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Integrin-β5 and zyxin mediate formation of ventral stress fibers in response to transforming growth factor β

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Abbreviations: BMP, bone-morphogenic protein; EMT, epithelial to mesenchymal transition; ECM, extracellular matrix; PCR, polymerase chain reaction, TGF-β, transforming growth factor beta; TNFα, tumor necrosis factor alpha; VSF, ventral stress fibers

Cell adhesion to the extracellular matrix is an essential element of various biological processes. TGF-β cytokines regulate the matrix components and cell–matrix adhesions. The present study investigates the molecular organization of TGF-β-induced matrix adhesions. The study demonstrates that in various mouse and human epithelial cells TGF-β induces cellular structures containing 2 matrix adhesions bridged by a stretch of actin fibers. These structures are similar to ventral stress fibers (VSFs). Suppression of integrin-β5 by RNA interference reduces VSFs in majority of cells (>75%), while overexpression of integrin-β5 fragments revealed a critical role of a distinct sequence in the cytoplasmic domain of integrin-β5 in the VSF structures. In addition, the integrity of actin fibers and Src kinase activity contribute to integrin-β5-mediated signaling and VSF formation. TGF-β-Smad signaling upregulates actin-regulatory proteins, such as caldesmon, zyxin, and zyxin-binding protein Csrp1 in mouse and human epithelial cells. Suppression of zyxin markedly inhibits formation of VSFs in response to TGF-β and integrin-β5. Zyxin is localized at actin fibers and matrix adhesions of VSFs and might bridge integrin-β5-mediated adhesions to actin fibers. These findings provide a platform for defining the molecular mechanism regulating the organization and activities of VSFs in response to TGF-β.

Introduction

Cell adhesion to extracellular matrix (ECM) is essential for embryonic development, wound healing and human diseases, providing physical support and a signaling platform in cell migration, proliferation and differentiation.[1] Integrins are major cell-surface receptors mediating cell–matrix adhesions and adhesion-induced signaling, which are composed of trans-membrane α and β integrin subunits.[2,3] Upon engagement of integrin–matrix interactions, the cytoplasmic domains of integrin subunits facilitate assembly of large protein complexes called adhesomes. Expression of integrins and adhesome components are frequently elevated in aggressive tumors, rendering these proteins as attractive targets for cancer therapy.[3]

Transforming growth factor β (TGF-β) cytokines have emerged as major regulators of ECM and cell–matrix adhesions in normal and pathological conditions. TGF-β cytokines acting upon epithelial cells induce epithelial to mesenchymal transition (EMT), while fibroblasts respond with a myofibroblast conversion. Both processes are important for embryonic development, wound repair, tissue fibrosis, and cancer.[4,5] TGF-β regulates the composition of adhesion complexes including integrins and adhesome components,[6-10] as well as expression and deposition of matrix proteins, including fibronectin and collagens.[4,9,11] On the other hand, cell–matrix adhesions mediate activation of latent TGF-β cytokines deposited in matrix.[12,13] Better understanding of how TGF-β regulates the assembly of cell–matrix adhesions will benefit in development of more effective therapies.

EMT induced by TGF-β results in the disintegration of the polarized epithelial architecture and remodeling of the cellular filamentous structures such as actin filaments.[14-16] The formation of linear actin fibers linked to focal adhesions is a distinct feature of TGF-β-induced EMT in epithelial cells.[15-18] TGF-β-induced actin remodeling and formation of actin stress fibers in epithelial cells requires de novo protein synthesis[17] and activity of Rac1 GTPase.[19] Importantly, canonical TGFβ-Smad signaling mediates upregulation of actin-stabilizing proteins such as tropomyosin and α-actinin[17] and the αvβ integrins (αv, β1, β3, β5, and β6),[8] which contribute to EMT and cancer progression.[3,8,20,21] In particular, integrin-β5 chain is required for TGF-β-induced EMT[8] and myofibroblast conversion.[22] Recent studies have also

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implicated integrin-β5 in the tumorigenic and metastatic capacities of breast carcinomas[23] and pancreatic cancer cells.[24]

The cytoplasmic domain of integrins serves as an anchor for actin fibers and a platform for the assembly of adhesomes and adhesion-mediated signaling.[25] Integrins lack enzymatic activity and promote intracellular signaling by recruiting and activating kinases such as focal adhesion kinase (FAK) and integrin-linked kinase (ILK).[2,26] A non-receptor tyrosine kinase FAK is activated at cell–matrix adhesions by auto-phosphorylation at Tyr397 and by Src or other tyrosine kinases phosphorylating FAK at several Tyr residues.[27] In particular, phosphorylation of Tyr861 by Src is important for integrin-β5-mediated signaling[28] and cell transformation by oncogenic Ras.[29] Breast tumors with high histologic grade and a triple-negative sub-type produce elevated levels of FAK.[30] Accordingly, integrin-β5-FAK-Src signaling contributes to tumorigenic potential of breast cancer cells.[23] In addition, integrin-β1 may also contribute to actin remodeling in response to TGF-β by mediating p38MAPK signaling.[21] Thus, integrins can mediate cell–matrix adhesion and actin remodeling induced by TGF-β, although the function of specific β-integrins and the organization of the TGF-β-induced actin fiber structures remain to be defined.

The present study investigates the molecular components of cell–matrix adhesions induced by TGF-β and examines the contribution of integrin-β5 in this response. The study shows that TGF-β cytokines induce actin fiber structures linked to 2 matrix adhesions, which are similar to ventral stress fibers (VSFs).[31-33] Knockdown of integrin-β5 disrupted TGF-β induction of these VSF structures in both human and mouse epithelial cells, while re-expression of full-length integrin-β5 restored VSFs. Complementation studies with integrin-β5-deletion constructs uncovered a critical role of the C-terminal 780–793 aa sequence of integrin-β5 in the formation of VSFs, cell adhesion, and signaling. In addition, this study identified important molecular and signaling components of TGF-β-induced VSFs.

Results

The induction of ventral stress fibers is specific to TGF-β cytokines

Formation of actin fibers is a major characteristic of TGF-β-induced EMT. Besides TGF-β, several other cytokines have been implicated in EMT in various cell systems.[4,34] Here, we compared the response to TGF-β-superfamily cytokines in mouse NMuMG mammary epithelial cells, an established model of cytokine-induced EMT.[14-16]

In untreated NMuMG cells, the continuous actin fibers (phalloidin, green) were observed at the cell–cell contacts, while vinculin (red), a focal-adhesion and actin-binding protein, was mainly located within the cytoplasm (**Fig. 1A**). Treatment with TGF-β1 disrupted this epithelial actin architecture and resulted in the formation of an extensive network of actin fibers and a focal localization of vinculin at the fiber endings (**Fig. 1A**). Three major types of actin fibers are recognized,[32,33] including (1) the arcs, containing no point of attachment to the matrix, (2) the dorsal fibers, with one point of attachment, and (3) the ventral fibers with 2 cell–matrix attachments (**Fig. 1E**). TGF-β1 induced in over 80% of cells the linear actin fibers with 2 cell–matrix attachments, indicating formation of ventral stress fibers (VSFs). Similar results were obtained in epithelial cell lines such as mouse kidney MCT cells, human breast MCF10A, and lung carcinoma A549 cells.[8,9,35] Next we examined the effects of tumor necrosis factor α (TNFα) and 2 members of TGF-β-superfamily cytokines, activin and bone-morphogenic protein-4 (BMP4), that have been also implicated in EMT.[34,36] None of the tested cytokines induced the disruption of the epithelial actin architecture, despite exhibiting specific activities: activin stimulated phosphorylation of Smad3 and downregulation of Id2, comparable to TGF-β1; BMP4 increased Id2, and TNFα increased Tnfaip3 levels (**Fig. 1B–C**). In comparison, the response to TGF-β3 was essentially indistinguishable from TGF-β1 (**Fig. S1**). Next, we examined phosphorylation of paxillin, an indicator of integrin signaling, that regulates cell–matrix adhesions and cell responses to ECM,[37] and TGF-β1 upregulates phospho-Tyr31-paxillin levels in various cell lines.[8,38] Both TGF-β1 and TGF-β3 induced phosphorylation of Tyr31-paxillin, while activin had no effect (**Fig. 1D**). Thus, the formation of actin fibers with 2 matrix adhesions, i.e., ventral stress fibers, and integrin signaling are specific features of EMT induced by TGF-β cytokine isoforms.

Integrin-β5 is essential for the formation of ventral stress fibers

Integrins mediate cell–matrix adhesions linked to actin fibers, and over 20 distinct integrin heterodimers could be generated by selective pairing of 18 α- and 8 β-subunits.[1] EMT induced by TGF-β requires de novo protein synthesis,[17] and TGF-β upregulates integrin αV and partnering β-subunits.[8] Here, we examined if depletion of integrin-β5 or -β1, 2 major β subunits in epithelial cells, would disrupt the TGF-β induction of VSFs. Knockdown of integrin-β1 by siRNA did not reduce TGF-β1-induced VSFs and phosphorylation of paxilin at Tyr31 (**Fig. 2A and B**). In contrast, depletion of integrin-β5 markedly reduced ventral fibers in over 75% of cells that was also associated with a reduction in phospho-Tyr31-paxilin levels (**Fig. 2A and C**). Similar results were obtained with MCF10A cells (**Fig. 2D and E**). Depletion of either β subunit does not affect the level and surface presentation of the other.[8,23] TGF-β-induced VSFs include one adhesion at the proximity of the cell nucleus and a second adhesion at the cell periphery (**Fig. 2A**, vinculin, red). The perinuclear adhesions were markedly reduced in integrin-β5-depleted cells: from 6–12 adhesions per cell in control to 1–3 in integrin-β5-depleted cells (**Fig. 2A**). Together these findings indicate a prominent role of integrin-β5 in TGF-β induction of VSFs, mediating formation of perinuclear adhesions.

