Combined Lysophosphatidic Acid/Platelet-Derived Growth Factor Signaling Triggers Glioma Cell Migration in a Tenascin-C Microenvironment

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Abstract

The antiadhesive extracellular matrix molecule tenascin-C abrogates cell spreading on fibronectin through competitive inhibition of syndecan-4, thereby preventing focal adhesion kinase (FAK) activation and triggering enhanced proteolytic degradation of both RhoA and tropomyosin 1 (TM1). Here, we show that simultaneous signaling by lysophosphatidic acid (LPA) and platelet-derived growth factor (PDGF) initiates glioma cell spreading and migration through syndecan-4–independent activation of paxillin and FAK and by stabilizing expression of RhoA, TM1, TM2, and TM3. By using gene silencing methods, we show that paxillin, TM1, TM2, and TM3 are essential for LPA/PDGF-induced cell spreading on a fibronectin/tenascin-C (FN/TN) substratum. LPA/PDGF-induced cell spreading and migration on FN/TN depends on phosphatidylinositol 3-kinase, RhoKinase, and mitogen-activated protein kinase/extracellular signal-regulated kinase kinase 1/2 but is independent of phospholipase C and Jun kinase. RNA microarray data reveal expression of tenascin-C, PDGFs, LPA, and the respective receptors in several types of cancer, suggesting that the TN/LPA/PDGF axis exists in malignant tumors. These findings may in turn be relevant for diagnostic or therapeutic applications targeting cancer. [Cancer Res 2008;68(17):6942–52]

Introduction

The extracellular matrix molecule tenascin-C is expressed in the stroma of most solid tumors. High expression of tenascin-C correlates with tumor progression of gliomas and other cancers (1) and is furthermore known as a pivotal marker of breast cancer metastasis (2). Tenascin-C stimulates tumor cell proliferation, invasion, angiogenesis, and suppression of the immune system (1, 3, 4); however, the underlying mechanisms of action are poorly understood. Tenascin-C inhibits cell spreading on fibronectin by two mechanisms. First, tenascin-C competes with syndecan-4

binding to fibronectin, which translates into enhanced proliferation of glioma cells (6), presumably through activation of Wnt and mitogen-activated protein kinase (MAPK) signaling (7). Second, tenascin-C induces endothelin receptor type A (EDNRA) expression, and EDNRA signaling maintains cell rounding on a fibronectin/tenascin-C (FN/TN) substratum. This is linked to inhibition of a minimal set of molecules, namely, focal adhesion kinase (FAK), RhoA, and tropomyosin 1 (TM1; ref. 8), which are crucial for cell spreading. Tenascin-C increases cell motility (9) and triggers glioma cell migration in an integrin $\alpha_2\beta_1$-dependent manner (10). Moreover, tenascin-C induced migration and invasion of colon carcinoma cells in conjunction with hepatocyte growth factor, which involves activation of epidermal growth factor receptor (EGFR) and Rac signaling (11).

Ligation of the integrin $\alpha_5\beta_1$ together with the proteoglycan syndecan-4 induces cell spreading on fibronectin (12). Integrins are linked to paxillin in complexes containing talin, FAK, among other molecules (13). Paxillin binds syndesmos, which itself interacts with the cytoplasmic tail of syndecan-4 (14), thus forming a molecular bridge between integrin $\alpha_5\beta_1$ and syndecan-4. Paxillin and FAK are critically involved in cell spreading and integrate signals from different transmembrane receptors, including integrins and the platelet-derived growth factor receptor (PDGFR; refs. 15, 16).

The small GTPase RhoA and its downstream targets RhoKinase (ROCK; ref. 17) and myosin light chain (MLC; ref. 18) are instrumental in actin polymerization, cell contractility, and migration. In addition, actin filaments are stabilized by TMs. Expression of the high molecular weight tumor suppressor–like TM1 and TM2 is down-regulated in cancer and on oncogenic transformation, and reexpression of TM1 causes reversion of the transformed phenotype (9, 19). Previously, we observed that TM1, TM2, and TM3, which form heterodimers, are down-regulated on a FN/TN substratum by a mechanism that involves down-regulation of TM2/TM3 mRNA levels, thus leading to proteasome-dependent degradation of TM1 heterodimers (8).

Signaling by lysophosphatidic acid (LPA) and PDGF is activated in human cancer (20–22) and in wound healing (23–25), where these factors seem to stimulate cell migration, as was shown in cultured cells (26, 27). Acquisition of a motile phenotype is a prerequisite for invasion and metastasis and thus important in promoting tumor progression. Tenascin-C, LPA, and PDGF also play a role in the brain stem cell niche as previously reported (28–30). PDGF and tenascin-C regulate oligodendrocyte progenitor cell (OPC) maintenance in the germinal subventricular zone (SVZ) of the brain, which is impaired in tenascin-C knockout mice (28). Tenascin-C stimulates PDGFRα expression in glioma cells (7),

whereas PDGFR signaling induces hyperproliferation and migration of stem cells in the SVZ (30). Tenascin-C (3), PDGF, and LPA (23–25) also regulate wound healing, again suggesting a potential synergistic effect of these factors on cell migration *in vivo*.

The aim of this work was to analyze expression of tenascin-C, PDGFs, LPA, and their receptors in cancer and to elucidate the mechanism by which PDGF and LPA affect tumor cell adhesion and migration on a tenascin-C substratum. Here, we show that signaling by PDGF-BB in conjunction with LPA blocks tenascin-C–induced cell rounding and induces cell migration by a mechanism that involves syndecan-4–independent activation of paxillin. A potential relevance of the identified mechanism in tumor progression is supported by high expression of tenascin-C, PDGF, LPA, and their receptors in several cancers as we deduced from RNA microarray analysis. Our results provide a novel mechanism by which tenascin-C promotes cell migration in conjunction with LPA/PDGF signaling. This mechanism might play an important role in wound healing, the brain stem cell niche, and in tumor progression.

Materials and Methods

Cell plating, inhibitor studies, and preparation of cell lysates. Human T98G glioblastoma and KRIB osteosarcoma cells, rat REF52 (American Type Culture Collection), and syndecan-4–deficient mouse embryonic fibroblasts (MEF; gift from Frank Echtermeyer, University of Münster, Münster, Germany) were grown as described (8). Cells were transferred into DMEM, 10% FCS 24 h before the experiment, serum starved for 18 h, and then transferred onto dishes (Falcon, BD Biosciences) coated with equimolar amounts of fibronectin and tenascin-C in serum-free medium (5, 7). Recombinant his-tagged human tenascin-C, FNIII13, and serum-derived horse fibronectin were purified as described (6, 8). Cells were plated in serum-free medium 1 h before incubation with 100 ng/mL EGF, 1 μmol/L LPA, and 80 ng/mL PDGF-BB (Sigma) or inhibitor UO126 (25 μmol/L; Calbiochem), wortmannin (20 μmol/L; Sigma), SP600125 (20 mmol/L; Sigma), and U73122 (20 nmol/L; Sigma) followed by lysis in sample buffer (6, 8) after an additional 4 h.

Immunoblotting and immunofluorescence. Immunoblotting and immunofluorescence were done as previously described (6, 8) with the following mouse monoclonal antibodies against TM1, TM2, and TM3 (TM311, 1:1,000; Sigma), vinculin (hVIN-1, 1:1,000; Sigma), paxillin (5H11, 1:1,000; Biosource), α-tubulin (Ab-1, 1:5,000; Oncogene), RhoA (26C4, 1:1,000; Santa Cruz Biotechnology), phosphorylated Akt (Ser[473]) and phosphorylated extracellular signal-regulated kinase (ERK) 1/2 (1:1,000; Cell Signaling and BD Biosciences), FAK and phospho-Y397-FAK (1:1,000; BD Biosciences), and MLC (1:1,000; Santa Cruz Biotechnology) and rabbit polyclonal antibodies against paxillin and phospho-Y118-paxillin (1:1,000; Abcam), Akt and ERK1/2 (1:1,000; Cell Signaling), phospho-S910-FAK (1:1,000; Biosource), and phospho-S19-MLC (1:1,000; Santa Cruz Biotechnology).

