribute to disease progression in humans [24]. It remains to be established whether another Smad, Smad4, which also contributes to TGFβ1 induction of MMP2 (data not shown), is also regulated by $\alpha_v\beta_6$. This signaling cascade appears consistent with a previously described pathway whereby a different $\alpha_v\beta_3$ integrin elevates levels of phosphorylated Smad5 in a Runx2-dependent manner in PrCa cells [82]. Overall, Gupta et al.'s study and our analysis allow us to conclude that differential integrin expression regulates specific downstream TGFβ signaling. In conclusion, in our model (Figure 8), we propose that expression of $\alpha_v\beta_6$ integrin by associating with TβRII promotes Smad3-mediated downstream signaling pathway and consequent upregulation of MMP2 and MMP2-dependent cell migration in response to TGFβ1.

Acknowledgments

We thank Drs. M. Trerotola and B. Zerlanko in Dr. Languino's laboratory for constructive discussion. We also thank Drs. L.W. Chung for providing PC3-high cells; I. Hart for pBABE-β_6 integrin construct; S.W. Hayward for providing the BPH cell line.

FUNDING

This work was supported by the following grants: NIH-R01 CA89720, CA109874 and PO1 CA140043-Project 2 (L.R. Languino). This project is also funded, in part, under a Commonwealth University Research Enhancement Program grant with the Pennsylvania Department of Health (H.R.); the Department specifically disclaims responsibility for any analyses, interpretations or conclusions. C. F. was supported in part by an American-Italian Cancer Foundation Post-Doctoral Research Fellowship. Research in this publication includes work carried out using the Sidney Kimmel Cancer Center *Cancer Genomics* and *Flow Cytometry* Shared Resources, which is supported in part by NCI Cancer Center support grant P30CA056036. Shelia Violette is an employee and shareholder of Biogen, Cambridge, MA.

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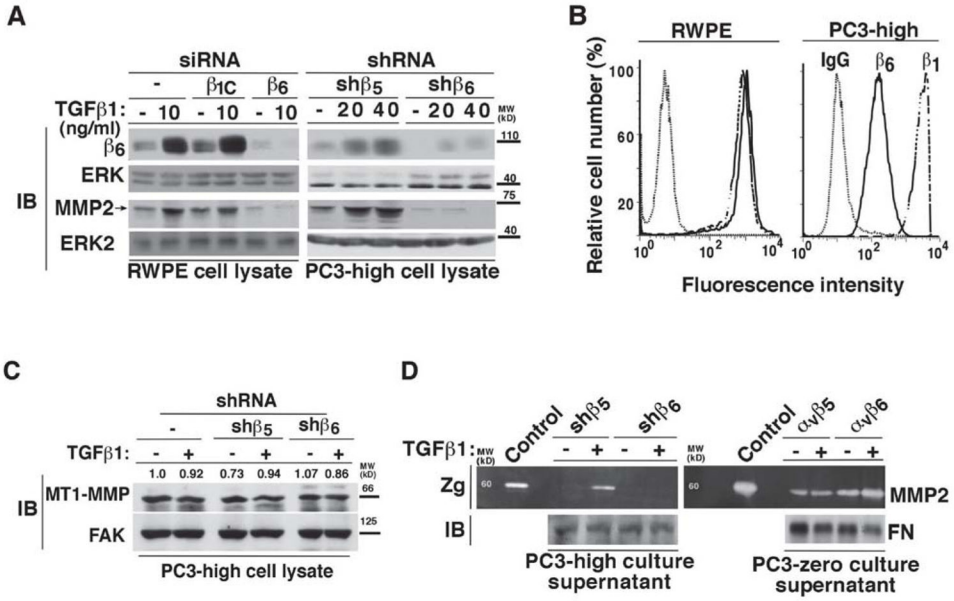