Expression of integrin-β5 is sufficient to induce ventral stress fibers

The mechanism of TGF-β induction of VSFs was further elucidated in breast carcinoma MDA-MB-231 cells in which integrin-β5 was depleted with shRNA. These cells have been extensively characterized.[23] In control MDA-MB-231 cells, TGF-β1 stimulated VSFs within 24 h, whereas integrin-β5-depleted (shB5) cells did not show the presence of VSFs in basal and TGF-β-treated conditions (**Fig. 3A**). Depletion of integrin-β5 does not affect integrin-β1 protein levels and its presentation on the cell surface,[23] suggesting that integrin-β1 cannot compensate for integrin-β5 in VSF formation. The shB5 cells overexpressing full-length integrin-β5 (shB5-oeB5-FL) showed numerous actin fibers with vinculin-containing adhesions at both ends, a feature of VSFs (**Fig. 3A**, bottom panels). Next, we examined whether phospho-Tyr31-paxillin is localized to integrin-β5-mediated adhesions. In control MDA-MB-231 cells, TGF-β1 induced actin fibers with phospho-Tyr31-paxillin at both ends, while in the integrin-β5 expressing cells (shB5-oeB5-FL) phospho-Tyr31-paxillin was found at the ends of actin fibers even in the absence of the cytokine (**Fig. 3B**). Accordingly, phospho-Tyr31-paxillin levels were reduced in integrin-β5 depleted cells (shB5) and increased in integrin-β5-expressing cells compared with control (**Fig. 3C**). These findings show that integrin-β5 is necessary and sufficient for induction of VSFs and phosphorylation of paxillin at the VSF adhesions.

The requirement of integrin-β5 cytoplasmic sequences for ventral stress fibers

The C-terminal cytoplasmic domain of β-integrin subunits (the β-integrin tail) facilitates the assembly and clustering of multimeric focal-adhesion complexes or adhesomes.[1] **Figure 4A** shows the alignment of β-integrin cytoplasmic sequences along with the arrangement of interacting partners, including molecules linking β-integrin to actin-regulatory proteins and signaling components.[25] The conserved HDRREF motif is proximal

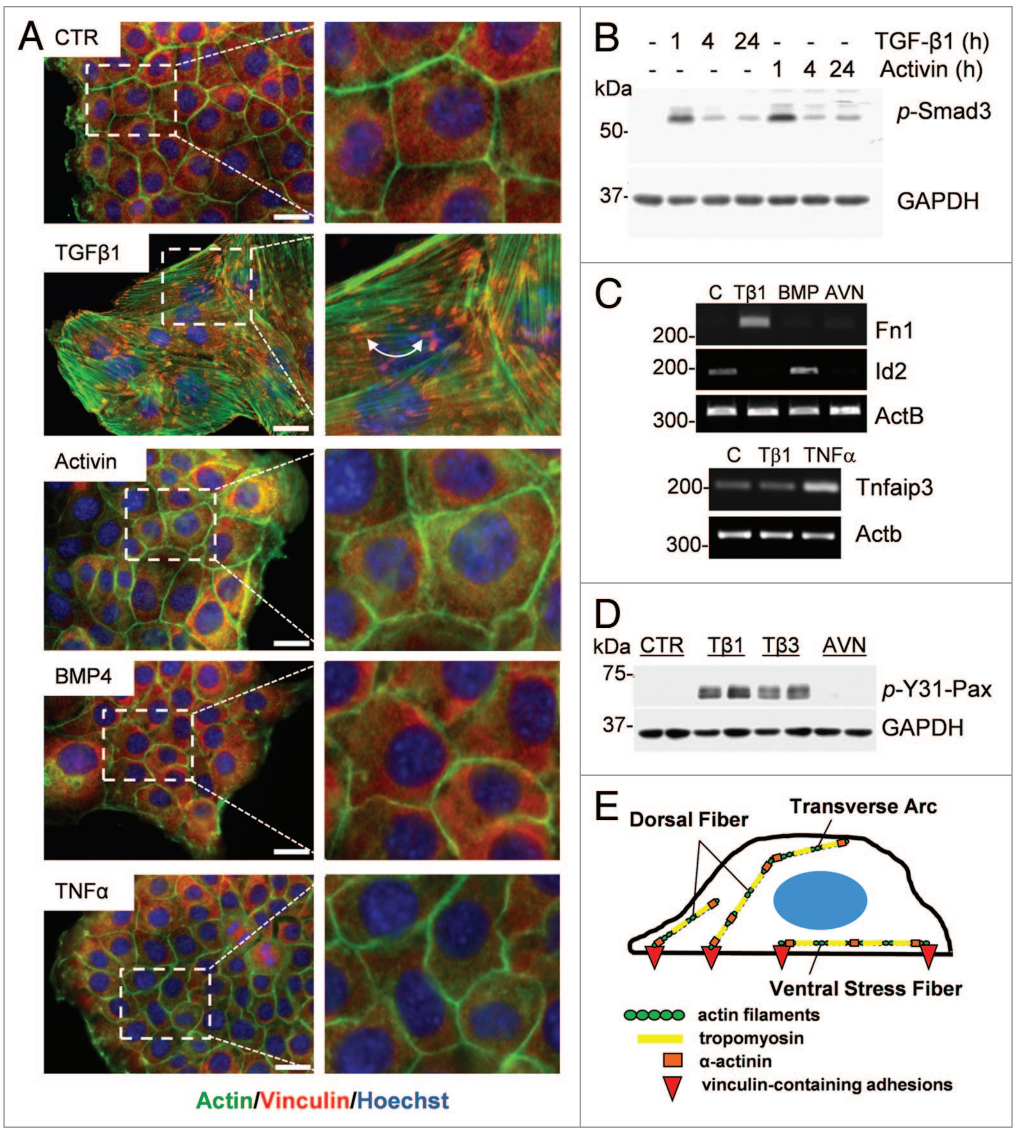

Figure 1. Induction of ventral stress fibers by TGF-β cytokines in epithelial cells. (**A**) NMuMG cells were treated with 2 ng/ml TGF-β1, 50 ng/ml BMP4, 50 ng/ml Activin, or 10 ng/ml TNF-α for 24 h. The overlay immunofluorescence microimages and their enlargements show actin filaments (phalloidin, green), focal adhesions (vinculin, red) and nucleus (Hoechst, blue). Scale bar, 20 μm. Linear actin stress fibers with 2 cell–matrix (focal) adhesions indicated by the arrows. (**B**) Immunoblotting with NMuMG cells treated with 2 ng/ml TGF-β1 or 50 ng/ml Activin for indicated time. Phospho-Smad3 indicates activation of Smad3, GAPDH was used as a loading control. (**C**) RT-PCR of fibronectin (Fn1), Id2, Tnfaip3, and Actb, a loading control, in NMuMG cells treated with TGF-β1, BMP4, Activin, or TNF-α. (**D**) Immunoblotting with NMuMG cells treated with 2 ng/ml TGF-β1, 4 ng/ml TGF-β3 or 50 ng/ml Activin for 24 h. (**E**) A schematic presentation of actin stress fibers with binding proteins (adapted from ref. 33).

to the membrane and interacts with α-subunit, FAK, and paxillin. The membrane-proximal NPXY and distal NXXY motifs are conserved among all β-integrin subunits and interact with phosphotyrosine-binding (PTB) domain-containing proteins, such as talin, tensin, kindling, and Shc. The human and mouse integrin-β5 includes 2 distinct features compared with β1 and β3-integrins: (1) a sequence separating the membrane-proximal NPXY and a distal NXXY motifs, and (2) 2 additional amino acids (aa) at the C terminus (**Fig. 4A**). To assess the contribution of various motifs in integrin-β5 to VSFs, we modified MDA-MB-231-shB5 cells by transducing EGFP-empty vector and shRNA-resistant integrin-β5 fragments: (1) the 1–793 aa (Δ793) fragment with a deletion of the last 6 aa disrupting the distal NXXY motif, (2) the 1–780 aa (Δ780) fragment, excluding the NXXY motif and a integrin-β5 unique sequence, and (3) the 1–772 aa (Δ772) fragment with deletion of both NPXY and NXXY motifs. Expression of constructs was confirmed by RT-PCR and by immunoblotting (**Fig. 4B and C**). Assessment by FACS of cell-surface presentation with the P1F6 antibody showed that all integrin-β5 constructs were expressed at comparable levels on the cell surface (**Fig. 4D**).

