Characterization of tenascin-C-induced signaling in tumorigenesis

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Research is the art of seeing what everyone has seen, and doing what no-one else has done"

Anonymous

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Abbrevations

ECM	Extracellular matrix
EDNRA	Endothelin receptor type A
EDNRB	Endothelin receptor type B
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ERK	Extracellular regulated kinase
ET1	Endothelin-1
FAK	Focal adhesion kinase
FN	Fibronectin
GPCR	G protein-coupled receptor
JNK	c-Jun amino –terminal kinase
LPA	Lysophosphatidic acid
MAPK	Mitogen-activated protein kinase
mDia	Mammalian homolog of Diaphanous
MEK	MAPK kinase
MLC	Myosin light chain
MMP	Matrix metalloproteinase
PDGF	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor receptor
PI3K	Phosphatidylinositol 3-kinase
PLC	Phospholipase C
ROCK	Rho kinase
RTK	Receptor tyrosine kinase
TM1	Tropomyosin-1
TN	Tenascin

1 Introduction

1.1 Summary

During cancer progression, the extracellular matrix (ECM) is extensively remodeled. The ECM molecule tenascin-C is an adhesion-modulating molecule, which is highly expressed in tumor stroma. Tenascin-C was shown to disrupt the interaction of cells with fibronectin, an adhesive ECM molecule, through inhibition of syndecan-4, the co-receptor of the fibronectin binding integrin $\alpha 5\beta 1$. Cells on a mixed substratum of fibronectin and tenascin-C failed to form cell adhesion structures and actin stress fibers and thus remained rounded. Focal adhesion kinase (FAK) (Huang et al., 2001; Orend, 2003) and the small GTPase RhoA (Wenk et al., 2000), two molecules with an important role in formation of focal adhesions and actin stress fibers, were downregulated in the presence of tenascin-C. Furthermore, the actin-binding and filament-stabilizing molecule tropomyosin-1 (TM1) was identified by Ruiz et al., (2004) to be downregulated by tenascin-C and its downregulation contributed to the lack of actin stress fiber formation on a fibronectin/tenascin-C substratum.

Here, we investigated the signaling events, that lead to cell rounding by tenascin-C. In particular, we wanted to understand how tenascin-C prevents the formation of focal adhesions and stress fibers, and how tenascin-C affects expression and function of the three downstream targets FAK, RhoA and TM1. First, we investigated whether inhibition of syndecan-4 by tenascin-C is linked to reduced expression of RhoA and TM1 and inhibition of FAK. By activating syndecan-4 on fibronectin/tenascin-C substratum we observed that indeed this is the case. Thus, repression of FAK, RhoA and TM1 could explain the lack of actin stress fiber formation on fibronectin/tenascin-C.

Whereas expression of TM1 was not regulated by tenascin-C at the transcriptional level, it turned out that tenascin-C repressed RNA levels of two other tropomyosins TM2 and TM3, which are far less expressed in T98G cells than TM1. Apparently, lowered levels of TM2 and TM3 affected TM1 protein heterodimer stabilization through proteasomal degradation that was largely enhanced on fibronectin/tenascin-C. This possibility was supported by our observation that inhibition of the proteasome restored TM1 expression on this mixed substratum. Our data suggest that tenascin-C does not only repress gene expression of tropomyosins but also enhances their proteasome-mediated protein degradation. Repression of TM1, a molecule with a tumor suppressor-like activity, by tenascin-C might be relevant in cancer, since low levels of this molecule can protect cancer cells from apoptosis.

To learn more about the underlying mechanism of tenascin-C-induced cell rounding, we searched for signaling pathways, that enabled cells to spread in the presence of tenascin-C. In addition, we used knockdown and overexpression studies together with chemical inhibitors. Our data suggest that concomittant restoration of the expression and function of all three downstream targets FAK, RhoA and TM1, is necessary to induce cell spreading on a fibronectin/tenascin-C substratum. In particular, we observed that activation of endothelin receptor type B (EDNRB) induced spreading in the presence of tenascin-C. This was dependent on PI3K, PLC and JNK, since chemical inhibitors of these enzymes blocked EDNRB-induced cell spreading on fibronectin/tenascin-C. Signaling by EDNRB was linked to activation of FAK and paxillin and restoration of TM1 and RhoA expression, again supporting our notion that inactivation of these molecules is critical for tenascin-C-induced cell rounding.

Based on the results from Ruiz et al., (2004), that described an enhanced expression of endothelin receptor type A (EDNRA) in the presence of tenascin-C, we wanted to know whether and how EDNRA signaling contributes to cell rounding by tenascin-C. EDNRA expression was triggered by tenascin-C upon contact with the substratum for more than 5 h. We demonstrated that EDNRA signaling is linked to cell rounding on a fibronectin/tenascin-C substratum through inhibition of FAK and repression of RhoA and TM1. Collectively, these data suggest that inhibition of syndecan-4 is responsible for initial cell rounding and that upon induction of EDNRA by tenascin-C, EDNRA maintains cell rounding on a fibronectin/tenascin-C substratum at later time points. In gliomas and other cancers we found a high expression of tenascin-C and EDNRA, that correlated with more advanced stages, which supports the possibility that tenascin-C potentially promotes tumor progression through EDNRA.

In addition to EDNRB, concomittant activation of the receptors for lysophosphatidic acid (LPA) and platelet-derived growth factor (PDGF) also enabled cells to spread on a fibronectin/tenascin-C substratum by a mechanism, which again involved restoration of the expression and function of FAK, paxillin, RhoA and TM1. By using cells lacking syndecan-4, we observed that LPA/PDGF bypassed the requirement for syndecan-4 in cell spreading on a mixed substratum. Knockdown of paxillin prevented LPA/PDGF-induced cell spreading on fibronectin/tenascin-C, which suggests an essential role of paxillin in LPA/PDGF induced cell spreading in presence of tenascin-C. In further support of an important role of paxillin in LPA/PDGF-induced cell spreading, we showed that ectopic expression of syndesmos, a molecule that binds syndecan-4 and paxillin, enabled cells to spread on fibronectin/tenascin-C. We showed that expression levels of TM1 are critical for cell rounding and cell spreading in the presence of tenascin-C, respectively. Whereas ectopic expression of TM1 restored cell

spreading on fibronectin/tenascin-C, knockdown of TM2/3 prevented LPA/PDGF-induced cell spreading on the mixed substratum. We also observed, that TM1 levels were tightly linked to expression of RhoA and activation of FAK, which suggests an interdependent regulation.

We observed that activation of the receptors for endothelin-1 (ET1) (EDNRB), and LPA/PDGF induced spreading in the presence of tenascin-C by distinct pathways. Whereas EDNRB-induced spreading was dependent on PI3K, PLC and JNK, but not on MEK, LPA/PDGF-induced cell spreading was dependent on PI3K and MEK, but not on PLC and JNK. Signaling by these factors was linked to activation of FAK and paxillin and, restoration of TM1 and RhoA expression. Together, our results suggest that at least two (and presumably many more) mechanisms exist that modulate the adhesive strength of a fibronectin/tenascin-C substratum. This supports the notion that combined signaling from the ECM and growth factors can determine cell adhesion and migration.

ET1 (through EDNRB) and LPA/PDGF did not only trigger cell spreading in the presence of tenascin-C but also stimulated cell migration on this substratum. In particular, LPA/PDGF-induced cell migration was PI3K and ROCK dependent. By using cells with ectopic expression of syndesmos and TM1, or reduced expression of TM1-3, we showed that both a strong as well as a loose adhesion blocked LPA/PDGF-induced cell migration on fibronectin/tenascin-C. These observations might be important for cancer diagnosis and may eventually allow to develop novel cancer treatments. In particular, we find that in gliomas a high expression of tenascin-C and of PDGF receptors a and b correlates with malignancy. Moreover, a high expression of syndesmos correlates with a bad 5 year survival prognosis and chemotherapy response rate in patients with oligodendrogliomas.

In summary, here it was shown that cell adhesion and migration on an anti-adhesive fibronectin/tenascin-C substratum can be modulated by additional signaling from growth factors. We identified a minimal set of critical targets of tenascin-C downstream of syndecan-4 that include FAK, paxillin, RhoA and TM1. Induction of EDNRA signaling by tenascin-C provides an additional mechanism that contributes to maintained cell rounding by a mechanism that again affects the same set of tenascin-C targets as those downstream of syndecan-4.

1.2 Extracellular matrix

The extracellular matrix (ECM) is a complex structural entity surrounding and supporting cells that are found within mammalian tissues. This matrix is composed of a variety of proteins and polysaccharides that are secreted locally and assembled into an organized meshwork in close association with the surface of the cell that produced them. The ECM has an active and complex role in regulating the behaviour of the cells that contact it. It determines their survival, development, migration, proliferation, cell shape and function. There are four prototypes of macromolecules of the ECM: collagens, elastin, proteoglycans and glycoproteins. One main family of the matrix glycoproteins are the nectins, such as fibronectin, vitronectin, thrombospondin and tenascin, which transducer signals to the cells embedded in the ECM. During cancer progression, the ECM of the tissue in which the tumor grows, is extensively remodelled, both by degradation of pre-existing ECM molecules and by neosynthesis of ECM components, which in many cases are not present in the ECM of normal tissue (Kaspar et al., 2006).

1.2.1 Fibronectin

Fibronectin (FN), a highly conserved multidomain adhesive glycoprotein, plays a prominent role in mediating ECM function. Secreted fibronectin usually forms dimers composed of two similar but not necessarily identical subunits of 250-280 kDa joined by a pair of disulfide bonds near their carboxyl terminal end (Ruoslathi, 1988). Each subunit contains three different types of repeating modular domains: 12 type I, 2 type II and 15 to 17 type III domains (**Fig. 1**), separated by regions of a flexible polypeptide chain. These domains are resistant to proteolysis, and may contain binding sites for ECM proteins such as collagen, cell-surface receptors (integrins), blood protein dervivatives (fibrin) and glycosaminoglycans (heparin). fibronectins play a critical role in the maintenance of normal cell morphology, cell adhesion, migration, hemostasis, thrombosis, wound healing, differentiation and proliferation (Hynes, 1990). A single gene encodes fibronectin but alternative splicing of pre-mRNA as well as posttranslational modifications allow formation of multiple isoforms, with up to 20 variants possible in human tissues (Schwarzbauer, 1991). Especially the fibronectin type III (FNIII) repeats are particulary widespread and one protein family that contains fibronectin type III repeats, is the tenascin family. The cell-binding domain contains a specific consensus tripeptide sequence, RGD sequence (Arg-Gly-Asp), which is recognised by several members of the integrin family of cell-surface matrix receptors, such as $\alpha_5\beta_1$ and $\alpha_v\beta_3$ (Pankov and Yamada, 2002; Clark and Brugge, 1995). The RGD sequence is located within the FNIII10 repeat. In addition, the adhesive region in FNIII9 has been called the synergy site (Obara et

al., 1988; Kimizuka et al., 1991) and is necessary for Integrin binding to FNIII10. Mutant mice, unable to produce fibronectin die early in embryogenesis because their endothelial cells fail to form proper blood vessels, which demonstrates an essential role of fibronecton in embryonic development.