Figure 1. $\alpha_v\beta_6$ is required for TGFβ1 induction of MMP2

(**A**) RWPE cells (left panels) transiently transfected with 100 nM β_{1C} siRNA, β_6 siRNA or non- transfected, were serum starved for 24 h, before being stimulated with 10 ng/ml TGFβ1 for 48 h. Cell lysates were analyzed by IB and probed with 2A1 Ab to β_6 or an Ab to MMP2. ERK was used as a loading control. PC3-high cells (right panels) stably transfected with β_5-shRNA (shβ_5) or β_6-shRNA (shβ_6) were serum starved for 24 h; then stimulated with 20 or 40 ng/ml TGFβ1 for 48 h. Cell lysates were analyzed by 10% SDS-PAGE, under non-reducing conditions and probed with 2A1 Ab to β_6, or under reducing conditions and probed with an Ab to MMP2. ERK was used as a loading control. (**B**) FACS analysis of β_6 (solid line) in RWPE and PC3-high cells is shown. β_1 (broken line) was used as positive control for each cell line. Dotted-lines represent staining with an isotype negative control Ab. (**C**) PC3-high cells, stably transfected with β_5- shRNA (shβ_5) or β_6-shRNA (shβ_6) were serum starved for 24 h; then stimulated with 20 ng/ml TGFβ1 for 48 h. Cell lysates were analyzed by 10% SDS-PAGE, under reducing conditions and probed with MT1-MMP Ab. FAK was used as a loading control. (**D**) MMP2 activity in culture supernatants of shβ_5- or shβ_6-PC3-high cells (left panels) and $\alpha_v\beta_5$- or $\alpha_v\beta_6$-PC3-zero (right panels) cells was analyzed by gelatin zymography (Zg). Cells were either stimulated by TGFβ1 (20 ng/ml) or left resting for 48 h. Serum-free culture medium from BPH1 cells was used as a control for MMP activity. Fibronectin (FN) protein levels, analyzed by IB, were used as a loading control.