Fluorescence microscopy of integrin-β5-depleted cells (shB5) showed the presence of actin filaments in lamellopodia-like structures, while vinculin (red) was diffused within the cytoplasm (**Fig. 4E**), indicating a significant reduction in actin fibers and focal adhesions. Similar results were obtained in cells expressing the Δ772 and Δ780 constructs (**Fig. 4E**). In contrast, the Δ793 cells exhibited numerous actin fibers, with 2 adhesions analogous to the cells expressing a full-length construct (**Fig. 4E**), indicating that the HTVDFTFNKFNKS sequence (780–793aa) in integrin-β5 is required for the formation of VSFs, whereas the distal NXXY motif is dispensable.

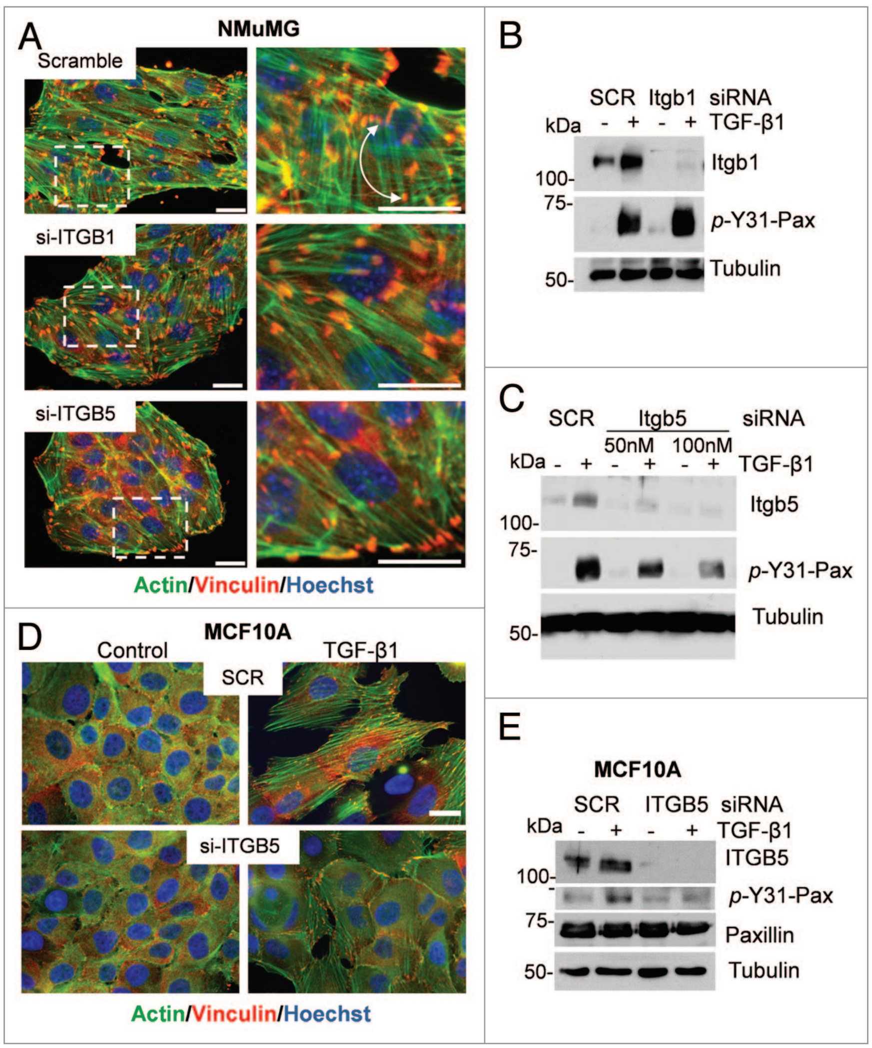

Figure 2. Depletion of integrin-β5 inhibits the TGF-β induction of ventral stress fibers. (**A–C**) NMuMG were transfected with siRNA to β1 or β5 integrins followed by treatment with TGF-β1 for 24 h. (**A**) Fluorescence overlay microimages and enlargements detect actin fibers with 2-point adhesions in scramble-control and β1-integrin depleted cells, whereas these structures are reduced in integrin-β5 depleted cells. (**B and C**) Immunoblotting for β1 or β5 integrins and phosphorylation of paxillin at Tyr31, GAPDH is a loading control. (**D and E**) MCF10A cells were transfected with siRNA to integrin-β5 followed by treatment with TGF-β1 for 48 h. (**E**) Immunoblotting for phospho-Tyr31-paxillin and integrin-β5. (**D**) Fluorescence microimages show actin filaments (phalloidin, green), focal adhesions (vinculin, red), and nuclei (hoechst, blue). Scale bar, 20 μm.

Integrin-β5-mediated adhesion and signaling

Depletion of integrin-β5 compromises the adhesive properties and colony formation of breast carcinoma cells.[23] These properties were examined in MDA-MB-231 cells expressing the integrin-β5 deletion constructs. As expected, cell adhesion to vitronectin, a cognate integrin-β5 substrate, was markedly reduced in integrin-β5-depleted cells (**Fig. 5A**, shB5). The Δ772 and Δ780 constructs only partially recovered adhesion on vitronectin, whereas the Δ793 fragment restored the adhesive capacity to the level of a full-length construct (**Fig. 5A**). Adhesion to fibronectin, for which integrin-β5 is not required, was not significantly affected by the constructs (data not shown). The clonogenic capacity of MDA-MB-231 cells was also reduced by integrin-β5 depletion (**Fig. 5B**, shB5). The Δ772 and Δ780 fragments did not recover the clonogenic capacity, whereas the Δ793 fragment conferred clonogenicity to the same extent as a full-length construct (**Fig. 5B**).

Next we examined whether adhesion on vitronectin would result in VSFs. Control MDA-MB-231 cells formed VSFs on vitronectin in the absence of TGF-β1, whereas the shB5 cells were unable to form VSFs even following exposure to TGF-β1 (**Fig. 5C and D**). This indicates that integrin-β1 cannot compensate for the loss of integrin-β5 in this activity, although integrin-β1 can mediate adhesion to vitronectin[3] and is upregulated by TGF-β1 in the shB5 cells.[23]

The contribution of integrin-β5 to adhesion-mediated signaling was assessed by comparing the signaling events in cells in suspension and after plating on cell culture dishes. Immunoblotting showed an increase in phosphorylation of Tyr397-FAK and Tyr31-paxillin in control cells after 2 h of plating, indicating activation of FAK and integrin signaling (**Fig. 5E**). These events were reduced in integrin-β5-depleted cells and in the Δ780 cells, whereas the Δ793 fragment increased phospho-Tyr397-FAK and phospho-Tyr31-paxillin levels (**Fig. 5E**). Thus, the C-terminal HTVDFTFNKFNKS sequence of integrin-β5 is required for VSF formation, adhesion-mediated signaling and clonogenic capacity of tumor cells.

Src activity and actin integrity is required for ventral stress fibers

To define the factors contributing to integrin-β5-mediated VSFs, we considered Src and the stability of actin fibers. Src could mediate phosphorylation of Tyr31-paxillin[23] and interact with the integrin-β5 tail.[1] Tropomyosin-promoted actin fibers enhance cell adhesion and phosphorylation of paxillin,[35] while suppression of tropomyosin hinders actin fibers and TGF-β1-induced EMT.[17] A role of Src in VSF formation was examined in MDA-MB-231 cells using Dasatinib, a Src inhibitor. Actin fibers (phalloidin, green) with 2-matrix adhesions (vinculin, red) were observed in control cells and were enhanced by TGF-β1, whereas Dasatinib completely dispersed these structures in both conditions (**Fig. 6A**). Immunoblotting showed a marked reduction in phospho-Tyr31-paxillin and phospho-Tyr861 levels by a Src inhibitor in MDA-MB-231 cells expressing a full-length integrin-β5 (**Fig. 6B**). Fluorescence microscopy was used to examine whether phospho-Tyr861-FAK is associated with TGF-β-induced VSFs. TGF-β1 increased phospho-Tyr861-FAK staining at the ends of actin fibers in the control cells, whereas this staining was absent in cells depleted for integrin-β5 (**Fig. 6C**), in agreement with a decrease in phospho-Tyr861-FAK levels in shB5 cells.[23] A contribution of actin filaments to the maintenance of adhesion sites was assessed using the actin-sequestration toxin latrunculin B in NMuMG cells that respond to TGF-β1 with robust levels of actin stress fibers (**Fig. 1**). Treatment with latrunculin B resulted in the disruption of TGF-β1-induced actin fibers and focal adhesions (**Fig. 6D**), as well as in the reduction of phospho-Tyr31-paxillin levels but did not affect vinculin and tropomyosin levels (**Fig. 6E**). This is consistent with downregulation of phospho-Tyr31-paxillin levels by tropomyosin depletion.[39] These observations show that Src activity and integrity of actin fibers are required for VSFs and adhesion-mediated signaling.