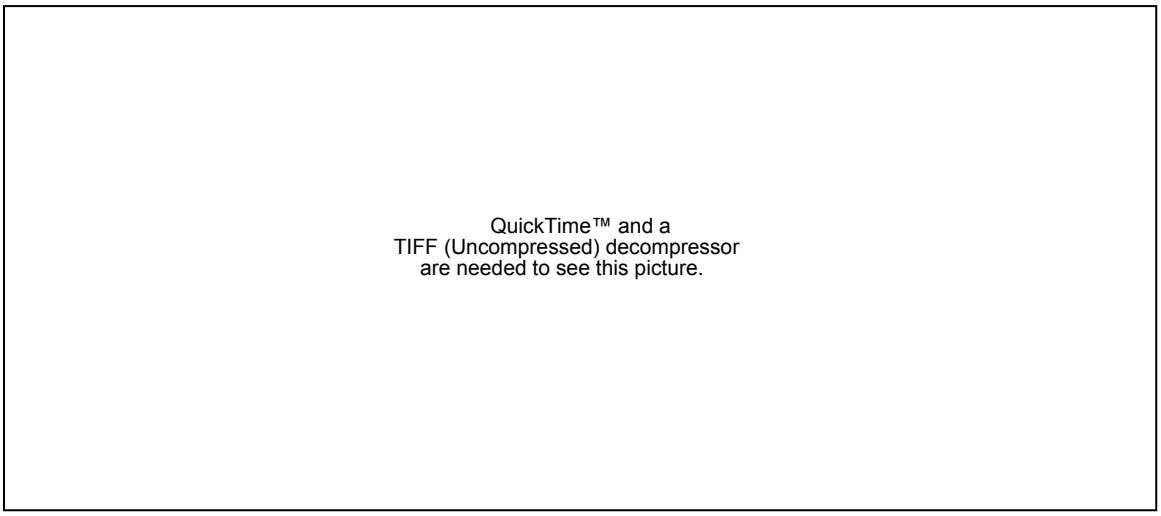


Fig.1: Overviev over domain composition of fibronectin and molecules binding to distinct domains.
In this scheme of fibronectin, narrow boxes, ovals, and wide boxes represent fibronectin type I, II, and III repeats, respectively. The heparin-binding sites (HepI-III), RGD, and the synergy site are marked and aligned with ineracting cell surface receptors (integrins; e.g., $\alpha 5\beta 1$), syndecan-4 (S4), and chondroitin of the recombinant fragments used and binding to tenascin-C are depicted above the model.
Huang et al., 2001

1.2.2 Tenascin-C

Tenascins are a family of large multimeric ECM proteins. Vertebrates express four tenascins termed tenascin-C, -R, -X and –W present in their connective tissues, with each tenascin having a specific expression pattern (Chiquet-Ehrismann, 2004). Tenascin-C (TNC) is an adhesion modulatory ECM molecule that is highly expressed in the microenvironment of most solid tumors, and indicates potential roles in tumor growth, metastasis, angiogenesis and immuno suppression (Orend and Chiquet-Ehrismann, 2003). Tenascin-C is highly expressed during embryogenesis, but in the adult organism its expression is restricted to a few sites under non-pathological conditions. Tenascin-C was also detected in stem cell niches (reviewed in Orend, 2005), as well as in T-lymphocyte-dependent zones in bone marrow, spleen, thymus and lymph nodes (Castanos-Velez et al., 1995). It is also induced in pathological conditions such as inflammation and wound healing (reviewed in Jones and Jones, 2000).

Tenascin-C is a huge molecule of about 300 kDa as an intact monomer and up to 1800 kDa when assembled into a hexamer. The multidomain molecule consists of an N-terminal assembly domain, followed by heptad- and 14 ½ EGF-like repeats, eight constant and up to 9 alternatively spliced fribronectin type III repeats and a C-terminal fibrinogen-like globular domain (see Fig. 2.2). Some studies have found certain alternatively spliced fibronectin type III repeats to be expressed in different cancer types (**Fig. 2**). Tenascin-C splice variants with extra repeats B/D are found in ductal carcinoma in situ (Adams et al., 2002) and with extra repeats A1/A2/A4 in invasive colorectal carcinoma (Dueck et al., 1999). The extra repeats AD1 were spliced in malignant human melanoma and ductal breast carcinoma (Derr et al., 1997), the extra repeat AD2 in malignant oral cancers (MIghell et al., 1997) and repeat C in malignant astrocytomas (Carnemolla et al., 1999; Viale et al., 2002).

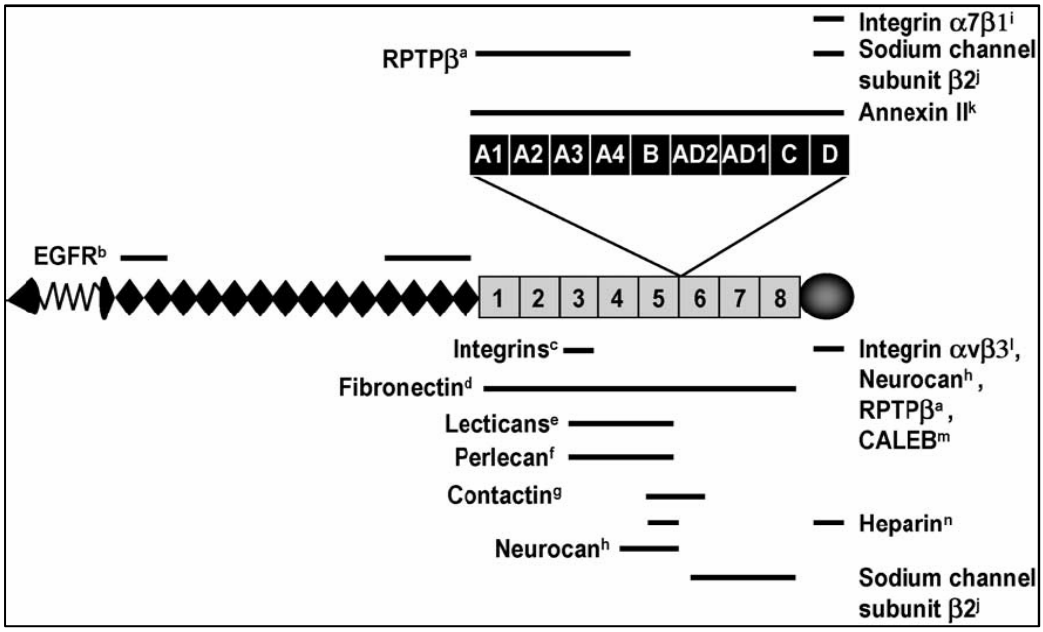


Fig. 2: Schematic representation of tenascin-C binding molecules (taken from Orend, 2005).
The N-terminal region, EGF-like, fibronectin type III anf fibrinogen-like domains are schematically depicted as triangle, rhombomeres, boxes and circle, respectively. The alternatively spliced fibronectin type III repeats A1-D found in human tenascin-C are highlited. Binding sites for interacting molecules within tenascin-C are shown: (a) Proteintyrosine phosphatase-β/ξ (RPTPβ) (Milev et al., 1997); (b) weak binding to EGF receptor (EGFR) in a mM K_D range (Swindle et al., 2001); (c) integrins αvβ3, αvβ6 (Yokosaki et al., 1996), and α8β1 (Denda et al., 1998) bind to RGD, α9β1 binds to IDG (Yokosaki et al., 1994); (d) fibronectin, fibronectin type III repeats 1-5 (Hauzenberger et al., 1999) and 6-8 (Chung et al., 1995); (e) the lecticans aggrecan, versican and neurocan bind to TNfnIII3-5 and to TNfnIII6-8 in a K_D range of 30-40 nM and 40-70 nM, respectively. Brevican binding to both regions within fibronectin was weaker in a μM K_D range (Day et al., 2004); (f) perlecan (Chung and Erickson, 1994); (g) contactin (Zisch et al., 1992); (h) neourocan (Rauch et al., 2001); (i) integrin α7β1 (Mercado et al., 2004); (j) sodium channel subunit β2 (Srinivasan et al., 1998); (k) annexin II (Chung and Erickson, 1994); (l) integrin αvβ3 (Yokoyama et al., 2000); (m) CALEB, chicken acidic leucine-rich EGF like domain containing brain protein (Schumacher et al., 2001); (n) heparin (Fischer et al., 1995; Jang et al., 2004; Weber et al., 1995).

Tenascin-C was shown to disturb the interaction of cells with fibronectin which leads to an increased proliferation of tumor cells (Chiquet-Ehrismann et al., 1988). The binding between tenascin-C and fibronectin does not involve the RGD-site of fibronectin. Recently it has been shown that tenascin-C binds to the fragments FNIII4-6 and FNIII13, but not to the fragment FNIII7-10 which includes the RGD site (Huang et al., 2001).

Apart from variations in the modular structure caused by alternative splicing, cleavage by matrix metalloproteinases (MMPs) can lead to specific domains being present in certain tissues. Tenascin-C has been shown to be cleaved by MMP7 and MMP2 (Siri et al., 1995). In melanoma cells, tenascin-C variants have been found that resemble the products of an *in vitro* cleavage of tenascin-C by the MMPs 1, 3 and 7 (Imai et al., 1994). The presence of tenascin-C leads to the induction or activation of certain MMPs.

Tenascin-C knock-out mice are viable, but show defects and deficits in the nervous system (reviewed in Chiquet-Ehrismann and Chiquet, 2003) and other tissues upon insults disrupting their homeostasis (Tucker and Mackie, 1999). Together with a very high conservation of its sequence throughout the kingdom of mammals, this demonstrates that tenascin-C is presumably essential foe life and its absence is compensated by several not well understood mechanisms.

1.2.3 Integrins

Cells interact with the ECM mainly via integrins, which display the major cell surface receptors for ECM ligands. They are widely expressed in all cell types in the body. Integrins comprise a heterodimer of two chains, termed α and β, which together form a ligand binding site and have short cytoplasmic domains that assemble a complex of kinases, adaptor proteins, and other signaling molecules following ligand binding, thus initiating downstream signaling pathways (Baron et al., 2004). The integrin family is comprised of 18 α and 8 β transmembrane subunits that form 24 different α/β-heterodimeric receptors for diverse ECM proteins (Hynes, 2002). Ligand binding to integrins initiates conformational changes in the integrin extracellular domain, integrin clustering and recruitment of cytoskeletal and signaling molecules (Dedhar and Hannigan, 1997; Shimoka et al., 2002). Besides structural functions, integrins transduce signals for the regulation of, for example, cell survival and proliferation in an outside-in and inside-out manner (Giancotti and Ruoslahti, 1999; Schwartz and Assoian, 2001; Watt, 2002; Brakebusch and Fässler, 2003).

The integrin α5β1 binds the ECM protein fibronectin and enables not only cell adhesion and cytoskeleton organization but also transduction of critical signals into the cells to promote survival, proliferation, differentiation, or migration programs. The primary binding site for this interaction is the RGD sequence in FNIII10, but within FNIII9 the PHSRN sequence provides synergistic effects for full α5β1 binding to fibronectin (Main et al., 1992; Aota et al., 1994).
A successful vascular development depends on fibronectin and its major receptor α5β1 integrin, but not on αvβ3, αvβ5, and α6β4 integrins that are instead central regulators of postnatal tumor angiogenesis (Serini et al., 2005). Tumor-associated blood vessels over-express fibronectin and its receptor α5β1, whereas the quiescent vasculature does not express these molecules, which confirms the importance of α5β1 integrins in pathological angiogenesis (Kim et al., 2000; Taverna and Hynes, 2001; Mettouchi and Meneguzzi, 2006).

1.2.4 Syndecan-4

The cell-surface heparin sulphated proteoglycan syndecan-4 acts in conjunction with the α5β1 integrin to promote the formation of actin stress fibers and focal adhesions in fibronectin-adherent cells, which will be discussed later. Syndecans are type I membrane proteins, with an N-terminal signal peptide, an ectodomain with several glycosaminoglycan attachment sites, a single transmembrane domain and a short C-terminal cytoplasmic domain. Syndecan core proteins range in size from 20 to 45 kDa. The ectodomains of syndecans display low sequence homology, whereas their transmembrane and cytoplasmic domains are highly conserved (reviewed in Woods, 2001; Woods et al., 1998; Carey, 1997; Bernfield et al., 1999). The cytoplasmic domain of each syndecan has two highly conserved regions, C1 and C2, proximal and distal to the membrane. A variable (V) region unique to each syndecan is flanked by the C1 and C2 regions (Yoneda and Couchman, 2003).
Although all members of the syndecans arose from a single ancestral gene, their expression patterns in tissues and during development are highly regulated (Woods et al., 1998; Carey, 1997; Bernfield et al., 1999). The terminal four amino acids (EFYA) of the cytoplasmic domain of all syndecan family members compose a binding site for the PDZ-containing proteins: synbindin, syntenin, CASK/LIN-2 and synectin (**Fig. 3**) (Ethell et al., 2000; Hsueh and Sheng, 1999; Gao et al., 2000; Grootjans et al., 1997). Syndesmos is another adaptor protein that binds to the cytoplasmic tail of syndecan-4 (Denhez et al., 2002). Syndecan-4 is the only family member to localize to sites of cell-matrix adhesions (Woods et al., 2000). Comparison of the localization of syndecan-4 with the focal adhesion marker protein vinculin suggests that syndecan-4 does not localize to newly formed contacts but with more established adhesion sites (Woods and Couchman, 1994).