RhoA activation assay. T98G cells were serum starved before plating onto fibronectin or FN/TN in six-well dishes (10 cm² per well, Falcon) for the indicated time points in serum-free or LPA/PDGF-supplemented medium and washed with PBS before lysis in "cell ice-cold lysis buffer" (GLISA, Cytoskeleton). Complexes of anti-RhoA and anti-mouse horseradish peroxidase antibodies were determined by measuring absorbance at 490 nm. Positive and negative controls were provided by the manufacturer.

Cell migration assay. Glass coverslips (10 mm²; medite Medizintechnik AG) were precoated with poly-L-lysine (0.01%, 70–150 kDa; Sigma) for 1 h before successive coating with fibronectin, tenascin-C, and bovine serum albumin. Serum-starved cells were plated onto the coverslips for 4 h in serum-free medium. Cells on the coverslip were transferred into FN/TN-coated six-well dishes (10 cm², Falcon) for the indicated time points in serum-free medium or in medium supplemented with EGF, LPA, PDGF, and LPA/PDGF, respectively. Pictures were taken from three areas and cells that had migrated from the coverslip into the six-well dish were counted.

Generation of gene-overexpressing and knockdown cells. T98G cells were stably transfected with short hairpin RNA (shRNA) constructs specific for αTM together with a pCIneo plasmid (Supplementary Table S3; SuperArray) and subcloned. Paxillin was transiently down-regulated with small interfering RNA (siRNA) oligonucleotides (Eurogentech; Supplementary Table S3). Down-regulation of αTM and paxillin was determined by real-time reverse transcription-PCR (RT-PCR) and immunoblotting. T98G cells stably expressing TM1 were described elsewhere (6). A plasmid with the chicken *syndesmos* sequence was cotransfected together with a pCIneo plasmid and G418-resistant cells were selected. Expression of the transgene was determined by semiquantitative RT-PCR that was done at 60°C annealing temperature with the indicated primers (Supplementary Table S1).

Quantitative and semiquantitative RT-PCR. Total RNA was isolated from three independent plates using the RNeasy Mini kit and RNase-free DNase set (Qiagen) following the manufacturer's instructions. cDNA was generated and expression of the respective gene was determined by quantitative RT-PCR as previously described (see primers in Supplementary Table S1; refs. 6, 8). Relative expression of the respective gene was determined after normalization to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as described (6, 8).

Oncomine data analysis. Expression of candidate genes was obtained from the indicated publications. Comparative metaprofiling was performed using various meta-analysis studies from differential microarray data sets present in the public database Oncomine.[6] Statistical analysis of differences was performed by Oncomine algorithm at a *P* value cutoff of 0.05 calculated by a two-sided *t* test as previously described (31).

Results

Cooperative signaling induced by LPA and PDGF restores cell spreading on a FN/TN substratum. We examined adhesion of T98G glioblastoma cells on FN/TN in the presence of different growth factors and observed that cells remained rounded in the presence of LPA, PDGF-BB (PDGF), and EGF during 4 h after plating as was observed in the absence of growth factors (Fig. 1*A*). This was in contrast to LPA/PDGF, where the majority of cells spread on FN/TN in comparison with fibronectin (Fig. 1*A*; Supplementary Fig. S1). To understand how LPA/PDGF induced cell spreading on FN/TN, we analyzed actin polymerization by immunostaining and observed that cells established actin stress fibers and formed vinculin-containing focal adhesions on stimulation with LPA/PDGF, in contrast to cells incubated in serum-free medium (Fig. 1*B*) or with LPA or PDGF alone (data not shown), where cells were rounded and lacked these structures. By immunoblotting, we determined expression of the tenascin-C targets RhoA, FAK, TM1, TM2, and TM3 (8). We observed that LPA/PDGF induced phosphorylation of FAK at Y397 and S910, indicative of FAK activation. LPA/PDGF also induced phosphorylation of ERK1/2 and restored expression of TM1, TM2, TM3, and RhoA to levels as on fibronectin. In addition, LPA/PDGF strongly induced phosphorylation of paxillin at Y118, which is indicative of paxillin activation. This was in contrast to stimulation by LPA or PDGF alone, where expression or phosphorylation of these molecules was significantly lower on FN/TN than on fibronectin (Fig. 1*C*).

Because TM1 heterodimer protein stability is reduced by tenascin-C–mediated down-regulation of TM2/TM3 mRNA expression (8), we examined whether LPA/PDGF affected TM1, TM2, and TM3 expression using quantitative real-time RT-PCR. On FN/TN, LPA/PDGF restored TM2/TM3 mRNA levels (αTM) in T98G cells, where TM1 is the most abundant high molecular weight

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[6] http://www.oncomine.org

TM (Fig. 1*D*). In contrast, TM1 (*βTM*) and RhoA mRNA levels were affected neither by tenascin-C nor by LPA/PDGF (data not shown). Thus, LPA/PDGF-induced cell spreading on FN/TN correlated with activation of FAK, paxillin, and ERK and with high protein levels of RhoA and TM1.

Syndecan-4 is upstream of RhoA, FAK, and TM1 but is dispensable for LPA/PDGF-induced cell spreading on FN/TN. Tenascin-C–induced cell rounding could be abolished by activation of syndecan-4 through an excess of recombinant fibronectin protein FNIII13 (6, 32). Here, we wanted to know whether activation of syndecan-4 by FNIII13 had an effect on RhoA, FAK, and TM1 function. We confirmed that FNIII13 restored spreading of T98G cells on a FN/TN substratum (Fig. 2*A*). As determined by immunoblotting, spreading correlated with restored expression of RhoA, phosphorylated FAK (P-FAK), and TM1 (Fig. 2*A*). By real-time RT-PCR, we observed that FNIII13 increased mRNA levels of TM2/TM3 (*αTM*; Fig. 2*B*), whereas TM1 (*βTM*) mRNA levels remained unchanged (data not shown).

To further determine whether activation of syndecan-4 is required for expression of RhoA, P-FAK, and TM1, we analyzed cell adhesion of wild-type (wt) MEFs and MEFs derived from synde-

can-4 knockout mice, where TM1 is far less expressed than TM2 and TM3, on the tenascin-C substrata 4 h after plating. In wt MEFs, FNIII13 restored cell spreading in the majority of cells plated on FN/TN, whereas FNIII13 failed to do so in syndecan-4–deficient MEFs (Supplementary Fig. S2*A* and *B*). This confirms that inhibition of syndecan-4 is the major mechanism of tenascin-C–induced cell rounding at this time point of adhesion. Similar to T98G cells, P-FAK, RhoA, TM1, TM2, and TM3 levels increased in wt MEFs in the presence of FNIII13 on FN/TN in comparison with its absence (Supplementary Fig. S2*C*). Collectively, these results suggest that syndecan-4 is important for activation of FAK and paxillin and for stable expression of RhoA, TM1, TM2, and TM3 in MEFs and T98G cells on FN/TN.