The role of zyxin in integrin-β5-mediated ventral stress fibers

The induction of actin fibers by TGF-β requires de novo protein synthesis and upregulation of actin-regulatory proteins including tropomyosin, α-actinin and calponin.[17] In addition, TGF-β1 regulates expression of proteins that could link actin filaments to integrin adhesions such as filamins A and B, dyxin, caldesmon, and zyxin.[39] Among these proteins, zyxin can localize to focal adhesions and actin fibers as well as physically interact with tropomyosin and α-actinin,[40] while caldesmon regulates actomyosin contractility. TGF-β1 induced caldesmon, zyxin and Csrp1, a zyxin-binding partner, in NMuMG cells (**Fig. 7A**). The induction was independent of de novo protein synthesis and was blocked by inhibition of transcription

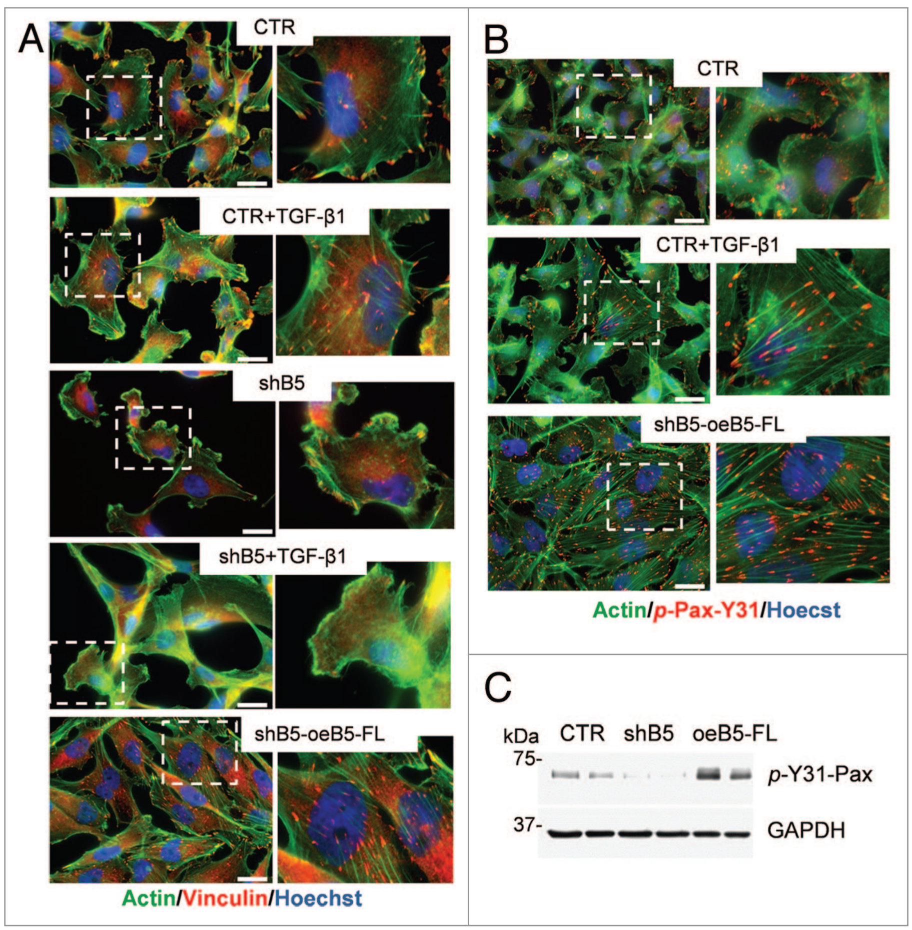

Figure 3. Modulation of the integrin-β5 expression affects ventral stress fibers and integrin signaling. (**A and B**) Control, shRNA-ITGB5 (shB5) and shB5 overexpressing a full-length human integrin-β5 (shB5-oeB5-FL) MDA-MB-231 cells were treated with 2 ng/ml TGF-β1 for 24 h where is indicated. Fluorescence microimages show actin filaments (phalloidin, green) and focal adhesions (vinculin, red) or phospho-Tyr31-paxillin (red), and cell nuclei (hoechst, blue). Scale bar, 20 μm. (**C**) Immunoblotting of phospho-Tyr31-paxillin and GAPDH (loading control) in MDA-MB-231 control (pSM2), shRNA-ITGB5 (shB5), and shB5-oeB5-FL cells. Forced expression integrin-β5 is sufficient to induce VSFs and phospho-Tyr31-paxillin.

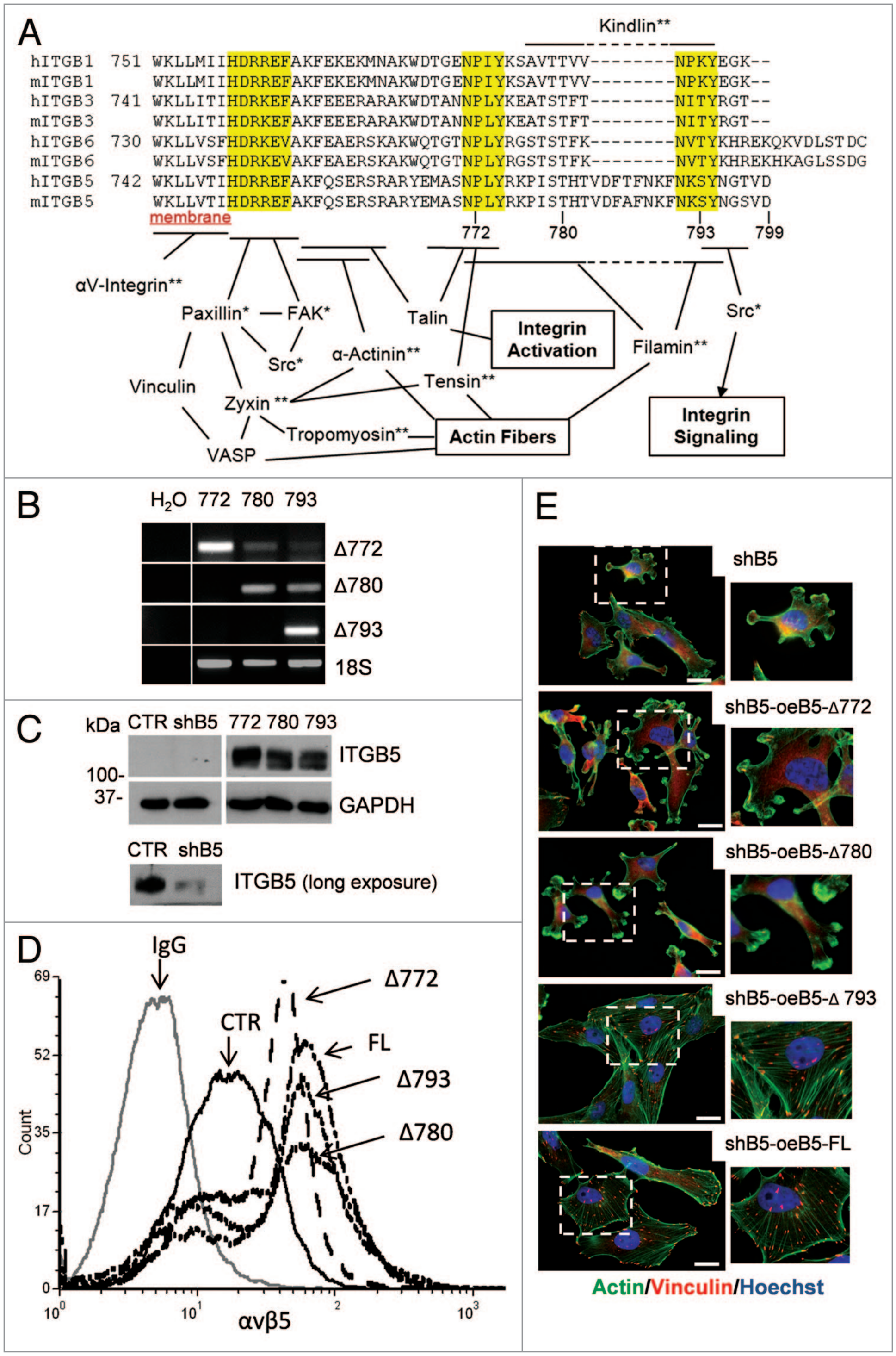

Figure 4. A specific sequence at the C-terminal domain of integrin-β5 is required for ventral stress fibers. (**A**) The scheme shows the C-terminal domain sequences of human and mouse β-integrin subunits and β-integrin interacting proteins. Asterisks indicate the TGF-β regulation of the activity or phosphorylation (*), and the expression (**) of interacting proteins. The conserved motifs are highlighted in yellow. Note the VDFTFNKFS sequence in integrin-β5 is not conserved among β-chains. This sequence splits the binding site for kindlin and filamin and spaces away a Src binding site from a FAK/paxillin interacting motif. (**B**) RT-PCR analysis of mRNA levels of integrin-β5 deletion constructs in MDA-MB-231-shB5 cells: (**A**) 1–772, Δ772; (**B**) 1–780, Δ780; (**C**) 1–793, Δ793. A common forward primer and a cloning reverse primer for each construct were used in PCR reactions. 18S rRNA was loading control. (**C**) Immunoblotting with cells overexpressing the integrin-β5 constructs with antibodies to integrin-β5 (Santa Cruz Biotechnology) and GAPDH as loading control. (**D**) Flow cytometry of cell-surface presentation of αvβ5 integrin in control (CTR) and shB5 cells expressing the integrin-β5 constructs (Δ772, Δ780, Δ793, and full-length). (**E**) Overlay images of actin filaments (green), vinculin (red), and nuclei (blue) in integrin-β5 depleted MDA-MB-231 cells (shB5) and cells expressing the integrin-β5 constructs. Scale bar, 20 μm.