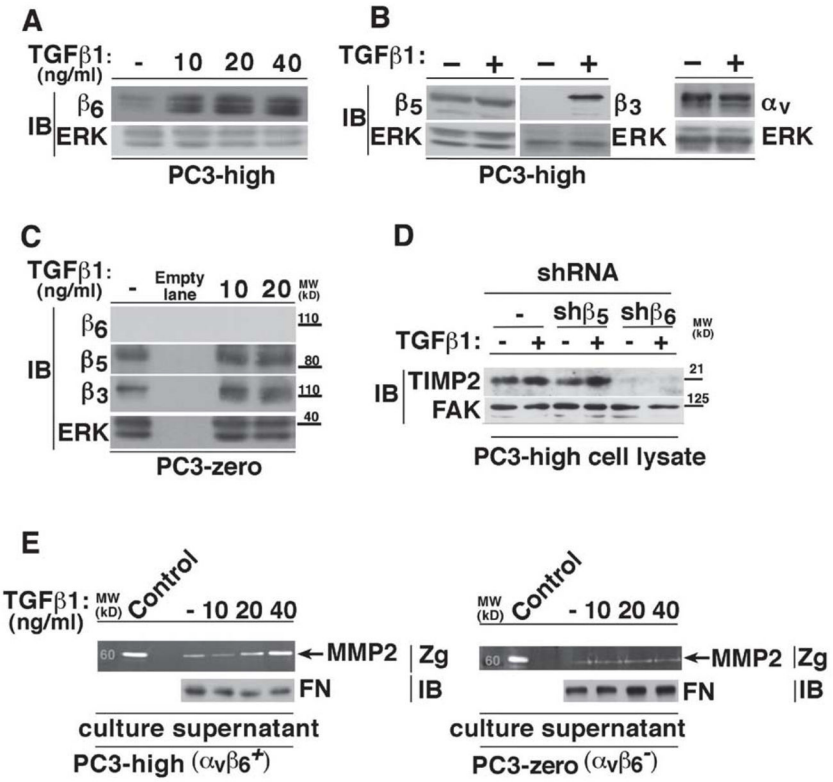

Figure 2. TGFβ1 upregulates MMP2 in $\alpha_v\beta_6^+$ - cells

(**A-B**) PC3-high cells were serum starved for 24 h; then stimulated with increasing concentrations of TGFβ1 for 48 h. PC3-high cell lysates were analyzed by IB and probed with 2A1 Ab to β_6 (10% SDS-PAGE under non-reducing conditions) (**A**) or with an Ab to β_5 (left panel; 10% SDS-PAGE under reducing conditions), to β_3 (middle panel; 10% SDS-PAGE under reducing conditions) or to α_v (right panel; 7.5% SDS-PAGE under non-reducing conditions) (**B**). ERK was used as a loading control. (**C**) Cell lysates from 10, 20 ng/ml TGFβ1 (48 h) stimulated or unstimulated PC3-zero cells were analyzed by 10% SDS-PAGE and probed with 2A1 Ab to β_6 (under non-reducing conditions), an Ab to β_5 or to β_3 (under reducing conditions). ERK was used as loading control. (**D**) PC3-high cells stably transfected with β_5-shRNA (shβ_5) or β_6-shRNA (shβ_6) were serum starved for 24 h; then stimulated with 20 ng/ml TGFβ1 for 48 h. Cell lysates were analyzed by 10% SDS-PAGE, under reducing conditions and probed with TIMP2 Ab. FAK was used as a loading control. (**E**) PC3-high ($\alpha_v\beta_6^+$; left panel) and PC3-zero ($\alpha_v\beta_6^-$; right panel) cells were serum starved for 24 h; then stimulated with 10, 20, 40 ng/ml TGFβ1 for 48 h or unstimulated (-). MMP2 activity in serum-free culture supernatants was analyzed by gelatin zymography (Zg). Serum-free culture medium from BPH1 cells was used as a control for MMP activity. Fibronectin (FN) protein levels, analyzed by IB, were used as loading controls.