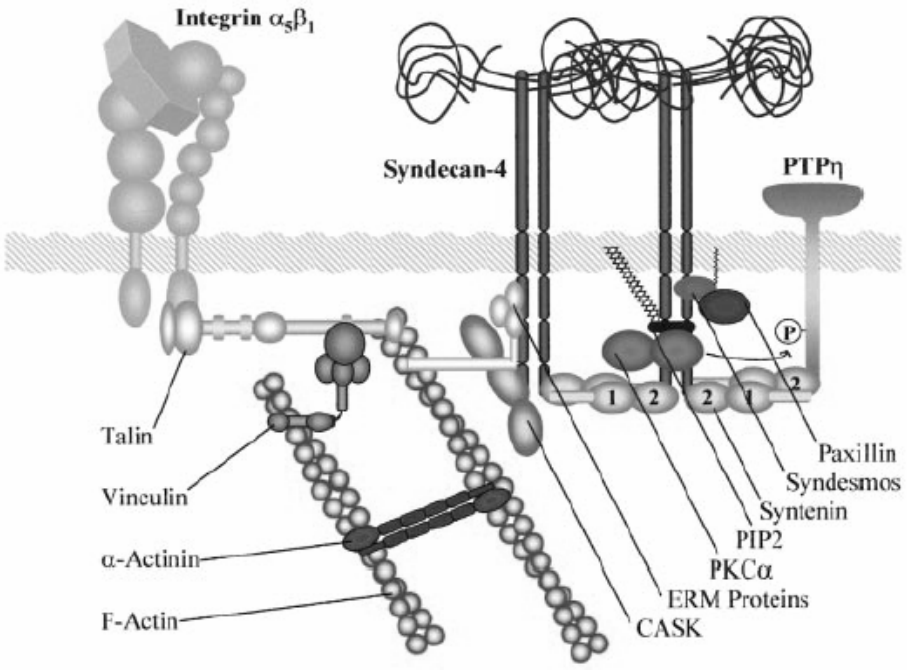


Fig. 3: Syndecan-4 acts as an organizing centre for transmembrane receptors and is anchored to the actin cytoskeleton.
The cytoplasmic domains of syndecan-4 interact with scaffold proteins, such as syntenin and CASK, that might in turn recruit additional transmembrane receptors (such as intergrins, phosphatases and growth factor receptors) to adhesions. Syndecan-4 is also linked to the actin cytoskeleton through CASK and the ERM family of actin-binding proteins. Clustering of syndecan-4 into focal adhesions is essential for adhesion formation in cells adhering via integrin $\alpha5\beta1$, and may depend on both a mechanical link between receptors and activation of signaling pathways. PTP, protein tyrosine phosphatase; 1 and 2 represent PDZ domains 1 and 2 respectively.
Bass and Humphries, 2002.

Mice, heterozygous or homozygous for a disrupted syndecan-4 gene (by homologous recombination) exhibit a statistically significant delay in the healing of skin wounds and show impaired angiogenesis in granulation tissue (Echtermeyer et al., 2000).

1.3 Formation of focal adhesions

Cell adhesion is important for such biological functions as cell growth, proliferation, survival, and migration. Cells adherent to ECM proteins (i.e. fibronectin) *in vitro*, cluster membrane adhesion receptors and intracellular cytoplasmic adaptor and signaling molecules into macromolecular complexes termed focal adhesions (Burridge and Chrzanowska, 1996). Focal adhesions are highly specialized type of attachment between actin filaments and the extracellular matrix that allows cells to pull on the substratum to which they bound. They create spots where the normal 50 nm gap between the bottom of the cell and the substratum is reduced to only 10-15 nm. At these sites, stress fibers, consisting of contractile bundles of actin and myosin II filaments, terminate at the plasma membrane, where clusters of intergrins are located. Their intracellular domains bind directly to bundles of actin filaments via the intracellular anchor proteins such as talin, vinculin, filamin, α-actinin, paxillin and focal adhesion kinase (FAK), which are recruited into focal adhesions. Previous studies have demonstrated that fibroblasts seeded onto an RGD-containing cell-binding domain (CBD) of fibronectin will attach but will not spread nor form focal adhesions and actin stress fibers (Saoncella et al., 1999; Woods et al., 1986). Denez et al., 2002, showed that syndecan-4, working in cooperation with the $\alpha 5\beta 1$ integrin, can stimulate a Rho-dependent pathway that will elicit focal adhesion and stress fiber formation in CBD-adherent cells. Thus, syndecan-4 and Integrin ligation is necessary for full cell spreading on fibronectin.

1.3.1 Focal adhesion kinase (FAK)

A large body of evidence has demonstrated that a rapid increase in the tyrosine phosphorylation of the non-receptor tyrosine kinase FAK is a prominent early event in fibroblasts stimulated by diverse signaling molecules that regulate cell proliferation, migration and survival, including mitogenic agonists that act via G protein-coupled receptors (GPCR), growth factors, integrin clustering by cell adhesion, and bacterial toxins (Zachary and Rozengurt, 1992; Rozengurt, 1995; Hanks and Polte, 1997; Schlaepfer et al., 1999; Schaller, 2001; Parson, 2003). FAK is an ubiquitinously expressed 125 kDa protein, which is composed of an N-terminal FERM (protein 4.1, ezrin, radixin and moesin homology) domain, a central kinase domain, prolin-rich regions and a C-terminal focal-adhesion targeting (FAT) domain (see Fig.). The best-characterized FAK phosphorylation event is autophosphorylation at Tyr397, which can occur in either cis or trans (Toutant, 2002). Phosphorylation of FAK at Tyr397 creates a motif that is recognized by various SH2-domain-containing proteins, such as Src-family kinases (SFKs), phospholipase Cγ (PLCγ), suppressor of cytokine signalling

(SOCS), growth-factor-receptor-bound protein-7 (GRB7), the Shc adapter protein, p120RasGAP, and the p85 subunit of phosphatidylinositol 3-kinase (PI3K) (**Fig. 4**) (Parson, 2003; Schlaepfer et al., 2004; Hanks et al., 2003; Schaller, 2001).

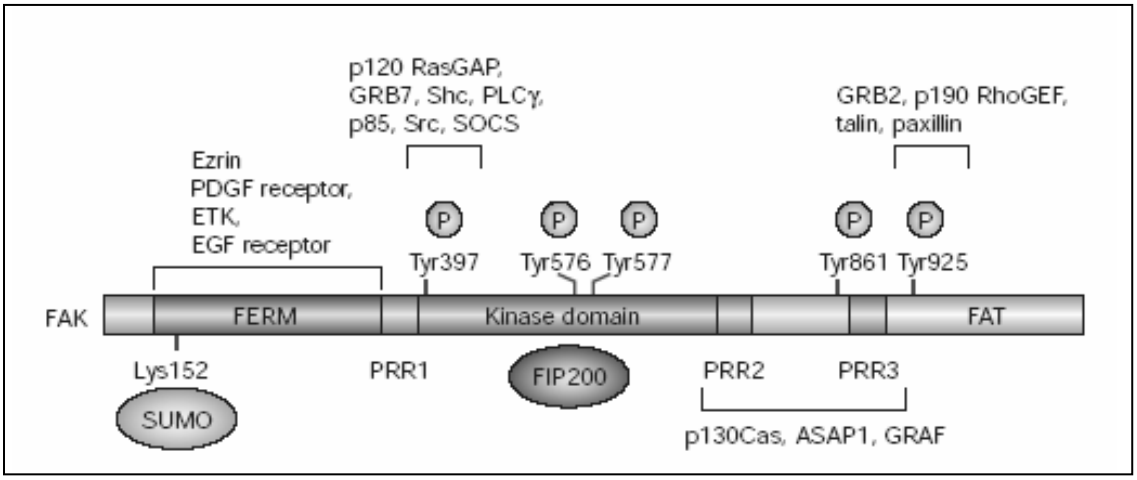


Fig. 4: Focal adhesion kinase domain structure and phosphorylation sites.
The FERM domain mediates interactions of FAK with the epidermal growth factor (EGF) receptor, platelet-derived growth factor (PDGF) receptor, the ETK tyrosine kinase and ezrin, and the FERM domain can be conjugated to SUMO (small ubiquitin-related modifier) at Lys152. The FAT domain recruits FAK to focal contacts by associating with integrin-associated proteins such as talin and paxillin. It also links FAK to the activation of Rho GTPases by binding to guanine nucleotide-exchange factors (GEFs) such as p190 RhoGEF. FAK contains three praline-rich regions (PRR1-3), which bind Src-homology-3 (SH3) domain-containing proteins such as p130Cas, the GTPase regulator associated with FAK (GRAF) and the Arf-GTPase-activating protein ASAP1. FAK is phosphorylated at (P) at several tyrosine residues, including Tyr397, 407, 576, 577, 861 and 925. Tyrosine phosphorylation on Tyr397 creates a Src-homology-2 (SH2) binding site for Src, phospholipase Cγ (PLCγ), suppressor of cytokine signalling (SOCS), growth-factor-receptor-bound protein 7 (GRB7), the Shc adaptor protein, p120 Ras GAP and the p85 subunit of phosphatidylinositol 3-kinase (PI3K). Phosphorylation of Tyr576 and Tyr577 within the kinase domain is required for maximal FAK catalytic activity, whereas the binding of FAK-family interacting protein of 200 kDa (FIP200) to the kinase region inhibits FAK catalytic activity. FAK phosphorylation at Tyr925 creates a binding site for GRB2. Reviewd in Mitra et al., 2005)

Ser910, a further important phosphorylation site within the FAT domain is phosphorylated during mitosis (Ma et al., 2001) and after growth factor stimulation of cells. Ser910 is phosphorylated by ERK2 and this is also associated with reduced paxillin binding to FAK (Hunger-Glaser et al., 2004).

FAK-mediated signal transduction is underscored by the fact that this tyrosine kinase plays a fundamental role in embryonic development (Ilic et al., 1995; Ilic et al., 1997)) and in the control of cell migration, cell cycle progression (Zhao et al., 2003) and apoptosis (Hungerford et al., 1996; Xu et al., 1996; Gervais et al., 1998; Golubovskaya et al., 2003). Furthermore, there is increasing evidence linking overexpression of FAK to the invasive properties of cancer cells (Owens et al., 1996; Mukai et al., 2002; Gabarra-Niecko et al., 2003; Hsia et al., 2003; Sood et al., 2004; Schlaepfer et al., 2004). However, focal contacts in in $FAK^{-/-}$ cells

form primarily around the cell periphery, enmeshed in a cortical actin ring, and do not undergo a normal maturation cycle (Sieg et al., 1999). Compensation by the FAK homologue Pyk2 might play a role in adhesion of FAK-/- cells (Gutenberg et al., 2004).

1.3.2 Paxillin

Paxillin is a 68 kDa phosphoprotein originally identified as a substrate for the non-receptor tyrosine kinase Src in Rous sarcoma virus-transformed fibroblasts (Glenny and Zokas, 1989; Turner at al., 1990). This multi-domain adaptor protein has previously been implicated in integrin signaling, cell motility (Turner et al., 2005) and disassembly of focal adhesions (adhesion turnover) in the protrusion of migrating cells (Horwitz et al., 2006). Functionally, paxillin has been implicated in the regulation of cell adhesion, spreading, muscle differentiation and gene expression through its ability to directly interact with multiple structural and signaling proteins involved in coordinating these events, such as tubulin, p120RasGAP, PKL, PTP-PEST, FAK, Src, Crk and Csk (Turner et al., 2005) (**Fig. 5**). Structurally, paxillin consists of an N-terminal region containing five LD protein-protein interaction motifs, a proline-rich region possibly involved in Src-SH3 binding, the SH2 binding phosphotyrosine residues 31 and 118. The C-terminal region containing four LIM domains responsible for focal adhesion targeting and binding to the phosphotyrosine phosphatase PTP-PEST microtubule protein tubulin (Brown et al., 1998; Cote et al., 1999; Herreros et a., 2000; Schaller and Parsons, 1995; Tumbarello et al., 2002; Turner, 2000).