with actinomycin D (**Fig. S2**). Smads are required for TGF-β-induction of actin stress fibers[17] and phosphorylation of Tyr31-paxillin.[39] Depletion of Smad4 blocked upregulation of caldesmon and zyxin at mRNA (**Fig. 7B**) and protein levels (**Fig. S2**). siRNA approach was used to assess the contribution of these proteins to adhesion-mediated signaling. Knockdown of zyxin in NMuMG cells markedly reduced tyrosine phosphorylation of proteins with molecular weight of 65–70 and 120 kDa, a signature of adhesion-mediated signaling and phosphorylation of paxillin and FAK.[8,39] Depletion of caldesmon and Csrp1 had no effect on tyrosine phosphorylation (data not shown). Next, we examined whether zyxin or caldesmon contribute to TGF-β1-stimulated cell–matrix adhesion. Depletion of zyxin blunted TGF-β1-induced cell adhesion to fibronectin (**Fig. 7D**) and to collagen and vitronectin (data not shown). Reduction of caldesmon had no effect on these TGF-β responses. To assess a link of integrin-β5 to zyxin, we examined zyxin location in control and shB5 MDA-MB-231 cells. In control cells, zyxin was found at the cell edges and the nucleus. TGF-β1 increased zyxin levels in the nucleus as well as zyxin localization to the

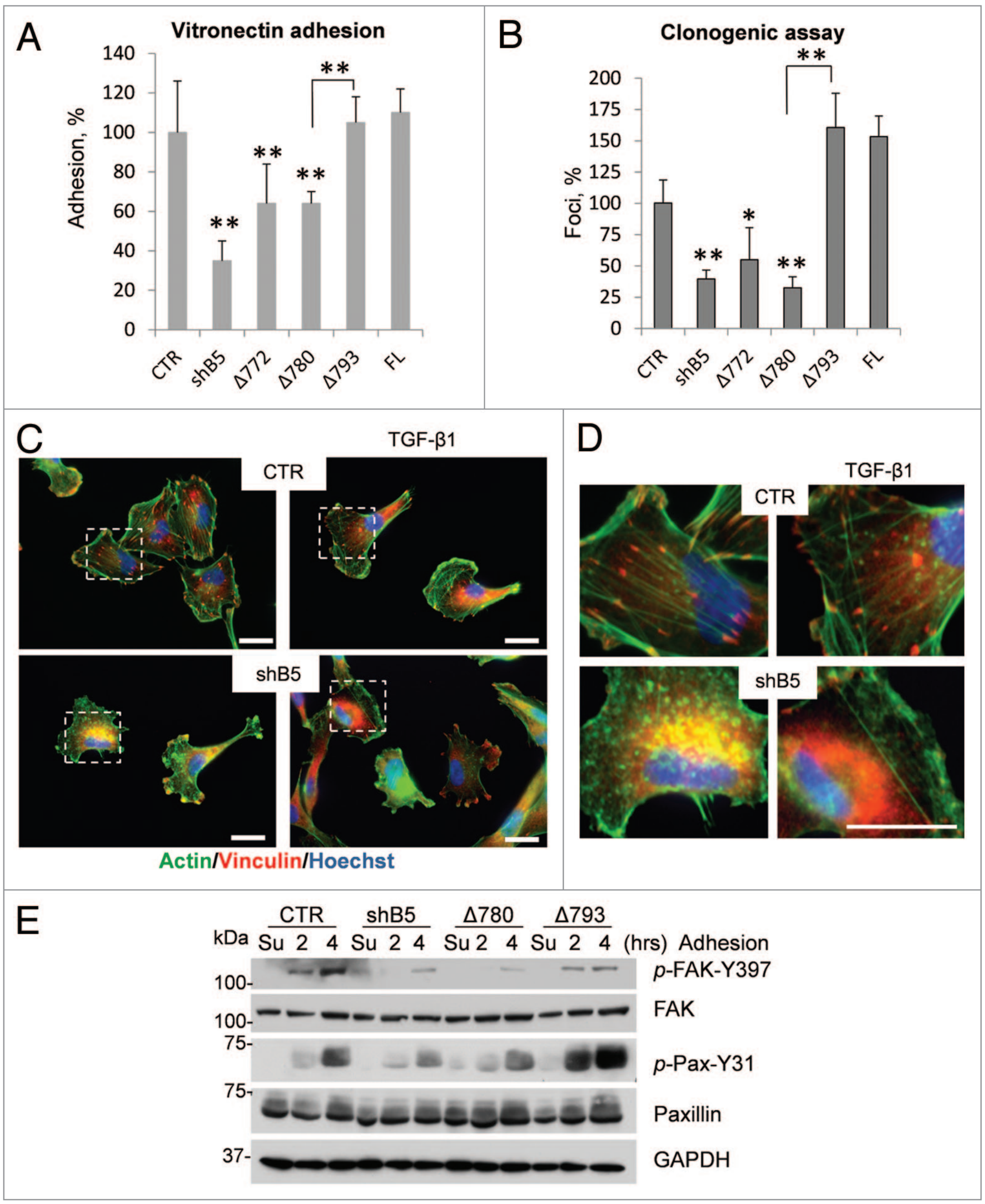

Figure 5. The HTVDFTFNKFNKS sequence of integrin-β5 is required for adhesion. (**A**) Adhesion to vitronectin-coated plates of MDA-MB-231 cells: control, shB5, and shB5 expressing the integrin-β5 constructs (Δ772, Δ780, Δ793, and full-length). Results are expressed as percentage of adhesion compared with control (**$P < 0.001$). (**B**) Colony-formation assays by MDA-MB-231 cells with modulated integrin-β5 as in (**A**). Experiments were performed in triplicates and repeated at least twice (*$P < 0.05$; **$P < 0.001$). (**C and D**) Fluorescence overlay images of control (CTR) and sh-ITGB5 (shB5) MDA-MB-231 cells cultured on coverslips coated with vitronectin and treated with TGF-β1 for 24 h. (**E**) Immunoblotting of control, shB5, Δ780, and Δ793 cells that were kept in suspension or after 2 or 4 h of adhesion to plastic. Membranes were probed for FAK, phospho-Tyr397-FAK, phospho- Ty31-paxillin, paxillin, and GAPDH as loading control.

ends of actin fibers, indicating a recruitment of zyxin to VSF adhesions (**Fig. 7E**). This is consistent with the ability of zyxin to shuttle between nucleus and cell adhesions.[40,41] In integrin-β5-depleted cells, zyxin was primarily in the nucleus and cell periphery (**Fig. 7E**). Thus, TGF-β-Smad signaling upregulates zyxin levels, while integrin-β5 facilitates zyxin localization to adhesion sites in VSFs, where zyxin contributes to cell–matrix adhesion and signaling.

Zyxin is an essential component of ventral stress fibers

To examine whether zyxin is required for the VSF formation, MDA-MB-231 cells were depleted for zyxin by siRNA and treated with TGF-β1 to induce ventral fibers. Knockdown of zyxin disrupted actin stress fibers with two-point adhesions and reduced phospho-Tyr31-paxillin levels (**Fig. 8A and B**). Likewise, knockdown of zyxin in MDA-MB-231 cells overexpressing integrin-β5 markedly reduced VSFs and phospho-Tyr31-paxillin levels (**Fig. 8C and D**). These findings demonstrate that zyxin is an essential component of VSFs and integrin-mediated signaling.