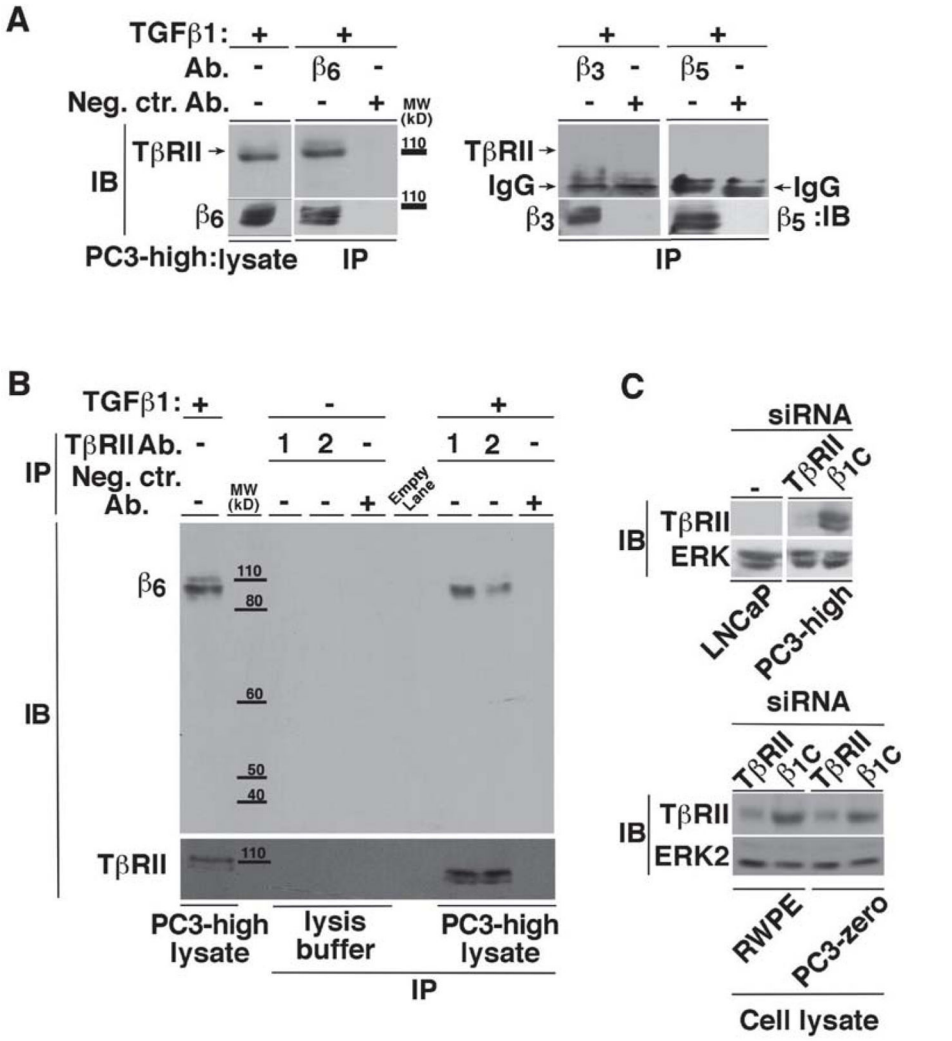

Figure 3. $\alpha_v\beta_6$ specifically interacts with TβRII

(**A**) $\alpha_v\beta_6$, $\alpha_v\beta_3$ or $\alpha_v\beta_5$ integrins were immunoprecipitated from 20 ng/ml TGFβ1 stimulated (48 h) PC3-high cell lysates and the immunoprecipitates were analyzed by 10% SDS-PAGE (under non-reducing conditions, left panels; or under reducing conditions, right panels) in order to detect TβRII. Mouse or rabbit IgG was used as a negative control (Neg.Ctr.) Ab. β_6 (left panel), integrin expression was analyzed in the immunoprecipitates by IB using 2A1 Ab to β_6. In the right panels an Ab to β_3 or β_5 was used. $\alpha_v\beta_6$ and TβRII expression was also detected in the cell lysate (left panel). (**B**) TβRII was immunoprecipitated from TGFβ1 stimulated PC3-high cell lysates using two different Abs against TβRII (1 and 2) and analyzed by 10% SDS-PAGE, under non-reducing conditions followed by IB to detect β_6. (**C**) PC3-high (top panels), RWPE and PC3-zero (bottom panels) cells were transiently transfected with β_{1C} or TβRII siRNA and the cell lysates were analyzed by 10% SDS-PAGE under non-reducing conditions and probed with rabbit Ab against TβRII. LNCaP cell lysate was used as a negative control. ERK was used as a loading control.