Importantly, phosphorylation of paxillin at Y31 and Y118 mediates the interaction with Crk and p120RasGAP (Schaller and Parson, 1995; Tsubouchi et al., 2002). These interactions are associated with cytoskeletal regulation through modulation of the Rho GTPases, Rac1 and RhoA respectively, and appear to perform cell specific roles in regulating integrin signaling and migration (Lamorte et al., 2003; Petit et al., 2000). Phosphorylation of paxillin at S85 and Y31 and Y118 regulates cell migration (Huang et al., 2004; Petit et al., 2000). The LD4 motif serves as a binding site for a number of signaling molecules, including focal adhesion kinase (FAK), G-coupled receptor kinase-interacting protein 1 (GIT1) and p95 paxillin-kinase linker (PKL) (Horwitz et al., 2005). Extracellular signal-regulated kinase (ERK), has recently been shown to localize to adhesions and to regulate paxillin disassembly (Fincham et al., 2000; Webb et al., 2004).

Paxillin is the founding member of a family of related proteins that now contains three members, paxillin, hic-5 and leupaxin. Hic-5 is 57% identical to paxillin and contains an organization of N-terminal LD motifs and C-terminal LIM domains similar to paxillin. Hic-5 localizes to focal adhesions and shares a number of binding partners with paxillin including FAK (Hagmann et al., 1998; Thomas et al., 1999; Ishino et al., 2000) and syndecan-4.

Leupaxin exhibits 37% identity with paxillin and contains the same domain organization as paxillin with N-terminal LD motifs and four C-terminal LIM domains (Lipsky et al., 1998). As expected from the sequence observation, leupaxin can associate with anumber of the same proteins that can bind paxillin including the FAK-related protein, Pyk2 (Lipsky et al., 1998). It is anticipated that some of the functions of paxillin, hic-5 and leupaxin overlap given the high degree of sequence identity within the LD motifs and LIM domains. However, within the N-terminal domains of these proteins there are regions of sequence divergence, suggesting that each protein might also perform unique functions. The phenotype of the paxillin-/- mice closely resembles that of fibronectin-/- mice, suggesting that paxillin is a critical transducer of signals from fibronectin receptors during early development (Hagel et al., 2001).

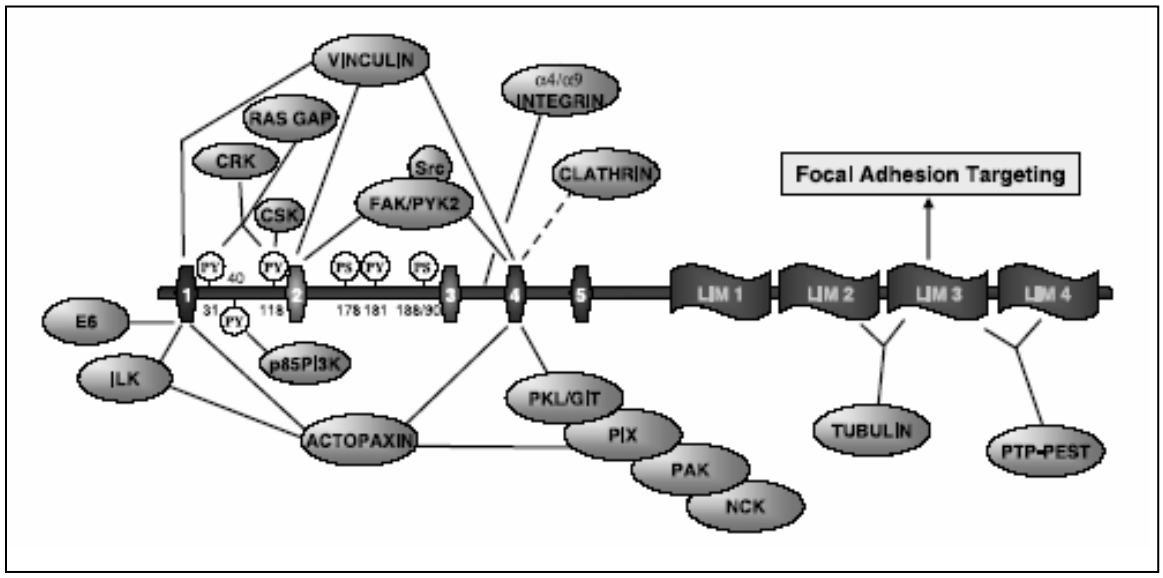


Fig. 5: Paxillin binding partners.
Paxillin is comprised of multiple protein binding motifs, including the amino-terminal LD motifs, the carboxy-terminal LIM domains, and several phosphotyrosine-SH2 domain docking sites. These range from structural actin binding proteins including actopaxin and vinculin to important signalling molecules such as FAK (focal adhesion kinase), ILK (integrin-linked kinase), and PTP-PEST, a tyrosine phosphatase. Many other potential paxillin binding partners have been identified in coprecipitation experiments, although in many cases it remains to be determined whether the interaction with paxillin is direct and which domain of paxillin is involved.
Brown and Turner, 2004

1.3.3 Syndesmos

Syndesmos is a cytoplasmic protein that interacts specifically with the cytoplasmic domain of syndecan-4, and co-localizes with syndecan-4 in focal contacts. The interaction of syndesmos involves both the C1 and the V subdomains of the cytoplasmic domain of syndecan-4 (Baciu et al., 2000). Syndesmos is ubiquitinously expressed and can be myristylated. Denhez et al., (2002) found that syndesmos interacts with the focal adhesion adaptor protein paxillin in a direct manner and also binds the paxillin homolog Hic-5. These interactions are triggered by the activation of protein kinase C (PKC). The connection of syndecan-4 with paxillin through syndesmos parallels the connection between paxillin and integrins and may thus reflect the cooperative signaling of these two receptors in the assembly of focal adhesions and actin stress fibers. When overexpressed in NIH3T3 cells, syndesmos accelerates cell spreading, enhances the assembly of actin stress fibers and focal adhesion formation in a serum-independent manner (Baciu et al., 2000).

1.4 Activated downstream signaling cascades

1.4.1 Enzyme-linked receptors

Receptor mediated signaling can occur through two different types of transmembrane receptors, the enzyme-linked and G-protein-linked cell-surface receptors. Enzyme-linked receptors are transmembrane proteins and five classes have thus been identified: receptor tyrosine kinases, tyrosine-kinase-associated receptors, receptor serine/threonine kinases, transmembrane guanylyl cyclases and histidine-kinase-associated receptors. The extracellular signals that act through receptor tyrosine kinases (RTKs) consists of a large variety of secreted growth factors and hormones including epidermal growth factor (EGF), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), insulinlike growth factor-1 (IGF-1) and vascular endothelial growth factor (VEGF) amongst others. Ligand binding to RTKs induces the receptors to cross-phosphorylate their cytoplasmic domains on multiple tyrosines. The autophosphorylation activates the kinases, as well as producing a set of phosphotyrosines that then serve as docking sites for a set of intracellular signaling proteins, which bind via their SH2 (Src homology region) domains. Some of the receptor-bound proteins serve as adaptors to couple the receptors to the small GTPase Ras, which, in turn, activates a cascade of serine/threonine phosphorylations that converge on a MAPK, which relays the signal to the nucleus by phosphorylating gene regulatory proteins there. Ras can also activate another protein that docks on activated RTKs, PI3K (phosphatidylinositol 3-

kinase), which generates specific inositol phospholipids that serve as docking sites in the plasma membrane for signaling proteins with pleckstrin homology domains (PH), including PKC (protein kinase B).

1.4.2 G protein-coupled receptors (GPCR)

G protein-coupled receptors (GPCRs), also known as seven transmembrane receptors, are a large family of transmembrane receptors that sense molecules outside the cell and activate inside signal transduction pathways. The extracellular loops contain two highly conserved cysteine residues which build disulfide bonds to stabilize the receptor structure. The G proteins are attached to the cytoplasmic face of the plasma membrane. There are various types of G proteins, e.g. the stimulatory G protein (Gs), which activates adenyl cyclase and thereby increases cyclic AMP concentration; the inhibitory G protein (Gi), which inhibits adenyl cyclase, but it mainly acts by directly regulating ion channels; and G protein Gq, which in turn activates phospholipase C-β. All G-proteins have the same structure and operate in a similar way. G proteins are composed of three protein subunits, α, β and γ. In the unstimulated state, the α subunit has GDP bound and the G protein is inactive. When stimulated by an activated receptor, the α subunit, which is a GTPase, releases its bound GDP, allowing GTP to bind in its place. This exchange causes the trimer to dissociate into two activated components, an α subunit and a $\beta\gamma$ complex. GTP binding causes a conformational change that affects the surface of the α subunit that associates with the $\beta\gamma$ complex in the trimer. The GTPase activity of the α subunit is greatly enhanced by the binding of a second protein, a regulator of G protein signaling (RGS), which acts as GTPase activating protein (GAPs). Activated G-protein-linked receptors themselves are phosphorylated by G-protein-linked receptor kinases (GRKs), which uncouple the receptors from G proteins and promotes receptor endocytosis. Some G-protein-linked receptors either activate or inactivate adenyl cyclase, thereby altering the intracellular concentration of the mediator cAMP. Others activate a phosphoinositide-specific phosphplipase C (PLCβ), which hydrolyzes phosphatidylinsositol 4,5-biphosphate (PIP_2). Protein kinase C and A (PKC and PKA) and Ca^{2+}/calmodulin-dependent protein kinase (CaM-kinase) phosphorylate specific target proteins on serine or threonines and thereby alter the activity of the proteins. One of this downstream phophorylations lead to the induction of the activation of myosin light chain kinase (MLCK), which is linked to actin polymerization.

1.4.3 MAPK and JNK signaling pathway

Mitogen-activated protein kinases (MAPKs) are important signal transducing enzymes that are involved in many facets of cellular regulation. MAPKs phosphorylate specific serines and threonines of target protein substrates and regulate cellular activities ranging from gene expression, mitosis, movement, metabolism, and programmed cell death (Johnson and Lapadat, 2002). MAPK activity is regulated through three-tiered cascades composed of a MAPK, MAPK kinase (MAPKK, MKK or MEK) and a MAPKK kinase or MEK kinase (MAPKKK or MEKK) (English, 1999). There are at least four distinctly regulated groups of MAPKs expressed, extracellular signal-related kinases (ERK1/2), c-Jun amino-terminal kinases (JNK1/2/3), p38 proteins (p38α/β/γ/δ) and ERK5. These MAPKs are activated by specific kinases, MAPKKs : MEK1/2 for ERK1/2, MKK3/6 for the p38 proteins, MKK4/7 for the JNKs, and MEK5 for ERK5 (Chang and Karin, 2001).

ERK1 and ERK2 are widely expressed and are involved in the regulation of meiosis, mitosis, and postmitotic functions in differentiated cells. Many stimuli, including growth factors, cytokines, virus infection, ligands for heterodimeric guanine nucleotide-binding protein (G-protein)-coupled receptors, transforming agents, and carcinogens, activate the ERK1 and ERK2 pathways. Both are components of a three-kinase phospho-relay module which can be activated by the proto-oncogene *Ras*. Oncogenic Ras persistently activates the ERK1 and ERK2 pathway through the MAPKKK activity of Raf-1, which phosphorylates and activates MEK1/2. MEK1/2 finally activate ERK1/2 by phosphorylation of threonine and tyrosine residues in the regulatory Thr-Glu-Tyr (TEY) motif (Robinson and Cobb, 1997). This contributes to an increased proliferative rate of tumor cells. For this reason, inhibitors of the ERK pathway are entering clinical trials as potential anticancer agents.