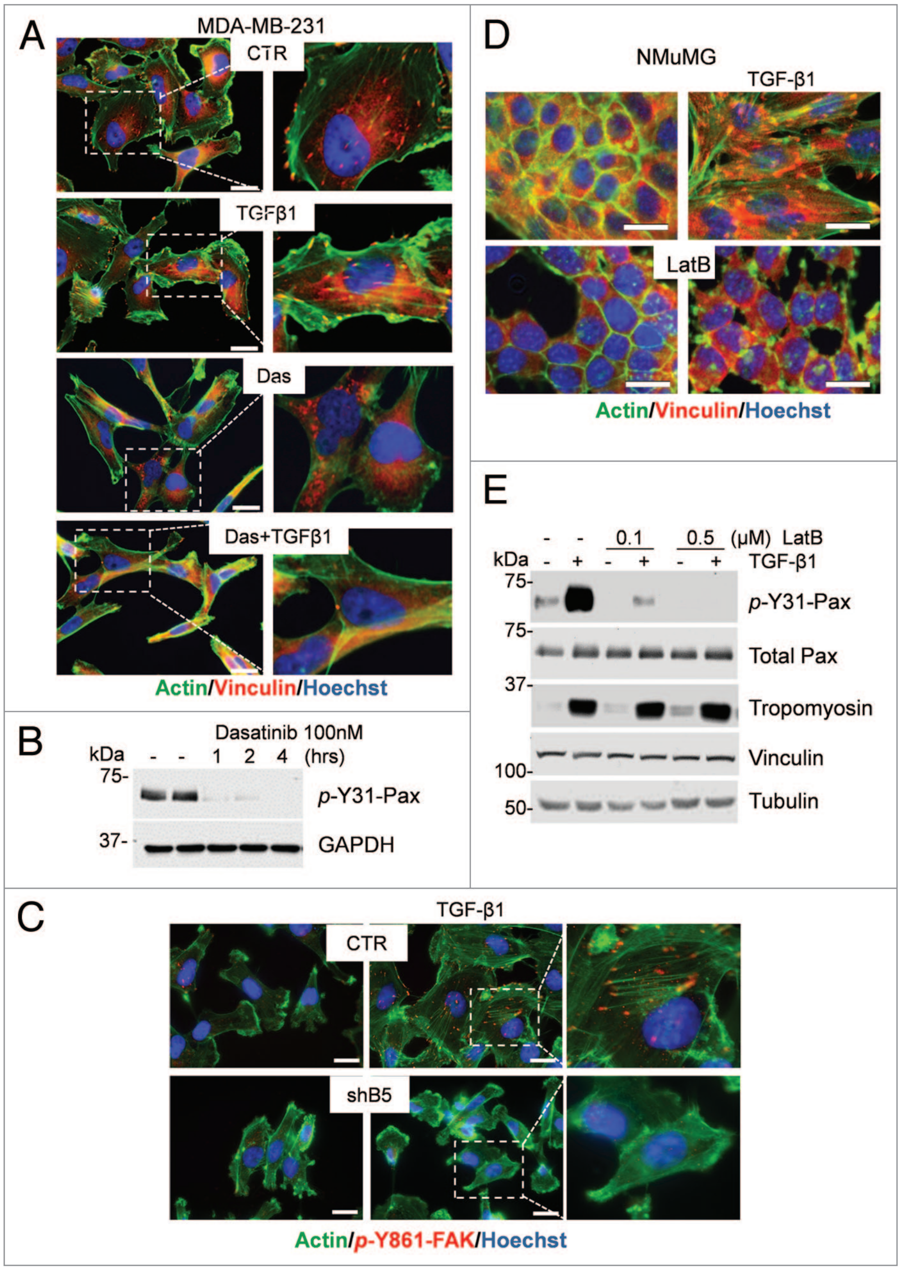

Figure 6. Activity of Src and the integrity of actin fibers in TGF-β-induced VSFs. (**A**) Fluorescence microimages of actin filaments (phalloidin, green) and focal adhesions (vinculin, red) in MDA-MB-231 cells treated with TGF-β1 for 24 h and dasatinib, a Src inhibitor. (**B**) Immunoblotting with MDA-MB-231-shB5-oeB5-FL cells treated with dasatinib. (**C**) Fluorescence microimages of actin filaments (phalloidin, green) and phospho-Tyr861-FAK (red) in control-pSM2 and shB5 MDA-MB-231 cells treated with TGF-β1 for 24 h. Enlargements of outlined areas are shown in a third row. Scale bar, 20 μm. (**D**) Fluorescence microimages of actin filaments (phalloidin, green) and vinculin (red) in NMuMG cells treated with TGF-β1 for 24 h and latrunculin B (LatB). Enlargements show the outlined areas in TGF-β-treated cells. Scale bar, 20 μm. (**E**) Immunoblotting with NMuMG cells treated with TGF-β1 for 24 h and latrunculin B.

Discussion

The current work demonstrates that TGF-β cytokines induce actin fibers with 2 cell–matrix adhesions, which are morphologically similar to ventral stress fibers (VSFs). The study revealed a critical role of integrin-β5 in the assembly of VSFs, whereas β1-integrin appears to be dispensable. A distinct sequence at the C terminus of integrin-β5 (marked red in **Fig. 9**) is required for formation of VSFs, contributing to the recruitment of structural and signaling components such as paxillin and FAK. The integrity of actin fibers and the kinase activity of Src are required for the assembly and maintenance of VSFs. TGF-β-Smad signaling upregulates actin-regulatory protein zyxin that contributes to VSF formation.

The induction of VSFs by TGF-β cytokines was observed in various epithelial cells, whereas neither activin nor BMP4 or TNF-α induced VSFs, consistent with previous studies for activin.[16] The TGF-β induction of VSFs occurs several hours following TGF-β addition to non-moving epithelial cells and requires de novo protein synthesis.[17] The TGF-β-induced VSFs contain one adhesion at the proximity of the cell nucleus and a second adhesion at the cell periphery, similar to ventral fibers in spreading cells. The mechanism of stress-fiber formation in spreading or moving cells involves 2 major steps: the establishment of focal adhesions and recruitment of actin and actin-regulatory proteins.[31] It is proposed that dorsal fibers are initiated in lamellipodia and then are slowly fused into ventral fibers. According to this idea, the cell–matrix adhesions in the perinuclear region of the cell originate in lamellipodia.[31-33] Rac1 appears to initiate dorsal stress fibers with 1 cell-matrix attachment, whereas RhoA mediates ventral stress fiber assembly and maintenance.[31] Recent study shows that dorsal fiber formation involves α-actinin,[42] which is a target of TGF-β-Smad signaling in epithelial cells.[17] Formation of stress fibers in TGF-β-induced EMT requires activity of RhoA[21] and Rac1.[19] Here, we report a major role of integrin-β5 in the formation of VSFs in mouse and human epithelial cells. Knockdown of integrin-β5 reduced VSFs, mainly affecting the formation of perinuclear adhesions, whereas suppression of integrin β1 had no effect on these structures. This is in agreement with inhibition of TGF-β-induced stress fibers in pulmonary endothelial cells by αvβ5 function-blocking antibodies.[43] Depletion of integrin-β5 does not affect expression of other β-subunits (β1, β3, and β6).[8,23] Importantly, re-expression of integrin-β5 restored VSFs and enhanced cell adhesiveness to matrix. Thus, integrin-β5 may mediate formation of perinuclear adhesions in TGF-β-induced VSFs, whereas the other β-subunits may contribute to some other aspects of EMT or myofibroblast conversion.[20,21,44]

The C-terminal HTVDFTFNKFNKS sequence of integrin-β5 is critical for the assembly of VSFs. This sequence may interact with a distinct set of proteins at the integrin-β5 adhesions, as it is absent in other β-subunits. Nearly 20 different proteins can bind directly to β-integrin tails, and over 200 proteins are found in the integrin adhesomes.[25] Our study identified vinculin, zyxin, phospho-Tyr31-paxillin, and phospho-Tyr861-FAK at the adhesions of the TGF-β-induced VSFs. Phosphorylation of these residues is mediated by Src and the activity of Src is required for VSF assembly. Both phospho-Tyr31-paxillin and phospho-Tyr861-FAK are present at focal and 3Dimensional (3D) adhesions but are reduced at fibrillar adhesions,[1] suggesting that integrin-β5 adhesions in the TGF-β-induced VSFs may have some features of focal and 3D adhesions.

TGF-β1 upregulates actin-regulatory proteins including tropomyosin and α-actinin that stabilize actin filaments and enhance cell–matrix adhesions.[17,35] Accordingly, actin-destabilizing agents or depletion of tropomyosin abrogates TGF-β-induced VSFs (**Fig. 6**; ref. 35). The current study showed upregulation of caldesmon, zyxin, and zyxin-binding protein Csrp1 by TGF-β-Smad signaling in epithelial cells. Depletion of zyxin blocks the TGF-β induction of VSFs, while suppression of caldesmon or Csrp1 had no effect. TGF-β via integrin-β5 facilitates zyxin localization at both actin fibers and adhesions sites of TGF-β-induced VSFs. In the focal adhesions, zyxin is positioned at the actin-regulatory layer and does not directly bind to the β-integrin tail.[45] The recruitment of zyxin to the adhesions could be mediated by integrin-binding proteins paxillin, filamin, or tensin. In turn, zyxin may link to actin filaments and actin-regulatory components such as vasodilator-stimulated phosphoprotein (VASP) and α-actinin.[1,25] The interaction of zyxin with