To investigate syndecan-4 dependence, we analyzed spreading of syndecan-4–deficient MEFs on stimulation with LPA/PDGF. Whereas syndecan-4–deficient MEFs were rounded on FN/TN in the absence of growth factors, the majority of cells spread on stimulation with LPA/PDGF (Fig. 2*C*). Thus, signaling by LPA/PDGF is sufficient to induce spreading on FN/TN independent of syndecan-4. As determined by immunoblotting, stimulation by LPA/PDGF strongly induced phosphorylation of paxillin and ERK
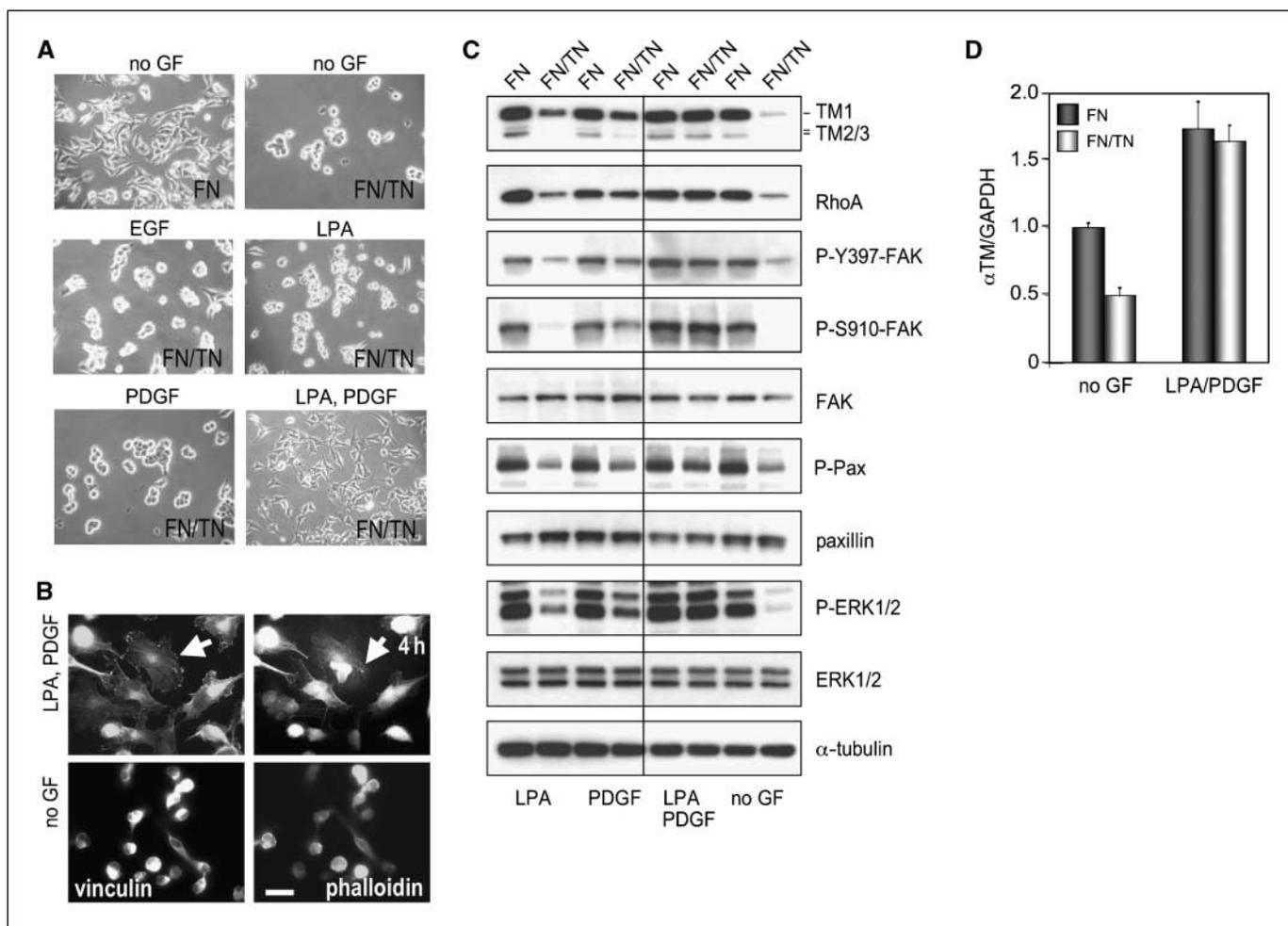
Figure 1. Cooperative signaling by LPA and PDGF induces cell spreading on FN/TN and restores adhesion signaling. T98G cells were plated on fibronectin or FN/TN in serum-free medium (*no GF*) or with EGF, LPA, PDGF, and LPA/PDGF for 4 h before documentation of cell shape (*A*), immunostaining for vinculin and polymerized actin (*B*), lysis and immunoblotting for the indicated molecules (*C*), and RNA extraction followed by real-time RT-PCR for *αTM* (*D*). Expression was normalized to GAPDH. Spread cells of three fields were counted. *Columns,* mean; *bars,* SD. *Scale bar,* 20 μm. A representative result of three independent experiments is shown. Notably, LPA/PDGF induced actin polymerization and focal adhesion formation on FN/TN, which correlated with high *αTM*, TM1, and RhoA levels and high phosphorylation of FAK, paxillin, and ERK1/2.
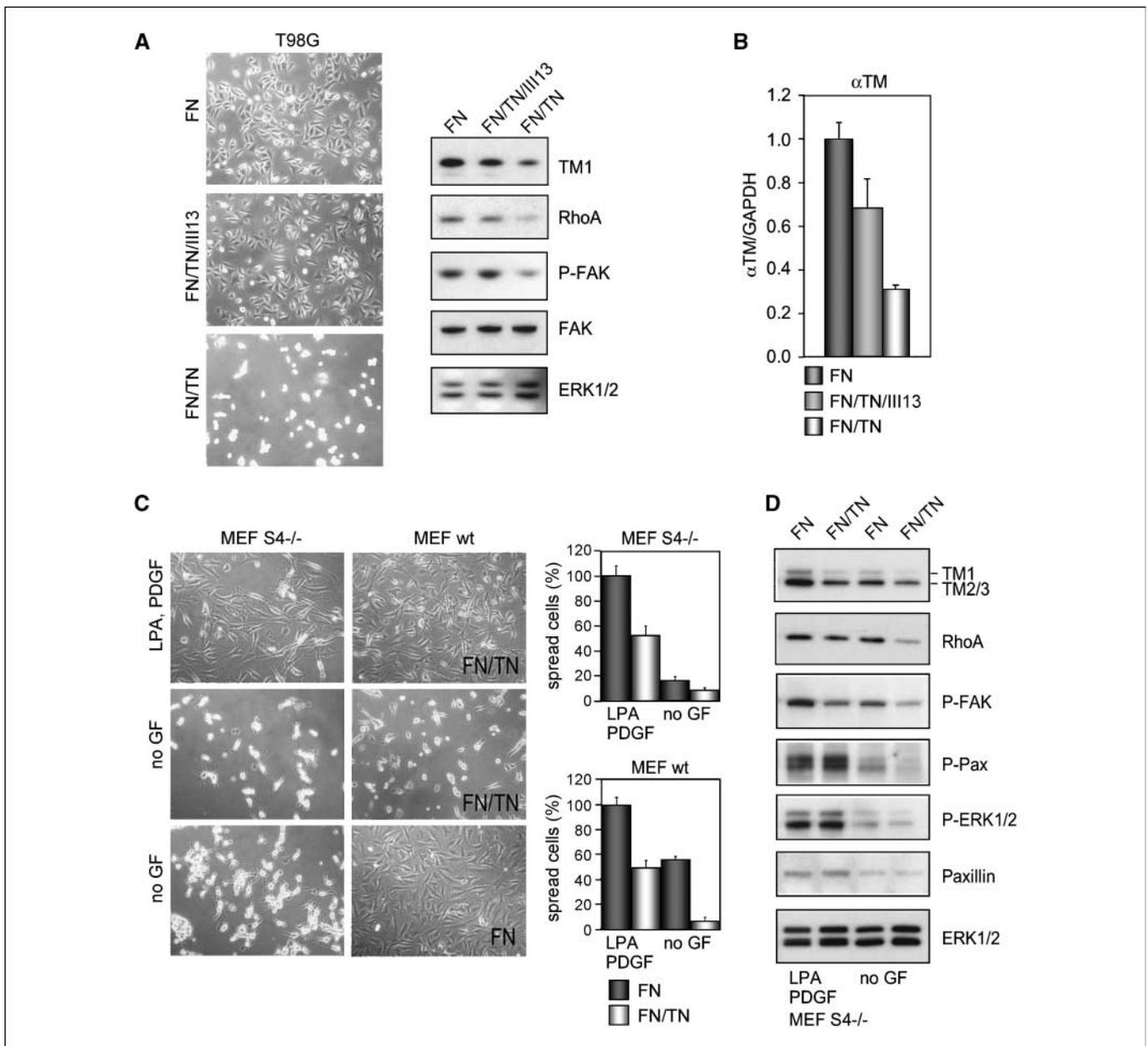
Figure 2. Role of syndecan-4 in LPA/PDGF-induced cell spreading on FN/TN. Cell adhesion (*A* and *C*) and expression of αTM (*B*) and of the indicated proteins (*D*) are shown for T98G (*A* and *B*) and syndecan-4–deficient MEFs (*C* and *D*). Note that syndecan-4 activation restores phosphorylation of FAK and expression of RhoA and TM1. LPA/PDGF also induced spreading in cells lacking syndecan-4 expression, which suggests that this signaling can bypass syndecan-4 requirement for cell spreading.

on FN/TN in syndecan-4–deficient MEFs (Fig. 2*D*). Our data confirm an essential role for syndecan-4 in the activation of paxillin and FAK (12). Moreover, we showed that syndecan-4 triggers stable expression of RhoA, TM1, TM2, and TM3 and that signaling by LPA/PDGF bypasses the requirement of syndecan-4 for cell spreading.