The JNKs were discovered to bind and phosphorylate the DNA binding protein c-Jun and increase its transcriptional activity. Regulation of the JNK pathway is extremely complex and is influenced by 13 MKKKs that regulate the stimuli of JNKs. JNKs are important in controlling programmed cell death or apoptosis (Tournier et al., 2000). Inhibition of JNKs enhances chemotherapy-induced inhibition of tumor cell growth, suggesting that JNKs may provide a molecular target for the treatment of cancer.

1.4.4 Endothelin-induced signaling cascade

Endothelins are a family of small, structurally related, vasoactive peptides that have a great number of physiological roles in many tissues. The 'endothelin axis' consitsts of three 21 amino acid peptides (ET1, ET2 and ET3), two G-protein-coupled receptors EDNRA and EDNRB, and two activating peptidases or endothelin-converting enzymes (ECE-1 and ECE-2). The three endothelin peptide isoforms derive from three separately regulated genes yet having a similar structure (Inoue et al., 1989). The gene for each endothelin has a distinct pattern of tissue expression: ET1 is expressed primarily by endothelial cells, ET2 in epithelial cells of the kidney and intestine, and ET3 is found in the brain (Saida et al., 2000). Endothelins and their receptors are also expressed by `mobile´ inflammatory cells such as monocytes and macrophages (Grimshaw et al., 2002; Ehrenreich et al., 1990). Binding of the ligands to their receptors, EDNRA and B, which are coupled to the G_s and $G_{q/11}$ proteins (Landry et al., 2005), may modulate several overlapping signalling pathways (**Fig. 6**). ET1 receptors can activate RhoA through the heterotrimeric G-protein α-subunits $G_{\alpha 13}$ (Hersch et al., 2004) and $G_{\alpha 13}/G_{\alpha q}$ (Mao et al., 1998; Yuan et al, 2001), respectively. The EDNRA is highly specific for ET1, whereas EDNRB also binds ET1, ET2, and ET3 with similiar affinity (Rubanyi and Polokoff, 1994).

Numerous tumors, including carcinomas of the lung (Ahmed et al., 2000), prostate (Nelson et al., 2000) and ovary (Bagnato et al., 1999) produce one or more of the endothelins and their receptors (Nelson et al., 2003). Endothelins play an autocrine and paracrine role in regulating growth of seceral tumor types and may also inhibit apoptosis (Grimshaw et al., 2002) and promote angiogenesis (Bagnato et al., 2002).

However, the actions of endothelins in cancer are unclear and appear to be tumor-specific. In prostate cancer, EDNRB is decreased or absent (Kopetz et al., 2002) and there is often methylation of the EDNRB gene (Nelson et al., 1997), while in lung cancer, EDNRA is down-regulated (Ahmed et al., 2000). ET1 enhances the secretion of MMPs, disrupts intracellular communications, and stimulates cell migration (Rosano et al., 2006). In ovarian carcinoma cells, where ET1 and EDNRA are overexpressed, ET1 induces loss of adherens and tight-junction protein expression, E-cadherin, beta-catenin, and gain of N-cadherin and vimentin expression. These results confirm the ability of ET1 to promote EMT, a metastable process involving sustained loss of epithelial markers and gain of mesenchymal markers. These findings provide evidence for a critical role for the ET1/EDNRA axis during distinct steps of ovarian carcinoma progression (Rosano et al., 2006).

In contrast to ovarian cancer with a high EDNRA expression, EDNRB is overexpressed in human cutanous melanomas, where it promotes tumorigenesis upon activation by ET1 or

ET3, thus representing a potential novel therapeutic target (Spinella et al., 2007). Spinella et al. (2007) showed that under normoxic conditions, EDNRB activation by ET1/ET3 enhances vascular endothelial growth factor (VEGF) up-regulation, cyclooxigenase (COX)-1/COX-2 protein expression and COX-2 promotor activity, prostaglandin E(2) (PGE(2)) production, and do so to a greater extent under hypoxia. These results identify a new mechanism whereby ET1/ET3/EDNRB axis can promote and interact with the HIF-1alpha-dependent machinery to amplify the COX-mediated invasive behaviour of melanoma cells (Spinella et al., 2007).

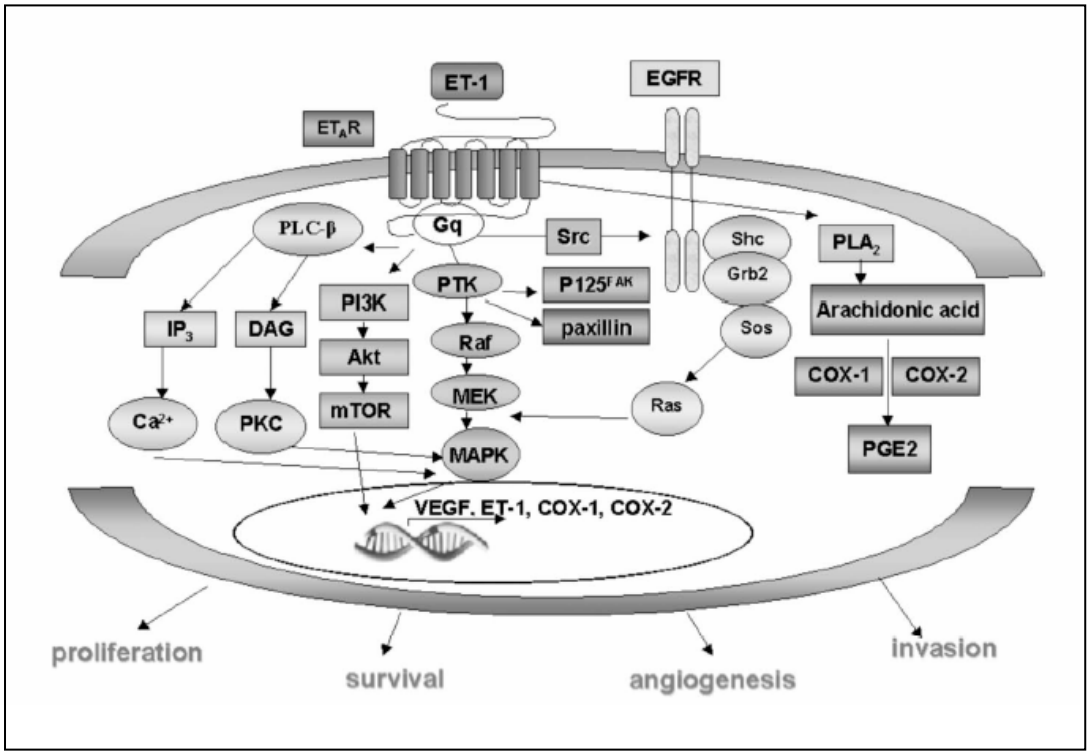


Fig. 6: ET1-induced signal transduction pathways in ovarian cancer cells.
Binding of ET-1 to EDNRA/EDNRB triggers signal transduction pathways through a pertussis-insensitive G protein that is coupled to the EDNRA/B intracellular domain. Activation of phospholipase C (PLC), protein tyrosine kinases (PTKs; such as FAK and paxillin) ultimately results in the activation of the RAF/MEK/MAPK pathway. ET1 also causes Src-mediated epidermal growth factor receptor (EGFR) transactivation that is in part responsible for MAPK activation. ET1 also stimulates phosphatidylinositol 3-kinase (PI3K)-mediated Akt activation. Parallel mobilization of intracellular calcium (Ca^{2+}), activation of protein kinase C (PKC), MAPK and Akt induces nuclear transcription of genes, such as VEGF, ET1, COX-1 and 2, leading to proliferation, survival, angiogenesis and invasion. Further analysis showed that ET1 promotes cyclooxigenase (Cox)-1 and -2 expression and, in turn, prostaglandine (PG) E2 production, and amplifying ET1 driven VEGF production. DG, Diacylglycerol; IP3, inositol 1, 4, 5 triphosphate; MAPK, mitogen activated protein kinase; mTOR, mammalian target of rapamycin; MEK, MAPK kinase; p125 FAK, focal adhesion kinase; PLA, phospholypase A; VEGF, vascular endothelial growth factor.
Bagnato et al., 2005

1.4.5 Lysophosphatidic acid (LPA)

Lysophosphatidic acid (LPA), which is generated from precursors in the plasma membrane, has numerous cellular effects including cell proliferation, calcium homeostasis, cytoskeleton reorganization, cell adhesion and migration, and ion transport regulation (Moolenaar, 1999; van Leeuwen et al., 2003; Sengupta et al., 2004). LPA is a serum-borne phospholipid with hormone and growth factor-like properties and has been shown to modulate tumor cell invasion and malignant cell growth (Stähle et al., 2003). LPA exerts its biological activities through its interaction with four identified LPA receptors, namely LPA_1, LPA_2, LPA_3, LPA_4, which additionally activate the G_i, $G_{12/13}$ and G_q subfamilies of G proteins **(Fig. 7)** (Moolenaar et al., 2004). While LPA_1 is expressed ubiquitously, recent studies have shown that the levels of LPA_2 and LPA_3 are elevated in various tumors such as ovarian, colon, breast and prostate cancers (Daaka, 2002; Fujita et al., 2003) Kitayama et al., 2004; Shida et al., 2004). LPA can promote the expression/activation of various invasion-associated proteases including metalloproteinases in ovarian cancer cells (Pustilnik et al., 1999; Fishman et al., 2001) and enhances proangiogenic factor production (VEGF, IL-6 and 8) by ovarian cancer cells (Schwartz et al., 2001; Hu et al., 2003). LPA activates Rac1 to promote cell spreading, lamellipodium formation and cell migration in fibroblasts (van Leeuwen et al., 2003), as well as RhoA through the $G_{12/13}$ subunits of G proteins (Kranenburg et al., 1999; Yuan et al., 2003). Bian et al. (2005), have shown that $G_{12/13}$-, but not G_q-associated signaling pathway mediated LPA-induced focal adhesion kinase (FAK) autophosphorylation and was important for efficient LPA-stimulated cell migration. Furthermore, LPA-induced ERK activation results in a transient translocation of the phosphorylated ERK to newly forming focal contact sites at the leading edge of the migrating cell (Stähle et al., 2003).

It has been shown that LPA-induced microtubule (MT) rearrangement was accompanied by accumulation of myosin IIB and polymerized actin at the base of retraction fibers. Fukushima and Morita, 2006, showed that LPA did not induce MT depolymerization and that LPA-induced actomyosin activation produced MT and neurofilament rearrangement, leading to neurite remodelling.