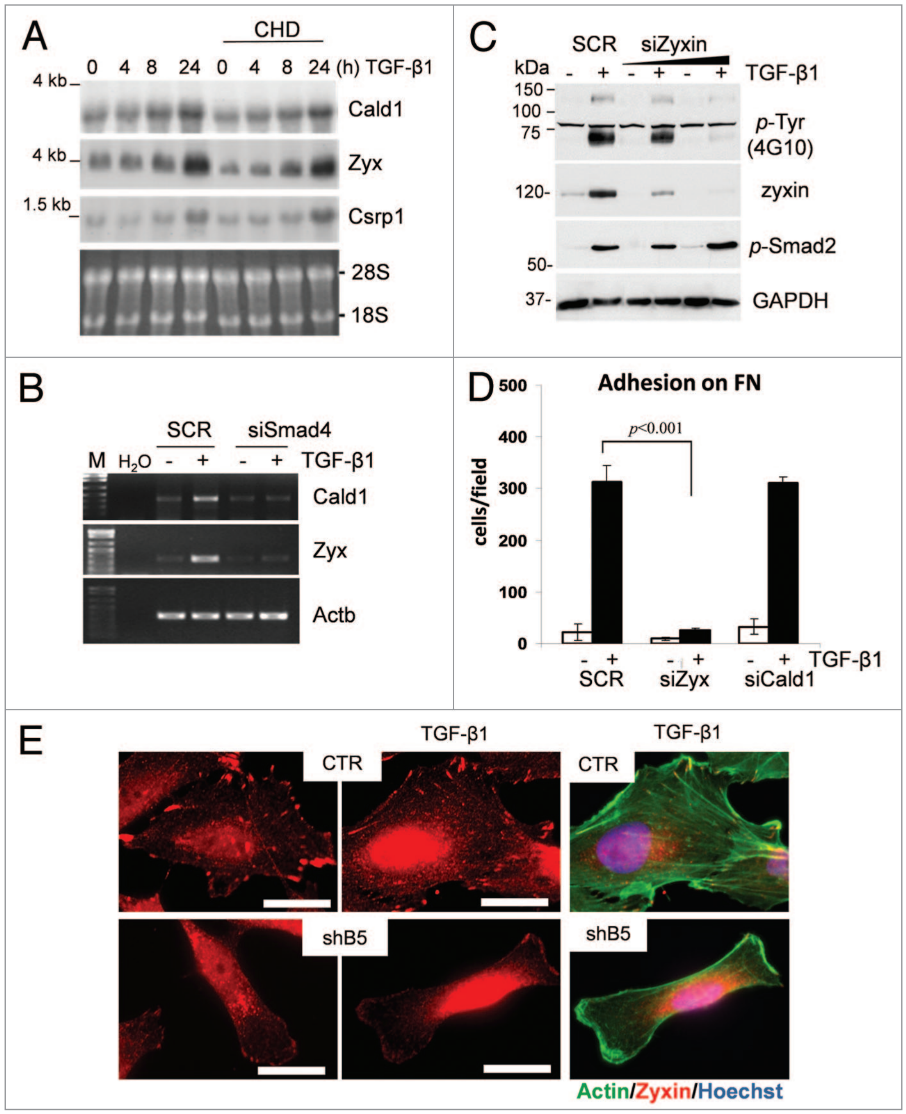

Figure 7. Zyxin is upregulated by TGF-β-Smad signaling and is localized to adhesions of ventral stress fibers. (**A**) Northern blot of total RNA from NMuMG cells treated with TGF-β1 for 24 h for caldesmon (Cald1), zyxin (Zyx), and Csrp1. Ethidium bromide staining is used as a loading control. (**B**) RT-PCR of caldesmon (Cald1), zyxin (Zyx), and actin-B (Actb) in NMuMG cells transfected with scramble-control or siRNA to Smad4 and treated with TGF-β1 for 24 h. (**C**) Immunoblotting with NMuMG cells transfected with scramble-control or siRNA to zyxin (100 and 200nM) and treated with TGF-β1 for 24 h. Membranes were probed with antibodies for zyxin, phospho-Smad2 and phospho-Tyr (4G10). (**D**) Adhesion to fibronectin-coated plates of NMuMG cells transfected with scramble-control or siRNA to zyxin. (**E**) Fluorescence microimages of control and shB5 MDA-MB-231 cells stained with antibodies to zyxin (red). Right panels show the overlay of phalloidin (green), zyxin (red) and cell nuclei (hoecsht, blue). Scale bar, 20 μm.

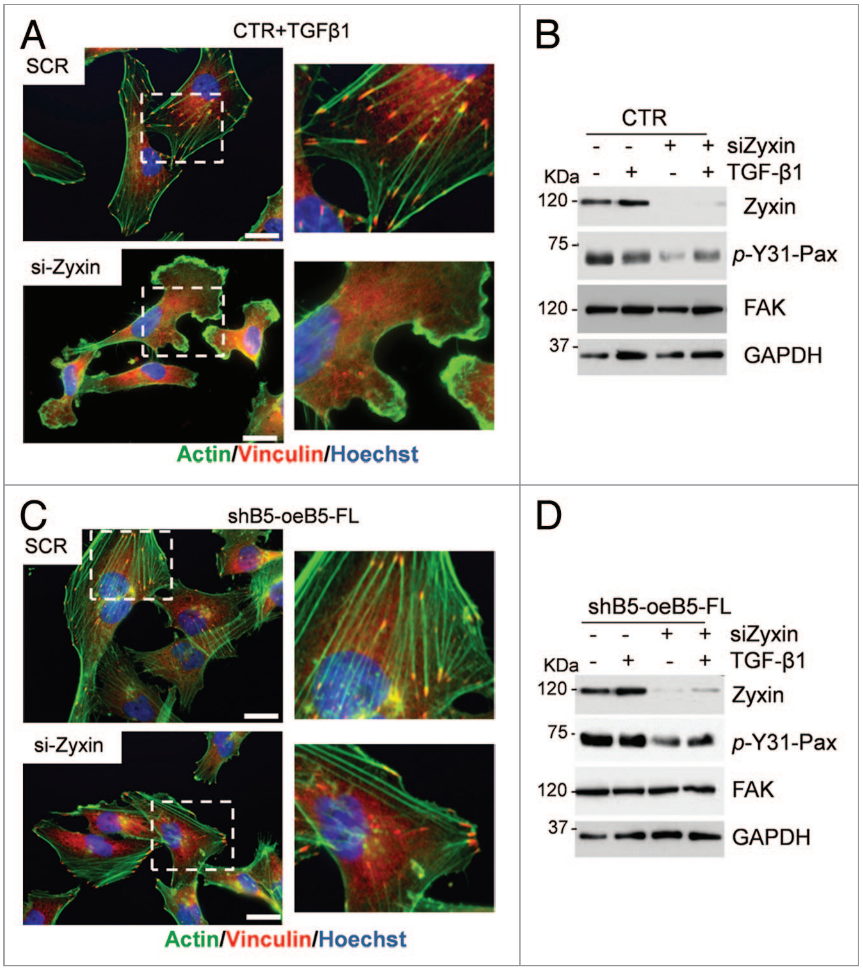

Figure 8. Depletion of zyxin impairs the formation of ventral SFs. (**A and C**) Fluorescence microimages of pSM2 and shB5-oeB5-FL MDA-MB-231 cells transfected with siRNA to zyxin. Actin filaments were visualized with phalloidin (green) and focal adhesions with antibody to vinculin (red). Cell nuclei were stained with hoechst (blue). Cells were treated with TGF-β1 for 24 h. Scale bar, 20 μm. (**B–D**) Immunoblotting of whole-cell lysates from MDA-MB-231 cells transfected with siRNA to zyxin and treated with TGF-β1 for 24 h. Membranes were probed with antibodies to zyxin, phospho-Tyr31-paxillin, FAK,g and GAPDH.

VASP and α-actinin could contribute to the stability of VSFs, as zyxin recruits these proteins to damaged stress-fiber sites for repair and maintenance of actin fibers.[46] In addition, zyxin may interact with kindlin/FERMT.[25] The kindlin and filamin binding sites are disrupted in the 780 fragment of integrin-β5 that is unable to restore VSFs, whereas the 793 construct includes these sites and restores VSFs (**Fig. 4**). The kindlin and filamin binding sites are intervened by the HTVDFTFNKFNKS sequence that is required for the VSF formation. As the paxillin, α-actinin, and tensin sites are included in both constructs, it is possible that zyxin interaction with kindlin and/or filamin could be critical for VSF formation. Intriguingly, filamin may compete with talin and kindlin for binding to β-integrin tail and regulate cell migration, adhesion, and deposition of fibronectin.[25,47] TGF-β upregulates filamin, kindlin, and fibronectin levels,[9,39] suggesting zyxin–filamin–kindlin interactions could regulate the dynamics of VSFs and matrix deposition. Zyxin may also have a specific function in the nucleus by regulating gene transcription directly or via interaction with transcription factors.[40]

In summary, the study identified integrin-β5 as a major component of specific cellular structures induced to TGF-β cytokines: actin stress fibers connected to 2 cell–matrix adhesions. The HTVDFTFNKFNKS sequence at the C terminus of integrin-β5 is required for the assembly of these structures, facilitating the recruitment to adhesion sites of structural and signaling components such as paxillin, zyxin, and FAK (**Fig. 9**). The integrity of actin fibers and the kinase activity of Src contribute to VSF assembly and maintenance. These findings provide a platform for defining the molecular mechanism regulating the organization and activity of TGF-β-induced VSFs as well as for developing therapeutic agents for treatment of cancer and human diseases associated with EMT and fibrosis.

Materials and Methods

Cell culture

Mouse mammary epithelial NMuMG, human mammary epithelial MCF10A, and human breast cancer MDA-MB-231 cell lines were obtained from American Tissure Culture Collection (ATCC). MDA-MB-231 and MCF10A cells were cultured as recommended by ATCC. NMuMG cells were cultured in Dulbecco modified Eagle medium (DMEM) (Invitrogen). Cells were kept at 37 °C under an atmosphere of 5% carbon dioxide (10% for MDA-MB-231). The medium was supplemented with 10% fetal bovine serum (FBS) or 5% horse serum (HS) for MCF10A (both were from Cellgro), 100 Units/ml penicillin and 0.1 mg/ml streptomycin (Invitrogen). The NMuMG media also contained 10 μg/ml insulin. MDA-MB-231 cells expressing shRNA to integrin-β5 and overexpressing integrin-β5 Δ772 and Δ780 constructs were cultured in tissue culture-treated flasks coated with collagen type I from rat tail (10 μg/ml) (BD bioscience).