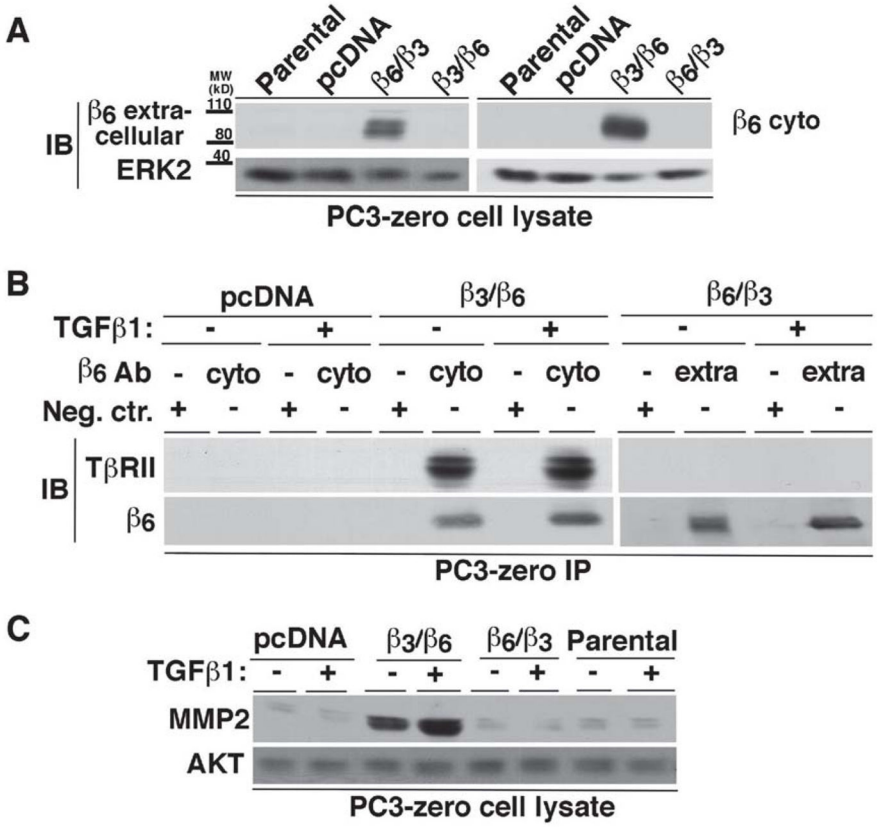

Figure 4. Association of TβRII with β_6 through the β_6 cytoplasmic domain induces MMP2

(**A**) PC3-zero cells were transfected as follows: pcDNA, β_6 extracellular and β_3 cytoplasmic (β_6/β_3) or β_3 extracellular and β_6 cytoplasmic (β_3/β_6) chimera. Non-transfected PC3-zero cells are designated as Parental. Expression of either extracellular (left panel) or cytoplasmic (right panel) domain of β_6 in Parental and PC3-zero transfectants was analyzed by IB. 2A1 Ab (10% SDS-PAGE under non-reducing conditions; left panel) was used to detect β_6 extracellular domain or C-19 Ab (10% SDS-PAGE under reducing conditions; right panel) to detect β_6 cytoplasmic domain. ERK was used as a loading control. (**B**) PC3-zero cells, transfected with pcDNA, β_6/β_3 integrin chimera or β_3/β_6 integrin chimera, were stimulated with 20 ng/ml TGFβ1 (48 h) or unstimulated. $\alpha_v\beta_6$ integrin was immunoprecipitated from lysates using either C-19 (which recognizes β_6 cytoplasmic domain (cyto); left panel) or 10D5 (specific for β_6 extracellular domain (extra); right panel) Ab. Goat IgG (left panel) or mouse IgG (right panel) was used as a negative control (Neg.Ctr.) Ab. The immunoprecipitates were analyzed by IB in order to detect TβRII. β_6 integrin expression was also analyzed in the immunoprecipitates by using C-19 (10% SDS-PAGE under reducing conditions; left panel) or 2A1 (10% SDS-PAGE under non-reducing conditions; right panel) as described above. (**C**) PC3-zero cells were transfected with pcDNA, β_6/β_3 integrin chimera, β_3/β_6 integrin chimera or were non-transfected (Parental). Cells were serum starved for 24 h before being incubated with 20 ng/ml TGFβ1 for 48 h. Cell lysates were analyzed by 10% SDS-PAGE under reducing conditions and probed with an Ab to MMP2. AKT was used as a loading control.