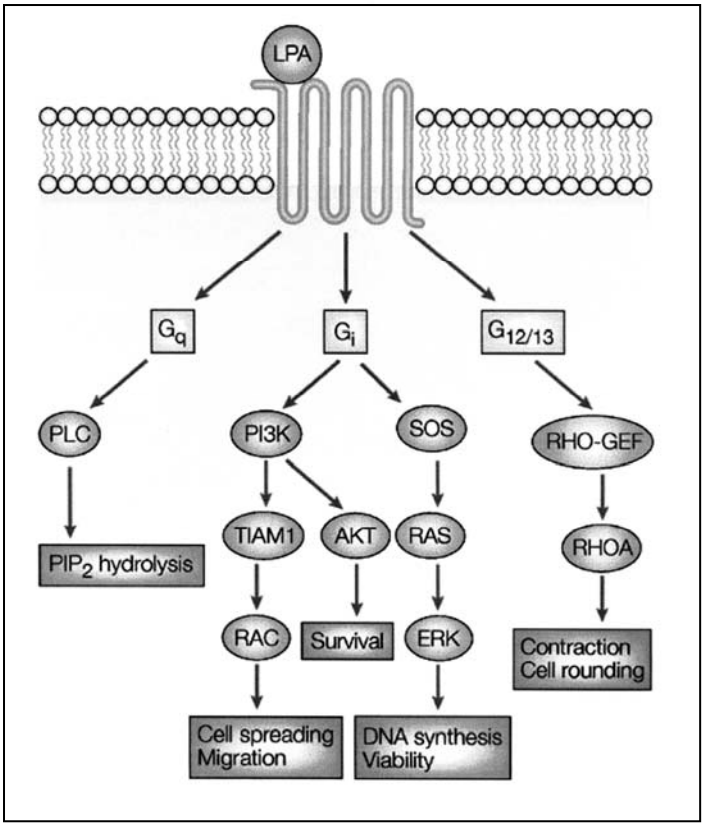


Fig. 7: The role of LPA
LPA signals through its own GPCR via at least three distinct classes of heterotrimeric G proteins- G_q, G_i and $G_{12/13}$- leading to activation of multiple downstream effector pathways. Among the main LPA-induced signaling pathways are: G_q (or/and G_i)-mediated activation PLC, which leads to the hydrolyses of PIP_2, with consequent calcium mobilization and PKC activation.; G_i-mediated activation of the Ras-ERK pathway, leading to cell proliferation; G_i-mediated activation of the PI3K-AKT 'survival' pathway, which suppresses apoptosis; and activation of the RHO and RAC GTPases via specific exchange factors, RHO-GEF and TIAM1. which leads to cytoskeletal remodelling (contraction and spreading), shape changes and cell migration.
Mills and Moolenaar. 2003

1.4.6 PI3K-Akt/PKB pathway

The signals set off by phosphoinositide 3-kinase (PI3K) at the membrane promote cell growth, cell cycle progression, migration, and cell survival. Phosphoinositide 3-kinases have been divided into three distinct classes (I, II and III). These kinases can be activated by receptor tyrosine kinases, as well as by many other types of cell-surface receptors, including G-protein-linked receptors. The so- called class IA isoforms of PI3Ks are tightly associated with the p85 regulatory subunit that contains two src homology 2 (SH2) domains. Once activated, the 110kDa-subunit of PI3K is brought in close proximity of the plasma membrane. There, PI3K catalyzes the phosphorylation of membrane-bound phosphatidylinositol-4,5-bisphosphate (PIP_2) at the 3´position of the inositol ring, producing the second messenger phosphatidylinositol-3,4,5-trisphosphate (PIP_3). This subsequently causes translocation of PIP_3-binding proteins to the plasma membrane (reviewed in Wymann et al., 2003) and they serves as docking site for a subgroup of proteins containing a pleckstrin homology (PH) domain, including the protein kinase B (also called Akt). After translocation to the membrane, PKB is phosphorylated by phosphoinositide-dependent kinase 1 (PDK1) on Thr308 and Ser473. The activation of class I PI3Ks is counteracted by phosphoinoside phosphatases, such as PTEN, which dephosphorylate PIP_3 to PIP_2 (Brazil and Hemmings, 2001). The

PI3K/PKB/TOR axis controls protein synthesis and growth, while PIP_3-mediated activation of Rho GTPases directs cell motility. PI3K activity has been linked to the formation of tumors, metastasis, chronic inflammation, allergy and cardiovascular diseases (Wymann and Marone, 2005).

1.4.7 PDGFR signaling

Platelet-derived growth factor (PDGF) exerts its stimulatory effects on cells by binding to two related protein tyrosine kinase receptors, PDGFRα and PDGFRβ. PDGF is a potent stimulator of growth and motility of connective tissue cells, such as fibroblasts and smooth muscle cells, but also for capillary endothelial cells and neurons (Zhang and Hutchins, 1997). It has important roles during embryonic development and in the adult. PDGF stimulates wound healing (Hanaoka et al. 2006) and has an important role in the maintenance of the interstitial fluid pressure (Heldin et al., 2004). Moreover PDGF has been implicated in the regulation of the tonus of blood vessels.

PDGF is a dimeric molecule consisting of disulfide-bonded A-and B-polypeptid chains. Homodimeric (PDGF-AA, PDGF-BB) as well as heterodimeric (PDGF-AB) isoforms exert, as well as the new identified isoforms C and D, that signal through the α and β receptor (PDGFR). The two receptors bind the PDGF isoforms with different affinities. The β-receptor binds PDGF-BB with high affinity (K_d, 0.5 nm) and PDGF-AB with lower affinity (K_d, 2.5 nm), but there is no appreciable affinity for PDGF-AA. The α-receptor binds all three PDGF isoforms with similar affinities (Claesson-Welsh, 1994). Ligand binding induces receptor dimerization and autophosphorylation, allowing binding and activation of cytoplasmic SH2-domain containing signal transduction molecules. PDGF-AA induces α-α homodimers, PDGF-AB induces α-α homodimers and α-β heterodimers, and PDGF-BB form all three types (α-α, α-β and β-β) of dimers. Dimerization of the receptors is the key event in PDGF receptor activation, which then allows phosphorylation in trans between the two receptors in the complex. One of the most important autophosphorylation sites, Y857, is located inside the kinase domain. More than 10 different SH2-domain-containing molecules have been shown to bind different autophosphorylation sites in the PDGF α- and β-receptors, including signal transduction molecules with enzymatic activity, such as PI3K, PLCγ, the Src family of tyrosine kinases, the tyrosine phosphatase SHP-2, GAP of Ras, as well as adaptor molecules such as Grb2, Shc, Nck, Grb7 and Crk and Stat5 **(Fig. 8)**. Thereby, Ras-Raf-ERK/MAPK, PI3K-Akt/PKB and PLCγ signaling pathways are initiated leading to cell growth, actin reorganization, migration and differentiation.

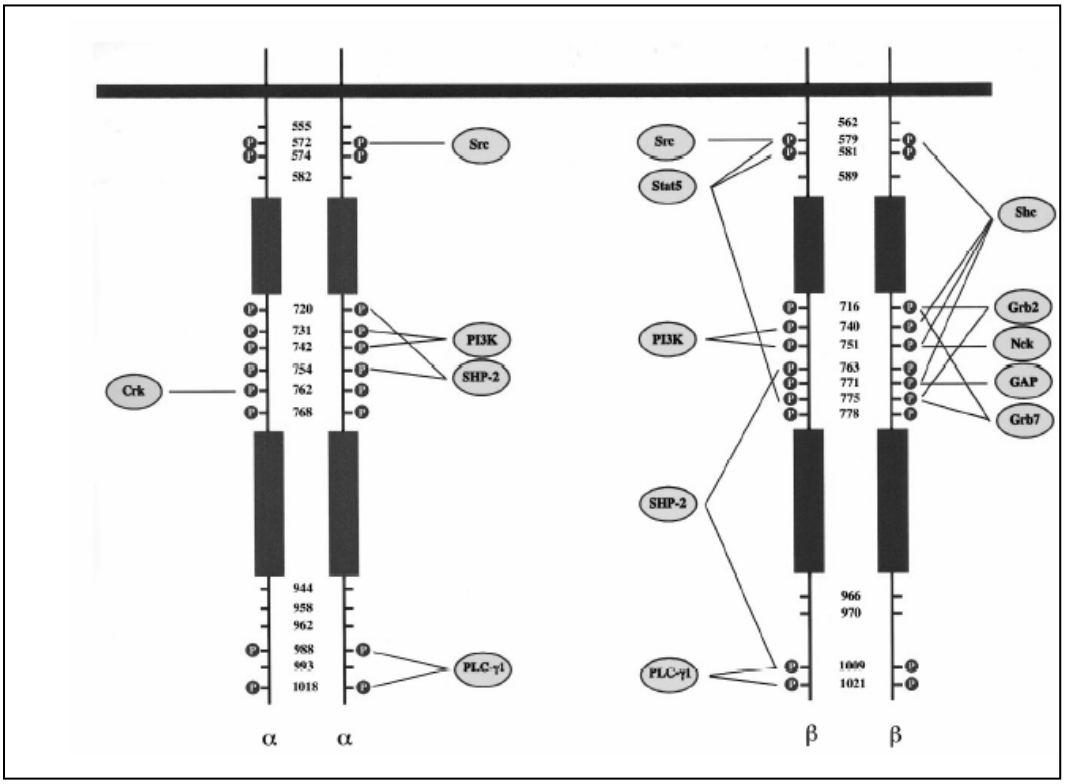


Fig. 8: Interaction between PDGF α- and β-receptors and SH2-domain containing signal transduction molecules. The intracellular part of homodimeric complexes of α- and β-receptors are depicted.
All tyrosine residues outside the catalytic domains and their numbers are indicated; known autophosphorylation sites are indicated by an encircled P. The conserved tyrosine residues in the kinase domains which are involved in the regulation of the catalytic activities are also indicated. The known interactions between individual phosphorylated tyrosine residues and different SH2 domain containing signal transduction molecules are shown.
Heldin et al., 1998

Recent observations suggest that extensive cross-talk occurs between different signaling pathways downstream of activated PDGFR, and that stimulatory signals are modulated by inhibitory signals arising in parallel **(Fig. 9)**.

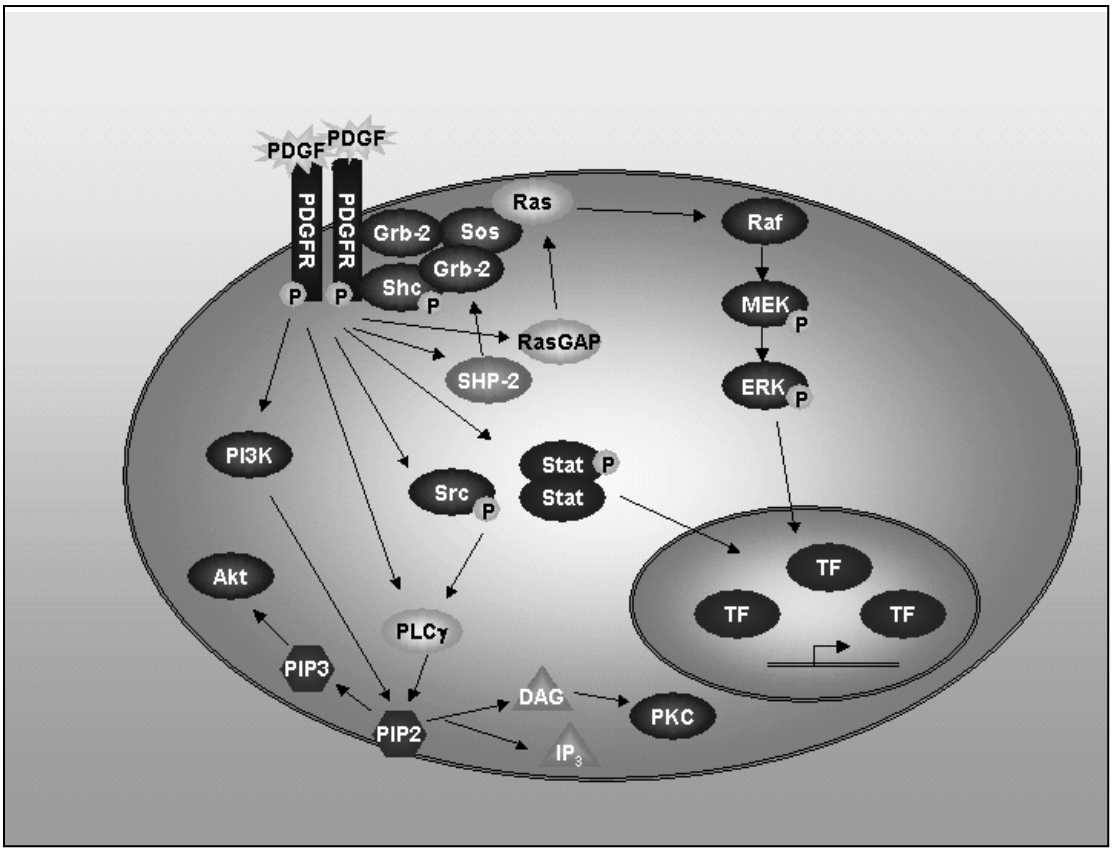


Fig. 9: Schematic illustration of certain signaling pathways that are initiated by PDGF and which trigger cell growth, migration and prevent apoptosis. www.grt.kynshu-u.ac.jp/spad/pathway/pdgf.html

One of the most common alterations in glioblastomas is the PDGF autocrine signaling, which is characterized by coexpression of PDGF and its receptor. Lokker and colleages showed 2002 low expression of PDGF-A,-C,-D, PDGFRα and β in the T98G cell line, but no expression of PDGF-B. In contrast to the T98Gs and other glioma cell lines, an increased expression of all PDGF isoforms and their receptors were found just in the A172, U251 and SF188 glioma cell lines. In contrast, Takeuchi et al.(1994) have shown high levels of PDGF-A and –B in malignant glioma cell lines U87-MG, D54 and T98G. The cooexpression of PDGF and its receptor is frequently detected in a variety of tumors including malignant glioma (Maxwell et al., 1990; Hermanson et al., 1992; Guha et al., 1995), lung cancer (Antoniades et al., 1992), and sarcoma (Smits et al., 1992), suggesting the existence of a PDGF autocrine loop. The more recently identified PDGF isoforms, PDGF-CC and –DD, have been implied in autocrine glioma signaling (LaRochelle et al., 2002; Lokker et al., 2002). Paracrine stimulation by PDGF can effect tumor stroma recruitment and growth, as evidenced by studies showing an increased rate or incidence of tumor formation upon transfection of PDGF into receptor-negative tumor cell lines (Forsberg et al., 1993; Skobe and Fusenig, 1998).