Essential role of paxillin and syndesmos in LPA/PDGF-triggered cell spreading on FN/TN. Next, we investigated whether paxillin is required for LPA/PDGF-induced cell spreading on FN/TN by silencing paxillin expression with siRNA oligonucleotides. Down-regulation of paxillin was confirmed by semiquantitative RT-PCR (Supplementary Fig. S3*A*) and immunoblotting (Fig. 3*A*). T98G:sipax and control T98G:siLuc cells were plated on

fibronectin and FN/TN 48 h after transfection in serum-free medium together with LPA/PDGF. T98G:sipax cells were mainly compromised in LPA/PDGF-triggered cell spreading on FN/TN, which was different to the transfected control cells, where >70% of cells spread on the tenascin-C–containing substratum (Fig. 3*B*). A similar result was also obtained with another siRNA oligonucleotide for paxillin (data not shown). Thus, activation of paxillin by LPA/PDGF is required for spreading on FN/TN.

Because LPA/PDGF-triggered cell spreading on FN/TN is syndecan-4 independent (Fig. 2*C*), we addressed the question of whether this process requires paxillin by testing spreading of syndecan-4–deficient MEFs on FN/TN on silencing of paxillin with siRNA oligonucleotides. By real-time RT-PCR (data not shown) and

immunoblotting, we confirmed depletion of paxillin from the cells (Supplementary Fig. S3*B*). Cells hardly attached and spread on fibronectin in serum-free medium (data not shown) and remained rounded on FN/TN with LPA/PDGF (Supplementary Fig. S3*C*). Thus, activation of paxillin by LPA/PDGF is essential for bypassing syndecan-4 dependence for cell spreading.

Ubiquitously expressed syndesmos binds paxillin (14) and overexpression of syndesmos enhances cell spreading (33). Thus, we examined whether ectopic expression of syndesmos had an effect on paxillin activation and cell rounding on FN/TN. T98G cells ectopically expressing chicken syndesmos were generated and expression of ectopic syndesmos was confirmed by semiquantitative RT-PCR (Supplemental Fig. S3*A*). In contrast to wt T98G cells, T98G:syndesmos cells spread on FN/TN in serum-free medium (Fig. 3*C*). Moreover, T98G:syndesmos cells formed vinculin-containing focal adhesions under these conditions, which were absent from the parental T98G cells (Fig. 3*C*). To address the mechanism of syndesmos-triggered spreading on FN/TN, we determined

expression of the tenascin-C targets FAK, paxillin, TM1, and RhoA. Whereas phosphorylation or expression of these molecules was very low in T98G cells (Fig. 1*C*), levels of phosphorylated paxillin (P-Pax), P-FAK, RhoA, and TM1 were as high on FN/TN as on fibronectin on ectopic expression of syndesmos (Fig. 3*D*). However, ectopically expressed syndesmos did not restore cell spreading nor expression and function of the tenascin-C targets on a pure tenascin-C substratum (Fig. 3*D*). It is possible that ectopically expressed syndesmos recruits syndecan-4 and ligated integrin $\alpha_5\beta_1$ into focal adhesions, thus overcoming the lack of syndecan-4 ligation on FN/TN. This would not be possible on tenascin-C, where $\alpha_5\beta_1$ remains unligated.

To determine whether syndesmos-triggered spreading on FN/TN requires paxillin, we knocked down paxillin expression with siRNA oligonucleotides (Supplementary Fig. S3*A*). T98G:syndesmos.sipax cells failed to spread on FN/TN, which was in contrast to the control siLuc transfected T98G:syndesmos cells that did spread on FN/TN (Fig. 3*C*). Together, these data show that paxillin is an
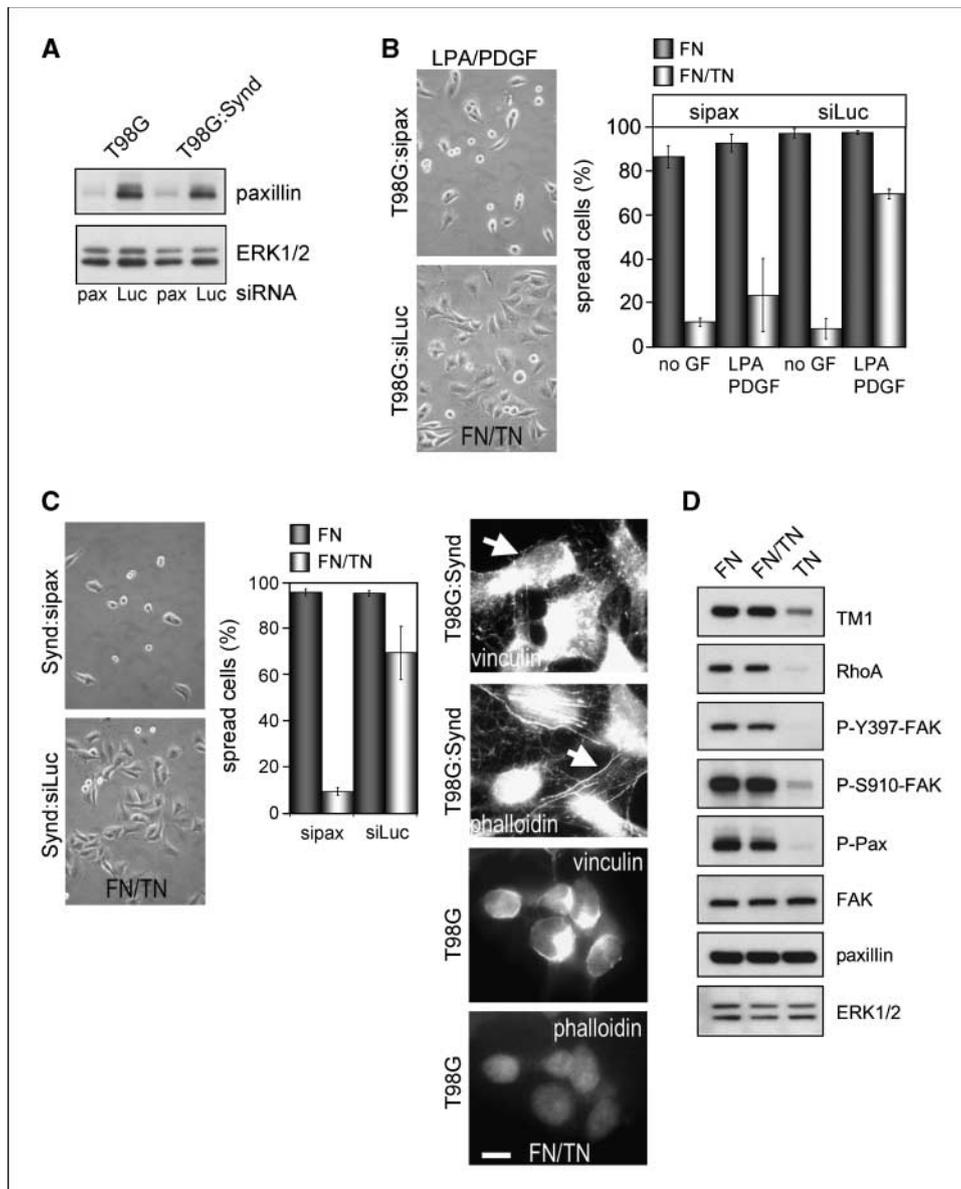
Figure 3. Effect of paxillin and syndesmos on cell adhesion to FN/TN. *A*, transient knockdown of paxillin 48 h after siRNA oligonucleotide transfection into T98G and T98G:syndesmos cells (stably expressing syndesmos) after 4 h on FN in serum-free medium was determined by immunoblotting for paxillin. Documentation of cell shape (*B*), actin stress fibers, and focal adhesions was documented by immunofluorescence (*C*), and expression of tenascin-C target molecules was determined by immunoblotting (*D*). Transfection of a siLuc oligonucleotide served as a negative control. Note that LPA/PDGF- and syndesmos-induced cell spreading on FN/TN was paxillin dependent.

essential factor for spreading, whose inhibition is instrumental for tenascin-C–induced cell rounding. Moreover, LPA/PDGF-induced spreading on FN/TN is dependent on paxillin. We conclude that syndesmos recruits syndecan-4, paxillin, and FAK into focal adhesions, thus initiating actin stress fiber formation. Our data also describe a novel role for syndesmos in cell adhesion, whereby it activates paxillin and FAK, thus triggering stabilization of RhoA and TM1.