Antibodies and other reagents

TGF-β1, -β3, BMP4, and activin were from R&D Systems; TNF-α was from Prospec. Vitronectin and fibronectin were from Sigma Aldrich. The following antibodies were used: mouse monoclonal to vinculin, tropomyosin (TM311), caldesmon, talin, and α-tubulin (Sigma); rabbit polyclonal to GAPDH, integrin-β5, integrin αv, and FAK (Santa Cruz Biotechnology); rabbit polyclonal to phospho-Y31-paxillin and phospho-Y861-FAK (BioSource International Invitrogen); mouse monoclonal to human integrin αvβ5, clone P1F6 (Millipore); rabbit polyclonal to phospho-Y397-FAK and Src, rabbit polyclonal to phospho-Smad3 (Cell Signaling); mouse monoclonal to paxillin and PE-conjugated rat to mouse IgG were from BD Biosciences. Phalloidins conjugated to Alexa-488 (green) and Alexa-568 (red) were from Molecular Probes. Goat-anti-mouse and goat-anti-rabbit antibodies conjugated to AlexaFluor 488 (green) and 568 (red) were from Invitrogen. Antibody 4G10 to phospho-Tyr was a gift from Dr Ray Mernaugh, (Vanderbilt University). Rabbit polyclonal to zyxin was a kind gift of Dr Mary Beckerle (University of Utah). Anti-rabbit or anti-mouse IgG antibodies conjugated to Horseradish Peroxidase (HRP) were from GE Healthcare. Cycloheximide was purchased from Calbiochem.

Short-interference RNA

Non-silencing scramble siRNA rhodamine-conjugated was from Qiagen. siRNA pools against human and mouse integrin β1 and β5, human zyxin were from Santa Cruz Biotechnology. Cells were transfected with 50–200 nM siRNA using Lipofectamine 2000 (Invitrogen) following the manufacturers protocol. The media was replenished 8 h after the transfection, and cells were grown for 1 d prior to further experiments.

Short-hairpin RNA

A retroviral vector encoding short-hairpin RNA (shRNA) to human integrin-β5 was obtained from Open Biosystems through the RPCI shRNA core facility. Retroviruses were prepared as described previously.[23]

Plasmid and retroviral constructs

Human integrin-β5 cDNA in a pCX-EGFP vector was a gift of Dr Raymond Birge (New Jersey Medical School). The integrin-β5 coding region (ORF) was shuttled into the retroviral pBMN-IRES-EGFP vector at BamHI and NotI restriction sites as previously described.[23] The integrin-β5 deletion constructs in the pBMN-EGFP vector were generated by PCR using high-fidelity Taq polymerase (Roche) and the primers containing BamHI (forward primer) or NotI (reverse primer) sites: FWD-BamHI (common for all): ACACAggatc c**ATG**C-CGCGG GCCCCGGCG; Δ772-REV-NotI: AGTCTgcggc cgc**TCA**TGGA TTTGAAGCCA TTTC; Δ780-REV-NotI: AGTCTgcggc cgc**TCA**CGTG GAGATAGGCT TTCT; Δ793-REV-NotI: AGTCTgcggc cgc**TCA**GGAT TTGTTGAACT TGTT. Retroviruses were prepared using Phoenix cells with 10 μg DNA per 100 mm dish and LipoD293 reagent (SignaGen Laboratories). Supernatants were passed through 0.4 μm filters and then used to infect MDA-MB-231 cells in the presence of 8 μg/ml Polybrene (Sigma). Three days later, GFP-positive cells were selected by flow cytometry.

Reverse transcription-PCR analysis

Cells were treated with 2–5 ng/ml TGF-β1 where it is indicated. Total RNA samples were prepared using the PerfectPure RNA purification system (5Prime) or with Trizol (Invitrogen). RNA pellets were resuspended in RNase-free distilled water and stored at –80 °C. First-strand cDNA synthesis was done using 1 μg of RNA in 20 μl with MMLV reverse transcriptase (Promega), according to the manufacturer's protocol. cDNAs were amplified with Go-Taq polymerase PCR system (Promega) using 2 μl of the cDNA reaction mix. The optimal number of the PCR cycles was determined for each primer set to ensure a linear range of amplification, typically 21–30 cycles.

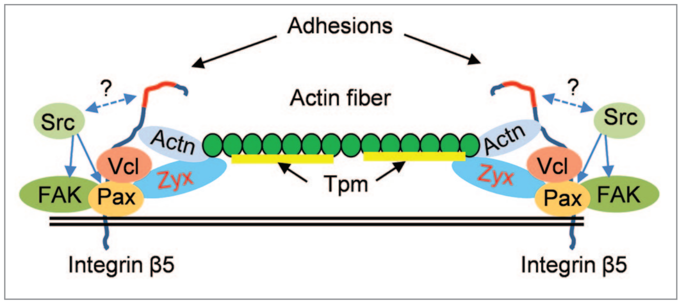

Figure 9. The model of ventral stress fibers mediated by integrin-β5. TGF-β promotes the formation of actin fibers with two matrix adhesions, i.e., ventral stress fibers. The cytoplasmic domain of integrin-β5 provides anchoring and scaffolding functions in adhesion complexes of VSF structures. A distinct sequence at the C terminus (red) as well as Src activity and the stability of actin fibers are required for VSF structures. Src phosphorylates FAK and paxilin (arrows). Tropomyosin (Tpm) and actinin stabilize actin filaments. Zyxin is upregulated by TGF-β and links adhesion complex to actin filaments.

Fluorescence microscopy

Cells were grown on glass coverslips (22 × 22 mm) untreated or coated with vitronectin or collagen type I (Sigma). Cells were treated with TGF-β1 (2–5 ng/ml) for the indicated time, fixed in 4% paraformaldehyde in PBS, and permeabilized with 0.05% Triton X-100 in PBS at room temperature (RT). After blocking with 3% milk in PBS, the cells were incubated for 1 h with antibody to vinculin (1/400 dilution), zyxin (1/500), phospho-Tyr31-paxillin (1/500), or phospho-Tyr861-FAK (1/250) in 1% milk in PBS, followed by incubation for 30 min with fluorescent secondary antibody (1/500) at RT. Actin filaments were stained with phalloidin-AlexaFluor-488 (1/200). Fluorescence images were captured using Plan Apo 60×/1.40 NA oil objective lens at ambient temperature using a Nikon TE2000-E inverted microscope equipped with a Roper CoolSnap HQ CCD camera (Photometrics). The images were acquired using MetaVue imaging software v7.3 (Molecular Devices).

Immunoblotting

Whole-cell extracts were prepared using NP40 lysis buffer containing 20 mM Tris, pH 7.4, 150 mM NaCl, 1% NP40, 10% glycerol, 20 mM NaF, supplemented with 1 mM Na orthovanadate, 1 mM PMSF, and a protease-inhibitor cocktail (Roche Molecular Biochemicals). Immunoblotting was done as described previously.[8]

Flow cytometry for surface expression

Cells were collected by mild trypsinization, washed once with PBS, and resuspended in cold FACS buffer (1× PBS, 2% FBS, 0.1% NaN_3, 1 mM $CaCl_2$, 1 mM $MgCl_2$). Cells were incubated for 15 min on ice with normal mouse IgG (200 μg/ml final) and transferred in 5 ml polystyrene round-bottom tubes (2 × 10^5 cells/tube). Cells were washed with FACS buffer, spun at 1200 rpm, 5 min, 4 °C and incubated with the primary antibody in 100 μl (P1F6, 1/250 dilution) for 30 min on ice. Cells were washed with PBS after each treatment. Cells were fixed with 2% PFA in PBS for 30 min on ice and incubated with the PE-conjugated secondary antibody (5 μg/ml) in 100 μl. Cells were resuspended in 1 ml of FACS buffer and 1 × 10^4 events were acquired with a

FacsCalibur instrument and analyzed by the FCS Express program (De Novo Software).

Cell adhesion

The 96-well plates were coated with 1 μg/ml vitronectin or 10 μg/ml fibronectin (both from Sigma) in PBS for 1 h at 37 °C and then blocked with 10 mg/ml BSA 30 min at 37 °C. Cells were seeded at 2×10^4 cells/well in 5% serum IMEM in 6 replicates. After 45 min, non-adherent and loosely attached cells were removed from the wells by gently washing with PBS. Attached cells were fixed and stained with 0.5% methylene blue in water/methanol (50:50) for 1 h at RT. After extensive rinsing in distilled water the plates were left to dry overnight. Cells were solubilized in 1% SDS in PBS for 10 min at RT, and the optical density of solutions was determined as the absorbance difference at 650 nm (methylene blue) and 540nm (background) using a SpectraMax fluorimeter (Molecular Devices LLC). The data were expressed as the mean value ± standard deviation.

Clonogenic assay

Cells (500 cells/well) were seeded in 6-well plates in complete media and grown for 12 d. Media was replenished every 3–4 d. Foci were fixed and stained with 0.5% methylene blue in water/methanol (50:50) for 1 h at RT, and the foci in each well were counted. Experiments were performed in triplicates and repeated at least twice. The data are expressed as the mean value ± standard deviation.

Statistical analysis

Experiments were repeated 2 or more times and data were compared using the Student *t* test or ANOVA. Statistical significance was accepted at *P* value ≤ 0.05.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Materials

Supplemental materials may be found here:
www.landesbioscience.com/journals/cc/article/26388

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