1.4.8 EGFR signaling

The epidermal growth factor receptor (EGFR) signaling pathway is one of the most important pathways that regulate growth, survival, proliferation and differentiation in mammalian cells. Following the identification of EGFR (ErbB1), three additional members of the same receptor family were identified, ErbB2, ErbB3, and ErbB4, which belong to the receptor tyrosine kinases (RTKs). Like all RTKs, this family is composed of an extracellular ligand binding domain, followed by a single transmembrane domain and a cytoplasmic domain containing a conserved protein tyrosine kinase (PTK) core, flanked by regulatory sequences (Schlessinger, 2002). The extracellular domaine is composed of four subdomains designated domain I, II, III, and VI or L1, S1, L2 and S2, respectively (Lax et al., 1989). Growth factor-induced receptor dimerization is followed by intermolecular autophosphorylation of key tyrosine residues in the activation loop of the catalytic PTK domain resulting in stimulation of its activity. Each dimeric receptor complex initiates a distinct signaling pathway by recruiting different SH2-containing effector proteins. Activated EGFR dimer complexes with the adaptor protein Grb_2/Sos-1 complex can bind directly to phosphotyrosine sites in the receptor or indirectly through Shc, which brings it into vicinity to Ras, allowing for its activation, followed by induction of ERK and JNK signaling pathways. Furthermore, the activation of EGFR leads to binding of PLCγ and PI3K as well. Activating mutations and overexpression of members of the EGFR family were implicated in a variety of cancers, including mammary carcinomas, squamous carcinomas, and glioblastomas, as well as other malignant diseases (Blume-Jensen and Hunter, 2001).

1.4.9 PLC signaling

The phosphatidylinositol-specific phosphplipase (PLC) family is a group of critical cellular signaling enzymes that hydrolyze PIP_2 to generate PIP_3 and diacylglycerol, which increase the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) and activate protein kinase C (PKC), respectively (Rhee, 2001; Rebecchi et al., 2000). Eleven isoforms of PLC, representing five distinct, differentially regulated classes, have been identified: PLCβ1 to β4; PLCγ1 and γ2; PLCδ1, δ3 and δ4; and PLCε and PLCζ. PLCβ is regulated by G-protein-coupled receptor (GPCR) activation of heterotrimeric G_q family G-proteins and Gβγ subunits. PLCγ is regulated by tyrosine phosphorylation by receptor tyrosine kinases (e.g. EGF, PDGF) and nonreceptor tyrosine kinases (e.g. Src) activated by immunoglobulins and cytokines. Regulation of PLCδ is less well understood but is propably regulated by changes in $[Ca^{2+}]_i$, possibly downstream from activation of other PLC isoforms, and by high molecular weight G-protein, G_h. PLCζ is also regulated by $[Ca^{2+}]_i$ (Kouchi et al., 2003). PLCε was discovered only recently and is the largest member of the PLC family (Kelley et al., 2001; Lopez et al., 2001; Song et al., 2001; Kelley et al., 2005). PLCε is regulated by the monomeric Ras (Song et al., 2002; Kelley et al., 2004) and Rho (Kelley et al., 2004; Seifert et al., 2004) families, the heterotrimeric G12 family and Gβγ subunits (Wing et al., 2001). Receptor tyrosine kinase agonists, EGF and PDGF have been shown to stimulate PLCε through Ras and Rap (Kelley et al., 2006). GPCR-mediated activation of PLCε by LPA occurs through $G\alpha_{12/13}$ and Rap (Kelley et al., 2004). The GPCR agonist, endothelin and LPA activate both endogeneous PLCε and PLCβ3 through endogenous receptors in Rat-1 fibroblasts (Kelley et al., 2006).

1.4.10 The proteasome pathway

Protein degradation via the proteasome is often deregulated in tumors (Spataroet al., 1998; Piva et al., 1999; Kato, 1999). One of the novel therapeutic targets for resistant tumors is the ubiquitin/proteasome pathway **(Fig. 10.)**, a key element in cell cycle progression and cell survival (Krek, 1998; Peters, 1999; Li and Dou, 2000; MacFarlane et al., 2002; Suzuki et al., 2001; Podust et al., 2000). Proteasome inhibition is very promising in controlling cell cycle and inducing apoptosis (Laurent et al., 2003), and an attractive target for anticancer therapy (Kisselev and Goldberg, 2001; Adams, 2002).

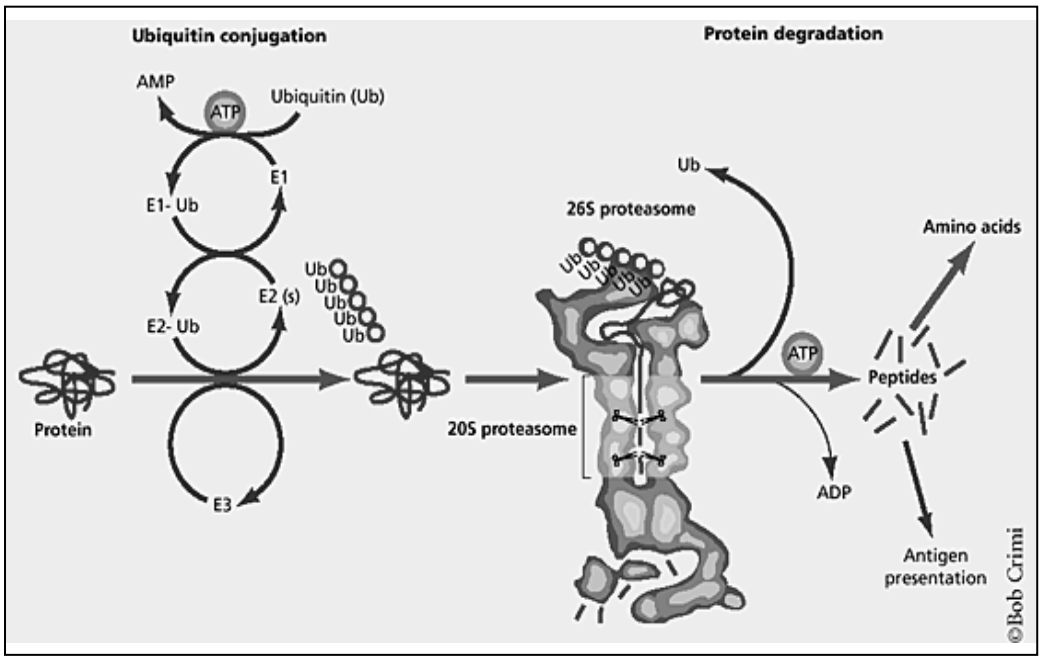


Fig. 10: Protein degradation through the ubiquitin (Ub)-proteasome pathway.
Most proteins that are destined for degradation through the Ub-proteasome pathway are first subjected to ubiquitination. This is accomplished in several stages. The E1 Ub-activating enzyme, is an ATP-dependent reaction, forms an activated complex with Ub and transfers it to the E2 Ub-conjugating protein. The E2 Ub-conjugating protein then transfers Ub to an E3 Ub-ligase protein, which has formed a complex with the target protein. In some cases an E3 Ub-ligase may not be necessary. After several cycles of ubiquitination, the polyubiquitinated target protein is recognized by the proteasomal cap proteins through its ubiquitin moieties, which are cleaved off by isopeptidases and recycled. In an ATP-dependent fashion the protein is then unwound and fed into the 20S core through an interior channel, where it is exposed to the active proteolytic enzymes. Oligopeptide digestion products are then released and degraded further to amino acids by oligopeptidases. Some proteins may be subject to proteasomal degradation without the need for prior ubiquitination (Orlowski et al, 2003; Goldberg, 2000).

Receptor degradation is preceded by ubiquitination and was blocked by proteasome inhibitors. C-Cbl, one of the numerous proteins that bind to the PDGFR (Bonita et al., 1997),

has recently been identified as a proteasomal E3-like ubiquitin ligase that is involved in down-regulation of tyrosine kinase receptors (Levkowitz et al., 1999; Joazeiro et al., 1999; Yokouchi et al., 1999). Decreased PDGFR levels in suspended cells correlated with ubiquitination of the PDGFR and was blocked by treatment with inhibitors of the proteasome pathway. Baron and Schwartz (2000), conclude that cell detachment results in cellular desensitization to PDGF that is mediated by degradation of the PDGFR via a novel ubiquitin-dependent pathway.

PDGF induced Src-dependent association between the multifunctional transcription/translation regulator hnRNP-K and the mRNA-encoding myosin regulatory light-chain (MRLC)-interacting protein (MIR), an E3-ubiquitin ligase that is MRLC specific. Down-regulation of MIR by RNA muting prevented the reorganization of actin structures and severely reduced the migratory and wound-healing potential of PDGF-treated cells. The PDGF-induced protein destabilization through the regulation of hnRNP-K controlled ubiquitin-ligase translation identifies a novel pathway by which external stimuli can regulate phenotypic development through rapid, organelle-specific changes in the activity and stability of cytoskeletal regulators (Nagano et al., 2006).

It has been reported that the small GTPase RhoA is targeted for degradation at the leading edge of migrating cells by the E3 ubiquitin ligase Smurf1, and that this is required for the formation of protrusions. Sahai et al. (2007), report that Smurf1-dependent RhoA degradation in tumor cells results in the downregulation of Rho kinase (ROCK) activity and myosin light chain2 (MLC2) phosphorylation at the cell peripherie. Smurf1 regulates cell polarity and protrusive activity via PKCζ-dependent recruitment to cellular protrusion sites, and subsequent ubiquitination and proteasomal degradation of RhoA in HEK293T cells (Zhang et al., 2004; Wang et al., 2003). This targeting leads to the activation of Rac1 and Cdc42, and the formation of membrane microspikes. Thus, Smurf1 generates an intracellular asymmetry of GTPase activity, thereby regulating cell polarity and cytoskeleton rearrangements (Bryan et al., 2005). In contrast, phosphorylation of Ser188 of RhoA protects RhoA, particulary its active form, from ubiquitin-mediated proteasomal degradation. This phosphorylation is induced by cAMP-dependent protein kinase (PKA) and c-GMP-dependent protein kinase (PKG), causing their relocalization in the cytosol (Lapetina et al., 1989; Quilliam et al., 1991; Lang et al., 1996). Results of Rolli-Derkinderen et al., 2005, suggest that Ser188 phosphorylation-mediated protection against degradation is a physiological process regulating the level of endogenous RhoA and this may define a novel function of RhoGDI, as an inhibitor of Rho protein degradation (Forget et al., 2002).