LPA/PDGF-induced cell spreading on FN/TN depends on elevation of TM1, TM2, and TM3 expression. Because suppression of TM1 is pivotal for tenascin-C–induced cell rounding (7), we investigated whether LPA/PDGF-induced cell spreading on FN/TN was dependent on restoration of TM1 expression. First, we confirmed that forced expression of TM1 in T98G:TM1D10 and REF52:TM1 cells promoted cell spreading on FN/TN, which is documented by formation of focal adhesions, actin stress fibers, and microtubules (Supplementary Fig. S4A and C). Thus, down-regulation of TM1 by tenascin-C is not a cell type–specific effect. Next, we wanted to know whether ectopically expressed TM1 had an effect on RhoA expression and phosphorylation of FAK. Levels of RhoA and of P-FAK were restored in T98G:TM1D10 cells on FN/TN back to levels as found on cells plated on fibronectin, which was different to control cells that exhibited very low expression levels (Supplementary Fig. S4B). These data support our notion that simultaneous high levels of P-FAK, RhoA, and TM1 are critical for cell spreading (8). Moreover, high TM1 levels have a positive effect on FAK function and RhoA expression.

To examine whether elevation of TM1 expression is required for LPA/PDGF-induced cell spreading, we generated cells stably suppressing TM2/TM3 mRNA levels (αTM) by shRNA oligonucleotides and plated cell clones expressing two different shRNAs on fibronectin and FN/TN followed by LPA/PDGF treatment. Cells with silenced αTM exhibited very low TM1 expression and thus mimicked TM1 protein down-regulation by tenascin-C (Fig. 4A; ref. 8). These cells hardly attached and did not spread on fibronectin in serum-free medium (Fig. 4B), which suggests that normal levels of TM1, TM2, and TM3 are required for cell spreading on fibronectin. In addition, cells with knockdown αTM did not spread on FN/TN on stimulation with LPA/PDGF, which was in contrast to control cells that spread on FN/TN under these conditions (Fig. 4B and C). Thus, elevation of TM1, TM2, and TM3 is required for LPA/PDGF-induced cell spreading on FN/TN.

By analyzing expression of the tenascin-C target molecules, we observed that in the shαTM3D2 cells, levels of P-FAK, RhoA, and TM1 remained very low on FN/TN on stimulation with LPA/PDGF. Most importantly, LPA/PDGF was unable to activate paxillin on silencing of αTM (Fig. 4D). This observation supports our notion that paxillin is an essential downstream effector of LPA/PDGF signaling on FN/TN. Together, activation of paxillin and FAK and stable expression of RhoA, TM1, TM2, and TM3 are tightly linked to cell adhesion and thereby seem interdependent. Whereas ectopic expression of TM1 restored expression and function of RhoA, FAK, and paxillin, forced down-regulation of TM1, TM2, and TM3 resulted in low levels of P-paxillin, P-FAK, and RhoA and blocked LPA/PDGF-induced cell spreading on FN/TN. In conclusion, LPA/PDGF-induced spreading on FN/TN is dependent on activation of paxillin and FAK and on a stable expression of RhoA, TM1, TM2, and TM3.

LPA/PDGF-specific expression of RhoA, P-FAK, and TM1 is phosphatidylinositol 3-kinase and MAPK/ERK kinase dependent. Next, we identified which are the signaling pathways by

which LPA/PDGF induces cell spreading on FN/TN. Cells were treated with chemical inhibitors for phosphatidylinositol 3-kinase (PI3K; wortmannin), MAPK/ERK kinase (MEK; UO126), phospholipase C (PLC; U73122), c-Jun NH$_2$-terminal kinase (JNK; SP600125), and ROCK (Y27632), and cell spreading and expression of P-paxillin as well as P-FAK, RhoA, and TM1 levels were determined. Inhibition of PI3K and MEK was documented by a lack of phosphorylation of downstream Akt and ERK1/2 (Fig. 5A). Wortmannin prevented cell spreading on FN/TN induced by LPA/PDGF (Fig. 5B). This was in contrast to the other inhibitors that did not interfere with LPA/PDGF-induced cell spreading (Supplementary Fig. S5A). On inhibition of PI3K by wortmannin, levels of P-paxillin, P-FAK, RhoA, and TM1 were lowered on fibronectin and were very low or absent in cells plated onto FN/TN together with LPA/PDGF (Fig. 5C). Inhibition of MEK by UO126 did not cause cell rounding but reduced expression of P-paxillin, P-FAK, RhoA, and TM1 (Fig. 5D), but expression levels remained high enough to induce cell spreading on FN/TN (Fig. 5B). Inhibition of PLC and JNK did not alter the high levels of RhoA, TM1, and P-FAK induced by LPA/PDGF (Supplementary Fig. S5B and C).

Because LPA/PDGF increased RhoA levels on FN/TN, we wanted to know whether LPA/PDGF had an influence on RhoA activation. We measured GTP loading of RhoA in T98G cells on fibronectin or FN/TN in serum-free medium by ELISA for rhotekin-bound RhoA. RhoA activation was 73%, 70%, and 90% of that on fibronectin at 15 and 30 min and 4 h after plating, in serum-free medium, respectively, which was in a similar range as the positive control. Stimulation by LPA/PDGF insignificantly elevated rhotekin-bound RhoA levels over those found in cells incubated without growth factors (Supplementary Fig. S6A). Thus, activation of RhoA is independent of matrix adhesion and growth factors in these cells.

To address the possibility that ROCK, a kinase involved in actin polymerization downstream of RhoA, is modulated by tenascin-C or LPA/PDGF signaling or by both, cells were treated with the ROCK inhibitor Y27632. Cells spread and exhibited protrusions resembling filopodia (Supplementary Fig. S6B), the latter observation confirming inhibition of ROCK by Y27632. Expression of RhoA, P-FAK, TM1, TM2, and TM3 was even enhanced on FN/TN on stimulation with LPA/PDGF and concomitant inhibition of ROCK. Whereas MLC, a downstream target of ROCK, was not phosphorylated on FN/TN in serum-free medium, MLC became phosphorylated on stimulation with LPA/PDGF, which was enhanced by the ROCK inhibitor Y27632 (Supplementary Fig. S6C). Thus, signaling by LPA/PDGF restored MLC phosphorylation on FN/TN by a ROCK-independent mechanism.

LPA/PDGF promotes glioma cell migration on a FN/TN substratum in a ROCK- and MEK-dependent manner with potential relevance for tumor progression. Given that tenascin-C promotes glioma cell migration (34), we asked whether LPA/PDGF affects cell migration on FN/TN. Migration of T98G glioblastoma cells on FN/TN was monitored on stimulation with EGF, LPA, and PDGF. In contrast to stationary cells in the absence of growth factors, or in the presence of EGF, LPA, and PDGF alone (data not shown), the combination of LPA and PDGF stimulated cell migration (Fig. 6A; Supplementary Fig. S7A). After 48 h, cells presumably secreted factors that induced migration under serum-free conditions on FN/TN and thus promoted cell migration at this time point with a rounded cell shape. This was different to the fibroblastoid cell shape observed on stimulation with LPA/PDGF (Fig. 6A; Supplementary Fig. S7A). Interestingly, EGF completely blocked cell spreading and migration in serum-free medium

(Fig. 6A). It is possible that EGF counteracted LPA/PDGF-induced cell spreading and migration, which is supported by our observation that LPA and PDGF in combination with EGF and other growth factors did not induce spreading of T98G cells (data not shown) or fibroblasts on FN/TN (32). Thus, LPA/PDGF triggered cell migration on the FN/TN substratum. We showed that growth factor signaling can enhance or block cell migration on a tenascin-C substratum.