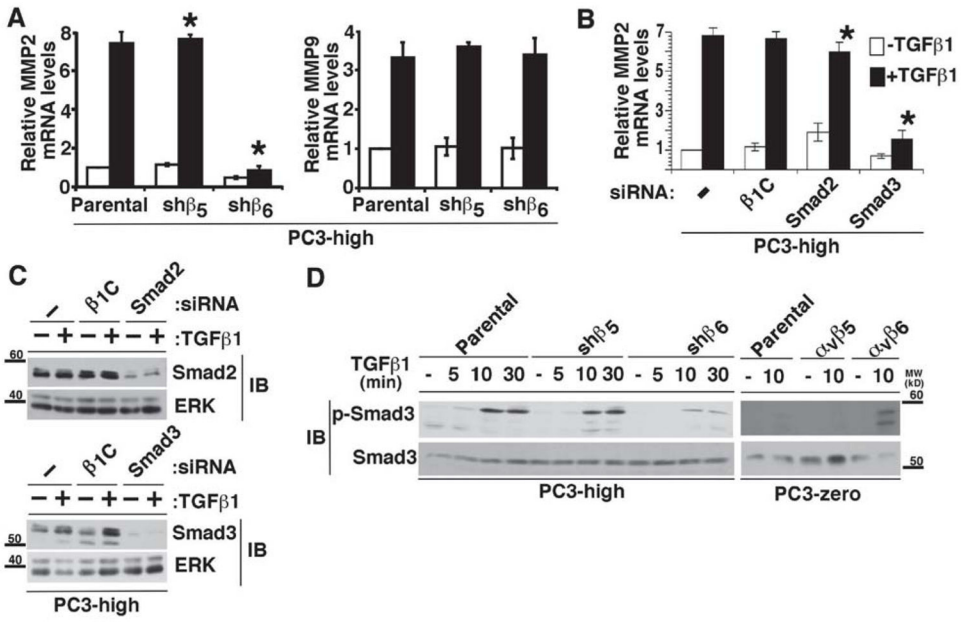

Figure 5. $\alpha_v\beta_6$ supports TGFβ1-induced Smad3 phosphorylation

(**A**) MMP2 (left) and MMP9 (right) mRNA levels were analyzed by qRT-PCR in Parental, shβ_5- and shβ_6-PC3-high cells. These cells were serum starved for 24 h, followed by incubation with or without 20 ng/ml TGFβ1 for 48 h. MMP2 and MMP9 mRNA expression levels were normalized to GAPDH. *, *P*=0.003. (**B–C**) PC3-high cells were transiently transfected with the following siRNAs: 100 nM β_{1C}, Smad2, Smad3 and starved for 24 h in serum-free medium before being incubated with 20 ng/ml TGFβ1 for 48 h. Cells were analyzed for MMP2 mRNA levels using total RNA by qRT-PCR; expression levels were normalized to GAPDH (**B**). *, *P*=0.014. Cells were also lysed (**C**) and analyzed by 10% SDS-PAGE under reducing conditions, followed by IB using an Ab to Smad2 or Smad3. ERK was used as loading control. (**D**) shβ_5-, shβ_6- and Parental PC3-high cells (left panels) were serum starved for 24 h, incubated with 20 ng/ml TGFβ1 for 0, 5, 10 or 30 min. Similarly, $\alpha_v\beta_5$ or $\alpha_v\beta_6$-transfected and Parental PC3-zero cells (right panels) were also serum starved for 24 h before being incubated with 20 ng/ml TGFβ1 for 0 or 10 min. Proteins in cell lysates were separated by 10% SDS-PAGE under reducing conditions and probed with Abs to phospho- Smad3 or Smad3.