Next, we asked whether LPA/PDGF-induced migration was affected by the adhesive strength between the cells and their substratum. Therefore, migration of cells with an altered expression of syndesmos, TM1, TM2, and TM3 was determined on a FN/TN substratum (Supplementary Fig. S7B). Whereas all cells did not migrate in the absence of growth factors, LPA and PDGF alone (data not shown), control T98G:pCIneo, and control shRNA-transfected cells migrated very well on FN/TN on stimulation with LPA/PDGF in a similar range as the parental T98G cells (Supplementary Fig. S7B). In contrast to the control cells, T98G:syndesmos, T98G:TM1D10, and T98G:shαTM3D2 cells did essentially not move in the presence of LPA/PDGF. Thus, cell spreading on FN/TN (through ectopic expression of syndesmos or TM1), as well as reduced adhesion to FN/TN (upon suppression of TM1, TM2, and TM3), is not compatible with LPA/PDGF-stimulated cell migration. In conclusion, inhibition of syndecan-4 (by tenascin-C) and normalized levels of TM1, TM2, and TM3 (by LPA/PDGF) are required for cells to migrate on a FN/TN substratum.

To address a potential role of ROCK and MEK, we analyzed cell migration on inhibition of these kinases. Although inhibition of ROCK did not block LPA/PDGF-induced cell spreading at the 4-h time point (Supplementary Fig. S6B), Y27632 largely compromised LPA/PDGF-stimulated cell migration on FN/TN (Supplementary Fig. S7C and D). Similarly, cells did not migrate on FN/TN in the presence of LPA/PDGF on inhibition of MEK (Supplementary Fig. S7C and D). In conclusion, LPA/PDGF-stimulated cell migration on FN/TN is dependent on ROCK and MEK. This information is potentially useful for blocking tenascin-C–induced cell migration in cancer.

Next, we compared gene expression levels of tenascin-C, LPA receptors, LPA, PDGFR, and PDGFs in 84 glioblastomas (GeneChip U133Plus, Affymetrix; ref. 35) and by statistically evaluating gene expression changes in gliomas using the Oncomine 3.2.2 platform (31). Expression of tenascin-C, PDGFRα, PDGFC, and PDGFD showed some correlation in glioblastomas with high expression in gliomas (Fig. 6B) and low expression in nontumor brain tissue (Supplementary Fig. S8A). In comparison with PDGFRα, expression of PDGFRβ was lower in the majority of glioblastomas; however, it was higher than in nontumor tissue (Fig. 6B; Supplementary Fig. S8A). PDGFA, PDGFB, PDGFC, and PDGFD were expressed in glioblastomas but to a variable degree, with PDGFC being highly expressed in glioblastomas (Fig. 6B; Supplementary Fig. S8A). LPA receptor 1 (EDG2) was significantly expressed in glioblastomas (Fig. 6B). Global expression of LPA turned out to be very low in all
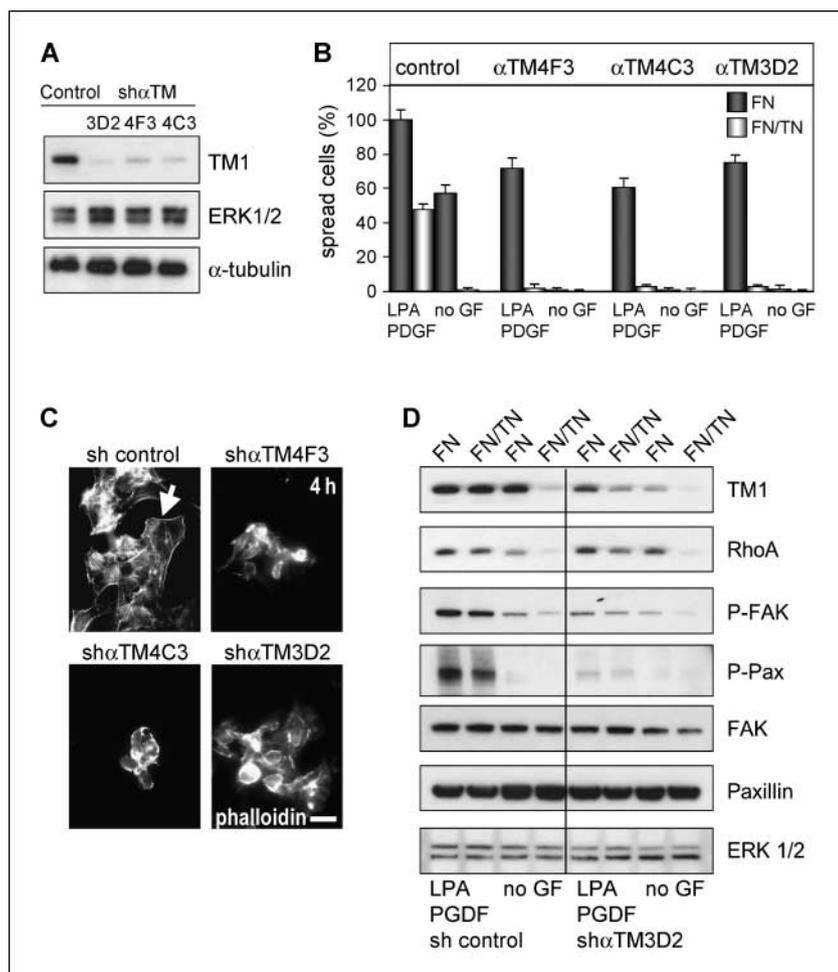
Figure 4. Effect of TM1 levels on LPA/PDGF-induced cell spreading on FN/TN. T98G cells with knockdown of αTM (clones 3D2, 4F3, and 4C3) were plated for 4 h before lysis and immunoblotting (A and D), counted (B), or analyzed by immunofluorescence for polymerized actin (C). Note that ectopic expression of TM1 induced spreading on FN/TN by presumably restoring expression and function of FAK and RhoA. In contrast, low TM1, TM2, and TM3 levels prevented LPA/PDGF-induced spreading on FN/TN correlating with inactive FAK and paxillin. Scale bar, 20 μm.
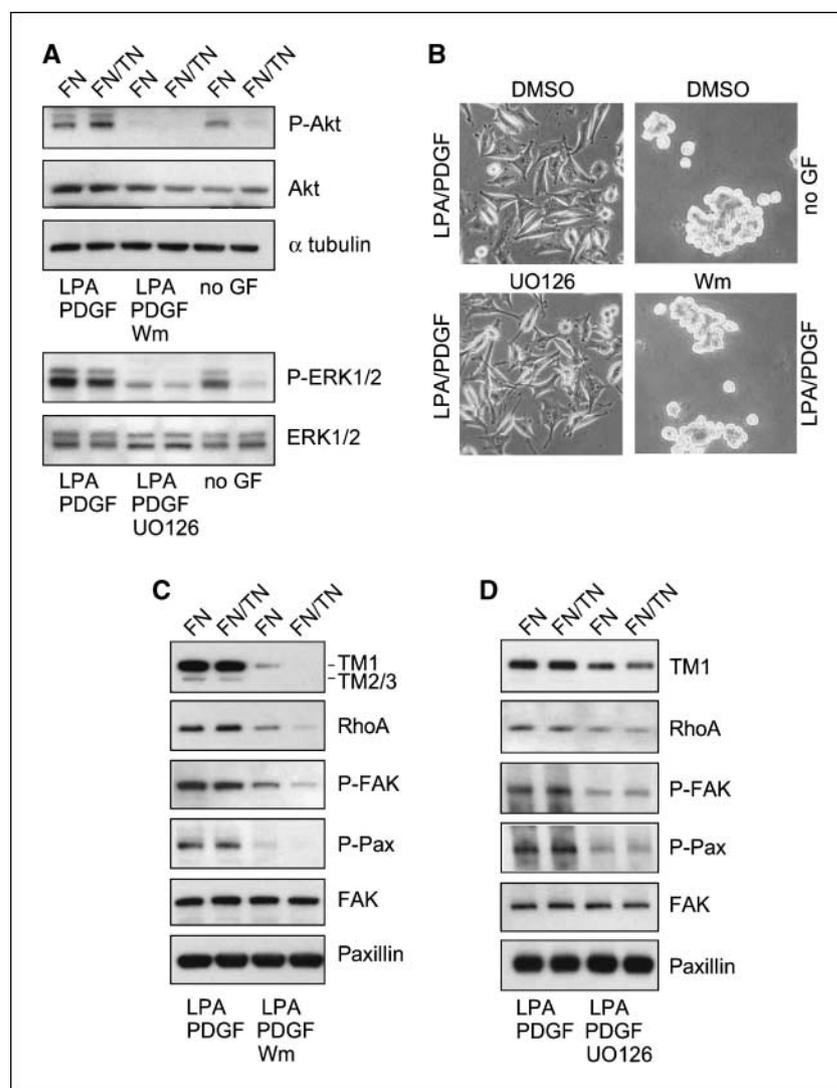
Figure 5. LPA/PDGF-induced adhesion signaling is PI3K and MEK dependent. Cells were plated onto the two substrata for 1 h before addition of wortmannin (*Wm*) or UO126 lasting another 4 h in serum-free or LPA/PDGF-supplemented medium. *A, C,* and *D,* immunoblotting for the indicated molecules. *B,* documentation of cell shape. Note that LPA/PDGF-triggered restoration of FAK, RhoA, and TM1 on FN/TN is dependent on PI3K and MEK.