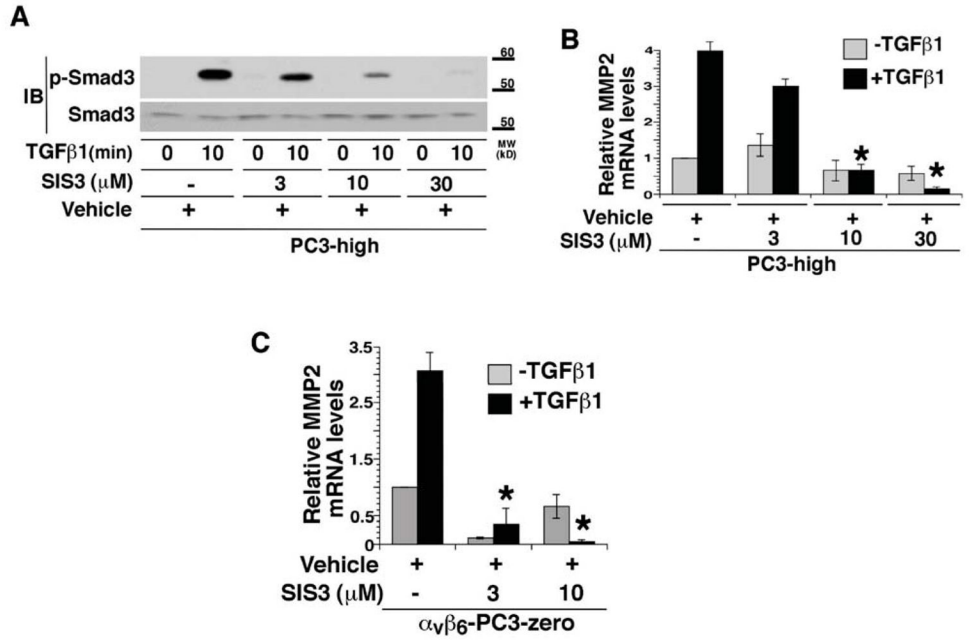

Figure 6. Smad3 phosphorylation is required for TGFβ1 induction of MMP2 mRNA

(**A–B**) PC3- high cells were serum starved for 24 h, pre-treated with 3, 10, 30 μM SIS3 or same volume of DMSO (vehicle) for 1 h before being incubated with 20 ng/ml TGFβ1 for 0 or 10 min. Cell lysates were analyzed by 10% SDS-PAGE under reducing conditions and probed with phospho-Smad3 Ab. Smad3 was used as loading control (**A**). MMP2 mRNA levels were analyzed by qRT-PCR (**B**). MMP2 mRNA expression levels were normalized to GAPDH. *, *P*=0.00078. **, *P*=0.00079. (**C**) $\alpha_v\beta_6$ stably transfected PC3-zero cells ($\alpha_v\beta_6$-PC3-zero) were pre-treated with 10 μM SIS3 or the same volume of DMSO (vehicle) for 1 h and incubated with 20 ng/ml TGFβ1 for 0 and 10 min. MMP2 mRNA levels were analyzed by qRT-PCR (**C**). MMP2 mRNA expression levels were normalized to GAPDH. *, *P*=0.0087. **, *P*=0.0079.