analyzed gliomas, but a potentially elevated expression of LPA at the invasive front cannot be detected by RNA analysis of whole glioma tissue. It is possible that TN/LPA/PDGF signaling plays a transient and spatial role in glioma invasion. By using the Oncomine platform to analyze expression of tenascin-C, LPA, PDGF, and the respective receptors, we found a correlation of high expression and malignancy in microsatellite instable and serrated colorectal carcinoma and metastatic lung adenocarcinoma (Supplementary Fig. S8*B–G*). Moreover, LPA/PDGF stimulated spreading of KRIB osteosarcoma cells on FN/TN (Supplementary Fig. S9). Together, the TN/LPA/PDGF signaling axis seems to be relevant in several cancer types and in multiple cells, including fibroblasts, glioma, and osteosarcoma cells. Our results are compatible with the possibility that PDGF and LPA in conjunction with tenascin-C have a pivotal role during cancer cell migration and tumor progression.

Discussion

Cell adhesion to an ECM substratum, such as tenascin-C, can be modulated by growth factors and provides a means to adjust the adhesive properties of the microenvironment within a tissue (36). A substratum of tenascin-C alone or in combination with fibronectin

is antiadhesive for most cells. Because tenascin-C stimulated expression of PDGFRα (7), we investigated how PDGF alone or in combination with other factors affected cell adhesion on FN/TN (32). Here, we showed that PDGF in combination with LPA induces spreading of T98G glioblastoma cells on FN/TN, which is in contrast to either factor alone, where cells remained rounded. We showed that LPA/PDGF-induced spreading was syndecan-4 independent because MEFs lacking syndecan-4 spread on FN/TN on stimulation with LPA/PDGF. We ruled out the possibility that increased levels of syndecan-1 and syndecan-2 potentially compensated for the absence of syndecan-4 because expression of these syndecans was not increased in syndecan-4–deficient MEFs compared with wt MEFs, as determined by real-time RT-PCR (data not shown). We confirmed that FAK, RhoA, TM1, TM2, and TM3 are a minimal set of critical targets of tenascin-C (8) and showed that the expression and function of these molecules is restored on stimulation with LPA/PDGF. We showed that paxillin is another critical target of tenascin-C. Activation of paxillin is essential for inducing cell spreading on FN/TN because silencing of paxillin by siRNA oligonucleotides blocked LPA/PDGF-induced cell spreading. We ectopically expressed syndesmos, which promotes cell spreading connecting syndecan-4 with paxillin (14, 33), resulting in
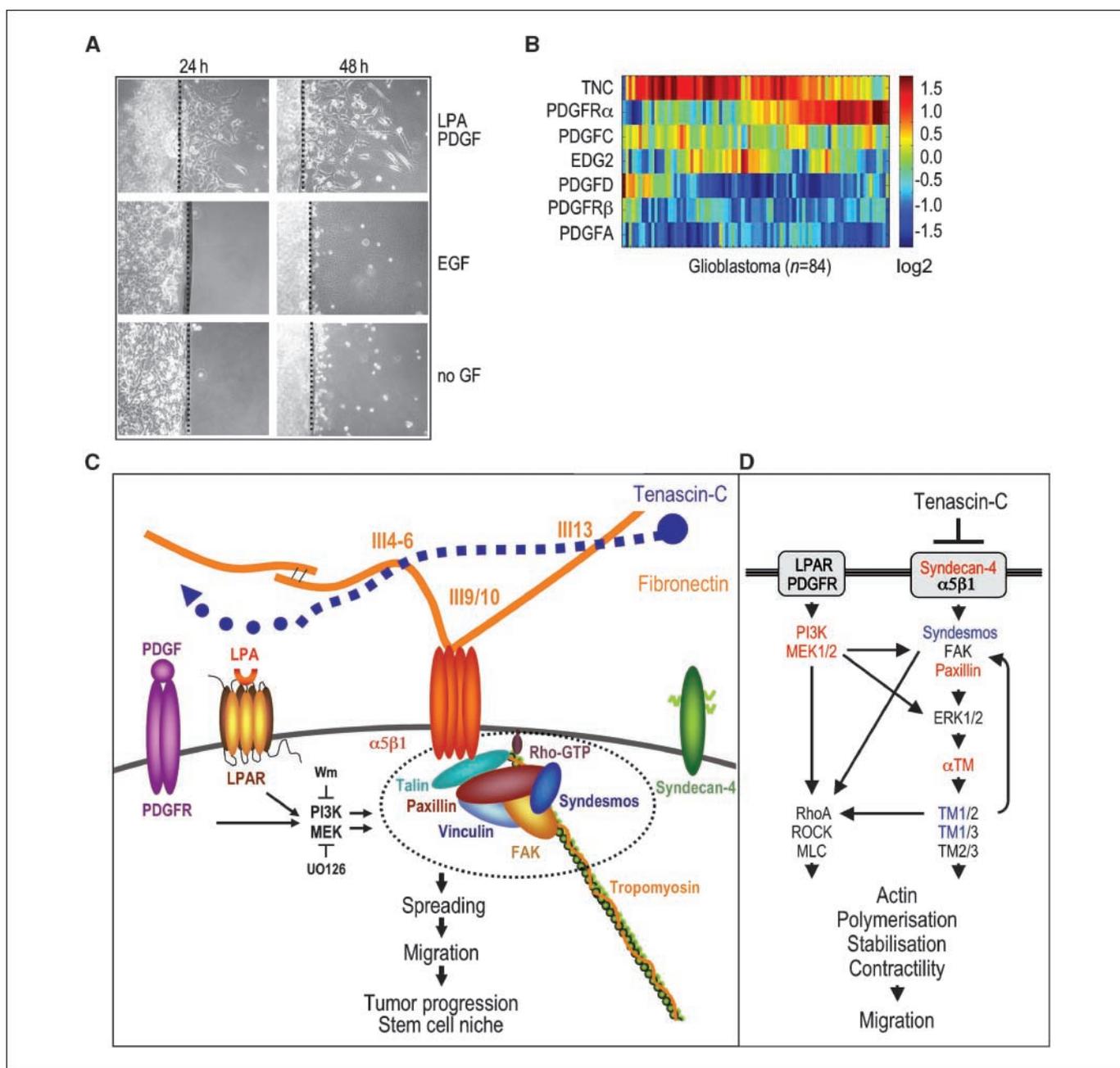
Figure 6. Induction of cell migration on FN/TN on stimulation with LPA/PDGF and expression of tenascin-C and PDGFR in gliomas. Migration of cells from a glass coverslip coated with poly-L-lysine, fibronectin, and tenascin-C onto a plastic surface coated with FN/TN is documented for T98G cells in the presence of the indicated supplements. Cell morphology (*A*) and numbers of migrated cells (Supplementary Fig. S7*A, B,* and *D*) are shown. Cells having passed the rim (margin highlighted by *dotted line*) from three areas were counted (Supplementary Fig. S7*A*). A representative result of two is shown. *B,* gene expression of tenascin-C and potential mediators is ordered by sorting points into neighborhoods analysis (34) for 84 glioblastomas. The ordering of points is iteratively permutated in search of a linear ordering. Expression, as determined on GeneChip 133Plus2, is centered and presented as a heat map (log2). Note that tenascin-C, PDGFRα, PDGFC, PDGFD, and LPA receptor 1 (EDG2) are highly expressed in most glioblastomas. This is in contrast to PDGFA and PDGFD, which exhibit a low global expression. *C,* on adhesion to fibronectin, simultaneous activation of integrin α5β1 [through FNIII repeats III9/10 (*III9/10*)] and syndecan-4 [through FN repeat III13 (*III13*)] leads to the establishment of focal adhesions (5). Paxillin and FAK are among the first effectors to become activated during cell spreading. RhoA, an important downstream effector of syndecan-4 (50), is targeted to the membrane, where it induces actin polymerization. Syndesmos provides a bridge between syndecan-4 and integrin α5β1 through its binding to paxillin (13). Tenascin-C competes with binding of syndecan-4 to FNIII13, thus blocking signaling from syndecan-4 (5), resulting in a lack of paxillin and FAK activation and enhanced proteolytic degradation of RhoA, TM1, TM2, and TM3 (7). Here, we showed that activation of paxillin through ectopically expressed syndesmos or through signaling from LPA/PDGF bypasses the requirement of syndecan-4 ligation for cell spreading. LPA/PDGF-induced spreading was dependent on PI3K and MEK but not on PLC and JNK. Inhibition of syndecan-4 by tenascin-C and remodeling of the actin cytoskeleton induced by LPA/PDGF trigger cell migration on FN/TN and, potentially, tumor progression. A balanced expression of tenascin-C, LPA, and PDGF and their receptors may also determine whether brain stem cells remain in their tenascin-C–containing niche or proliferate, differentiate, and migrate out of the niche. *D,* actin polymerization, filament stabilization, and contractility are induced by LPA/PDGF signaling and thus trigger cell spreading and migration on FN/TN, which is independent of syndecan-4. LPA/PDGF-induced spreading depends on paxillin, TM1, TM2, and TM3 expression because it was blocked on knockdown of paxillin and αTM and was restored on ectopic expression of syndesmos and αTM. Ectopic expression of TM1 restored cell spreading on FN/TN, presumably through a feedback mechanism, which leads to FAK activation and RhoA stabilization on FN/TN. *Red,* experimental inhibition or down-regulation; *blue,* activation or overexpression. Modular organization of tenascin-C: oligomerization domain (*triangle*), EGF-like repeats (*small circle*), FNIII repeats (*rectangles*), and fibrinogen globe (*big circle*). Note that the actual number of domains in tenascin-C is not reproduced.