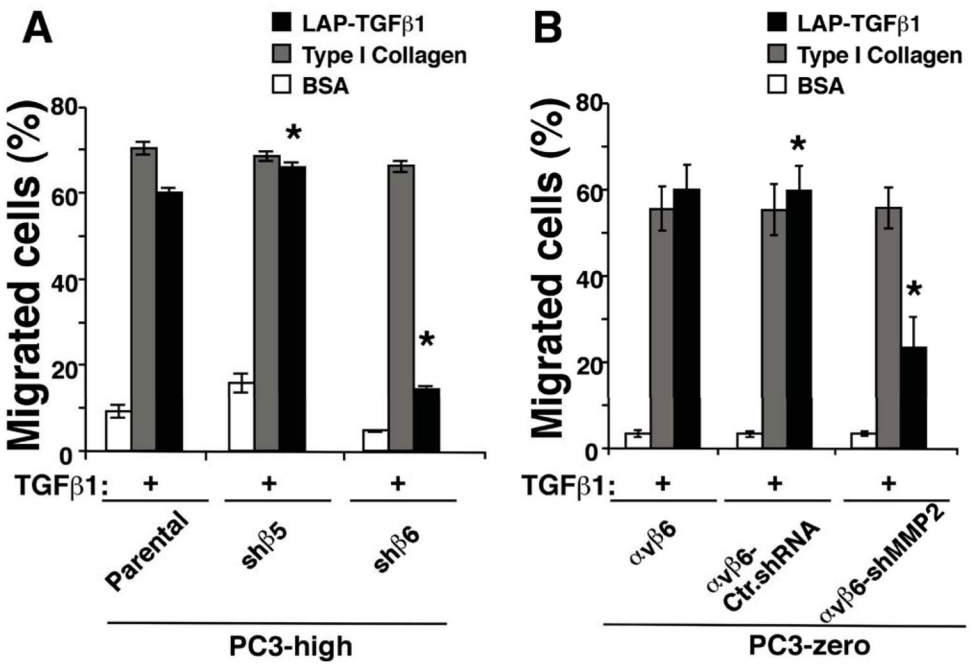

Figure 7. MMP2 promotes cell migration in $\alpha_v\beta_6$-expressing cells

Migration assays were performed using TGFβ1 pre-stimulated cells seeded on BSA, type I Collagen or LAP-TGFβ1-coated transwell chambers. Cells were allowed to migrate on different matrix ligands for 6 hr in the presence of TGFβ1. The differences in cell migration between $sh\beta_5$- and $sh\beta_6$-PC3-high cells (**A**) as well as between $\alpha_v\beta_6$-Ctr.shRNA-PC3-zero and $\alpha_v\beta_6$-shMMP2-PC3-zero (**B**) on LAP-TGFβ1 are statistically significant. *, P=0.004 and **, P=0.002.

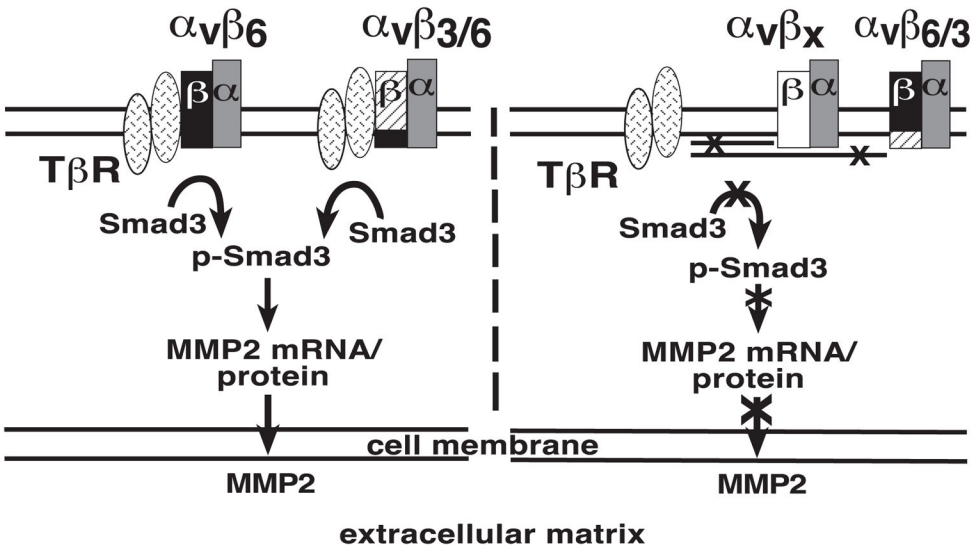

Figure 8. $\alpha_v\beta_6$ increases MMP2 levels through Smad3 activation

Our model shows that the $\alpha_v\beta_6$ integrin, through its cytoplasmic domain, interacts with TβR, initiates the Smad3-mediated downstream signaling cascade. As a consequence, tumor cells produce MMP2, which is released in the extracellular matrix (left). On the other hand, other related α_v-containing integrins, $\alpha_v\beta_x$, as well as the $\alpha_v\beta_{6/3}$ integrin, which contains a chimeric form of β_6 with a β_3 cytoplasmic domain, fail to show similar responses due to the lack of association with TβRII (right).