restored cell spreading and function of paxillin, FAK, RhoA, and TM1 on FN/TN. These experiments provide novel insight into the role of syndesmos in cell spreading: syndesmos activates paxillin and triggers stabilization of RhoA and TM1.

Although separate signaling by either PDGF (37, 38) or LPA (39) alone induces FAK activation, on FN/TN both factors were required simultaneously to activate FAK and paxillin. It was shown that PDGF can act as an amplifier or attenuator of LPA-induced cell migration (27). At the molecular level, differential use of the G protein $G\alpha13$ by PDGF and LPA (40) might be involved in stimulation of nonredundant additive signaling by LPA and PDGF. Nonredundant signaling might include activation of c-Met (41) and Wnt signaling (40) by LPA, signal transducer and activator of transcription (Stat) signaling by PDGF, among other pathways (42).

On a tenascin-C substratum, cells are unable to form actin stress fibers, which is due to inhibition of actin polymerization (43) and involves destabilization of actin stress fibers through repression of TM1, TM2, and TM3 (7). In T98G and REF52 cells, repression of TM1, TM2, and TM3 seems to be the major mechanism accounting for a lack of actin stress fibers on FN/TN because ectopic expression of TM1 was sufficient to restore actin stress fiber formation (7). Now, we have linked TM1 expression to FAK autophosphorylation and stable RhoA expression on FN/TN. This observation suggests a positive feedback mechanism, by which TM1 sustains actin filament stabilization, thus promoting FAK activation and stabilization of RhoA by a mechanism that needs to be addressed in future studies. We showed that TM levels are critical for cell spreading as has been shown by others (44, 45). Silencing expression of TM2/TM3 resulted in low TM1 protein expression in T98G cells and thus phenocopied repression of TM1 by tenascin-C (7). Conversely, induction of high TM2/TM3 RNA levels by LPA/PDGF was required to induce cell spreading on FN/TN because cells with knockdown αTM did not spread on the tenascin-C substratum on stimulation with these growth factors. Thus, LPA/PDGF signaling induces high TM2/TM3 RNA levels, which subsequently promote stabilization of TM1 homodimers and TM1, TM2, and TM3 heterodimers (8). Whether this affects transcription or is regulated at another level remains to be determined. In conclusion, TM2/TM3 RNA levels and TM1 protein stability are regulated by tenascin-C and endothelin-1 (8) and by syndecan-4 and LPA/PDGF. We identified PI3K- and MEK-associated signaling as essential for LPA/PDGF-induced cell spreading on FN/TN. The possibility that highly activated ERK1/2 via LPA/PDGF stimulation contributes to TM1 stability has also been shown by others (46). In contrast, the PLC and JNK pathways were not implicated (Supplementary Table S4). Although PLC is important in syndecan-4–mediated spreading (12), its activation does not seem to require the presence of LPA/PDGF. Spreading induced by LPA/PDGF was different from that induced by EDNRB. Whereas EDNRB-induced spreading was PLC and JNK dependent, but MEK independent (8), LPA/PDGF-induced spreading was PLC and JNK independent but MEK dependent.

Several mechanisms seem to exist that induce migration on a tenascin-C substratum. In addition to an involvement of integrin $\alpha_2\beta_1$ in tenascin-C–induced glioma cell migration (10), activation of c-Met, EGFR, and Rac in tenascin-C–induced colorectal carcinoma cell invasion (11), inhibition of syndecan-4 by tenascin-C, as well as remodeling of the actin cytoskeleton by LPA/PDGF facilitated motility of glioma cells on a tenascin-C substratum (Fig. 6*C* and *D*). Although tenascin-C (34), LPA, and PDGF (26) alone stimulated cell migration, only the combined signaling from both growth factors strongly induced cell spreading and migration on FN/TN, which involved RhoA, TM1, TM2, TM3, and phosphorylated MLC. This may contribute to cell migration during wound healing and tumor progression. The presence of tenascin-C, LPA, PDGFs, and their receptors in the more malignant stages of several cancer types supports this possibility (Supplementary Fig. S8). Signaling by LPA and PDGF also induced spreading in fibroblasts, which might be relevant for invasion of colon (11) and squamous carcinoma cells (47), which is driven by cancer-associated fibroblasts in a tenascin-C–dependent manner. Thus, tenascin-C in conjunction with LPA and PDGF might play a pivotal role in tumor progression.

Signaling by LPA/PDGF in combination with tenascin-C might also be relevant for brain stem cells residing in the SVZ, where tenascin-C expression determines maintenance of the OPC progenitor status and responses toward PDGF, processes which were impaired in tenascin-C knockout mice (48). In addition, stem cells in the SVZ express LPAR1 (29) and PDGFRα (30), and enhanced signaling through PDGFRα was shown to induce a hyperproliferative and invading phenotype in the SVZ (30). Together, these observations suggest that all three molecules, tenascin-C, LPA, and PDGF, largely contribute to identity of the SVZ stem cell niche. Because tenascin-C induces oncogenic signaling, expression of PDGFRα, and down-regulation of genes involved in chromosomal stability (9), it is possible that hyperproliferation and migration is induced in the stem cells of the SVZ on elevated expression of tenascin-C during tumorigenesis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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Combined Lysophosphatidic Acid/Platelet-Derived Growth Factor Signaling Triggers Glioma Cell Migration in a Tenascin-C Microenvironment

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