1.5 The actin cytoskeleton

The cytoplasm of eukaryotic cells is spatially organized by a network of protein filaments known as the cytoskeleton. This network contains three principal types of filaments. *Intermediate filaments* provide mechanical strength and resistance to shear stress. *Microtubules,* long hollow cylinders made of the protein tubulin, determine the position of membrane-enclosed organelles and direct intracellular transport. *Actin filaments* are most concentrated just beneath the cell membrane, and are responsible for resisting tension and maintaining cellular shape, forming cytoplasmic protuberances and participation in some cell-to-cell or cell-to-matrix junctions. Around 5-9 nm in diameter, this filament is composed of two-stranded helical polymers of the protein actin. They appear as flexible structures, which are organized into a variety of linear bundles, two-dimensional networks, and three-dimensional gels. Actin filaments have polarity and the actin monomers all orient with their cleft toward the same end of the filament (designated the minus end) and therefore they play a major role in cell migration.

Cell structures that involve actin and are important for cell migration are filopodia, lamellipodia and pseudopodia. *Filopodia* contain a core of long and thin, bundled actin filaments, which are oriented toward the tip, out of the cell surface. *Lamellipodia* are two-dimensional, dynamic sheet-like structures at the leading edge of a mobile cell. They contain an orthogonally cross-linked mesh of branched arrays of actin filaments, most of which lie in parallel to the solid substratum. *Pseudopodia* are stubby three-dimensional projections filled with actin-filament gel.

The motor protein myosin II in stress fibers is responsible for their ability to contract. In contractile bundles, loose packing allows myosin II to enter the bundle, whereas in parallel bundles a tight packing prevents myosin II from entering the bundle. Another important actin-filament binding protein is cofilin, which destabilizes actin filaments along their length, distorting the helical twist of filamentous actin.

1.5.1 The Rho family GTPases

For the actin cytoskeleton, global structural rearrangements in response to external signals are triggered through diverse cell-surface receptors. But all of these signals seem to converge inside the cell on a group of closely related monomeric GTPases. Rho GTPases comprise a large subfamily of the Ras superfamily and include Cdc42, Rac and Rho (Ras-homologous) proteins, as well as Rnd and RhoBTB (Burridge and Wennerberg, 2004). They are guanine nucleotide binding proteins that cycle between an active, GTP-bound and inactive, GDP-bound state. The activity of Rho proteins is controlled by guanine nucleotide

exchange factors (GEFs) and GTPase-activating proteins (GAPs). In cells, Rho GTPases exist predominantly in their inactive form, in complex with GDP dissociation inhibitors (GDIs) (Malliri et al., 2003). Rho GTPases also capture and stabilize microtubules (MT) through their effectors (e.g. mDia and Par6) next to the cell cortex, leading to polarized cell morphology and directional cell migration (Fukata et al., 2003). Activation of Rho (RhoA, RhoB, RhoC) leads to assembly of actin-myosin filaments (stress fibers) and of associated focal adhesion complex. It acts as a molecular switch to control a signal transduction pathway that links membrane receptors to the cytoskeleton. Activated Rac (Rac1, Rac2, Rac3, RhoG) induces actin polymerization, followed by the assembly of a meshwork of actin filaments at the cell periphery to produce lamellipodia and membrane ruffles. Activation of Cdc42 (Cdc42, TC10, TCL, Wrch1, Chp/Wrch2) was shown to induce actin polymerization which leads to actin-rich surface protrusions called filopodia (Alan Hall, 1998).

1.5.2 Rho/ROCK signaling pathway

The first molecular explanation for how Rho affects the actin cytoskeleton came from the discovery that its downstream target ROCK (Rho kinase) phosphorylates and thereby inhibits the regulatory subunit of myosin light chain (MLC) phosphatase. ROCK can also directly phosphorylate MLC. In this way, it increases the level of phosphorylated MLC, which promotes interaction of myosin filaments with actin filaments and increases contractility (Amano et al., 1996; Ridley, 2001). Several cytoskeletal targets have also been identified downstream of ROCK, such as LIM kinase (LIMK). LIM kinase phosphorylates the actin depolymerising protein, cofilin/ADF, thus inhibiting its function (Maekawa et al., 1999). The inhibition of cofilin stabilizes actin filament arrays such as stress fibers and the cell cortex, and promoted focal adhesion formation **(Fig. 11)**.

In contrast, stress fibers could also be induced by coexpression of activated forms of the mammalian homolog of diaphanous (mDia), another Rho effector (Watanabe et al., 1999). Expression of activated forms of mDia promote assembly of fine arrays of thin stress fibers that appear to be less bundled than the stress fibers induced by Rho activation or expression of constitutively active ROCK (Burridge and Wennerberg, 2004).

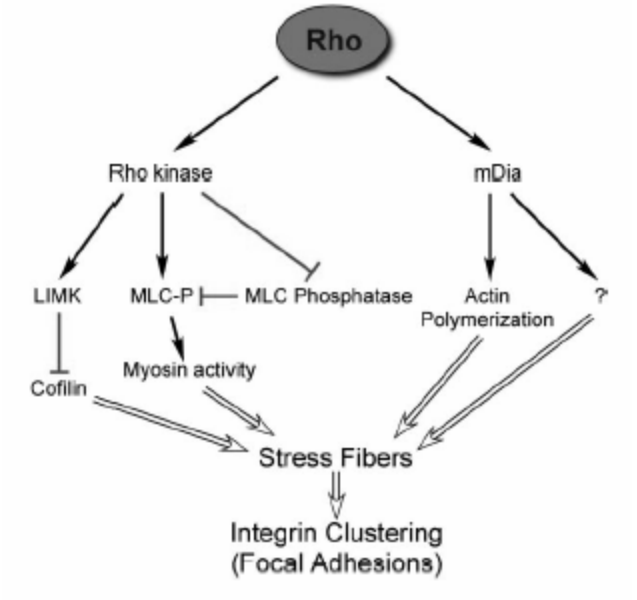


Fig. 11: Signaling from Rho to the cytoskeleton.
The pathways from Rho to the formation of actin stress fibers are described in the text.
Direct activating signals are presented by solid arrows. Inhibitory signals are depicted as red bars. Double-lined arrows and bars represent the net result of a signalling pathway.
Abbrevations used: LIMK, LIM kinase; MLC-P, phosphorylated myosin II regulatory light chain; MLC phosphatase, myosin light chain phosphatase.
Burridge and Wennerberg, 2004

1.5.3 Tropomyosins

Actin filaments in most cells are stabilized by the actin binding molecule tropomyosin, which binds simultaneously to seven adjacent actin subunits in one protofilament. It plays the key role in the regulation of muscle contraction and comes in a rod-like form and binds to the groove of each side of double-helical actin filaments **(Fig. 12)** (Vilfan, 2001). Together with the associated protein troponin, tropomyosin on actin can switch between two laterally shifted conformations (Lehmann et al., 2000). The equilibrium between these two confirmations is strongly influenced by the concentration of Ca^{2+} ions.

There exist three isoforms of high molecular weight (HMW) tropomyosins, TM1, 2 and 3, as well as low molecular weight (LMW) isoforms TM4 and 5. In vertebrates, more than 10 different isoforms of HMW tropomyosins are expressed from TPM1 (α-TM) and TPM2 (β-TM) genes, and by alternatively RNA splicing (Pittenger et al., 1994). The HMW tropomyosins are found along stress fibers and are thought to play a role in stabilizing the organization of actin filaments, which in turn plays an important role in the maintainance of cell shape, cell motility, and cell-cell and cell-matrix interactions (Button et al., 1996).

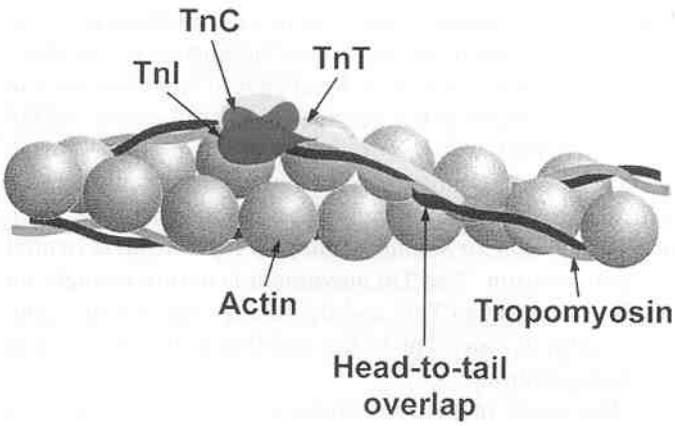


Fig. 12: Visualization of interaction of tropomyosin and actin.
A model of the arrangement of tropomyosin, actin and three troponin subunits (Tn) in a skeletal muscle thin filament .
Note the adjacent tropomyosin molecules overlap head to tail with the N-terminus of Tn-T lying along the overlap region. The C-terminus of Tn-T interacts with Tn-C and Tn-I, and Tn-I also interacts with actin.
Tropomyosin a-subunit brown, b-subunit orange, actin grey,TnI green, TnC red, TnT yellow.
Gordon et al., 2000.

In endothelial cells, oxidative stress quickly activates the MAPK, which results in the phosphorylation of tropomyosin. Houle et al. (2007), identified death-associated kinase 1 (DAP kinase 1) as the kinase that phosphorylates TM1 in response to ERK activation by hydrogen H_2O_2. They also report that the phosphorylation of TM1 mediated by DAP kinase occurs on S283.

Silencing of TMs with short interfering RNAs (siRNAs) blocks stress fiber assembly, whereas ectopic expression of TMs results in stress fibers. Ectopic expression and siRNA experiments showed that Smads mediate induction of TMs and stress fibers. TGF-β induction of TMs and stress fibers are significantly inhibited by Ras-ERK signaling in metastatic breast cancer cells. Inhibition of the Ras-ERK pathway restores TGF-β induction of TMs and stress fibers and thereby reduces cell motility. These results suggest that induction of TMs and stress fibers play an essential role in TGF-β control of cell motility, and the loss of this response is a critical step in the acquisition of a metastatic phenotype by tumor cells (Bakin et al., 2004).

1.5.3.1 Tropomyosins in cancer

One of the most prominent features of malignant cells is the presence of an altered actin cytoskeleton, arising from the suppression of several key actin-binding proteins, such as profiling, α-actinin, and tropomyosins (TMs) (Lin et al., 1997; Pawlak and Helfman, 2001). Tropomyosin isoform-1 (TM1) is consistently suppressed in breast cancer cell lines (Martin and Leder, 2001; Bhattacharya et al., 1990) and breast tumors (Raval et al., 2003), and is downregulated in urinary bladder tumors (Pawlak et al., 2004), suggesting that the loss of TM1 may contribute to neoplastic transformation. Restoration of TM1 expression in several oncogene-transformed (Prasad et al., 1993, 1999; Bravermann et al., 1996) and spontaneously transformed breast cancer cells (Mahadev et al., 2002; Raval et al., 2003) induces microfilaments, and actin stress fibers in a Rho-kinase-dependent fashion (Shah et al., 2001). This suppressed the anchorage-independent growth of the tumor cells. Further investigations have revealed that TM1 induces anoikis in breast cancer cells, and thus may suppress their malignant behavior (Mahadev et al., 2002; Raval et al., 2003). Since TMs stabilize the actin cytoskeleton (Cooper, 2002) and regulate actin-myosin interactions (Marston et al., 1998), Bharadwaj et al. (2004), have hypothesized that TM1-mediated cytoskeletal reorganization is critical for the antineoplastic effects of TM1. A tenascin-C induced downregulation of TM1 expression in T98G glioblastoma cells was observed by Ruiz et al. (2004). Pawlak et al.(2004), observed a high level of the isoform TM5 in four different transitional cell carcinoma (TCC) cell lines, whereas TM5 was expressed at very low levels in normal bladder mucosa.

Bharadwaj and Prasad (2002), showed that hypermethylation of TM1 gene and chromatin remodelling are the predominant mechanisms by which TM1 expression is down-regulated in breast cancer cells. This finding indicates that hypermethylation and chromosomal compaction by histone deacetylation are major mechanisms by which TM1 expression is silenced in breast cancer cells. Thus, while the βTM gene is generally inactivated by gene methylation in cancer cells, it appears that down-regulation of αTM gene (which codes for TM2 and TM3) via promoter methylation may occurs upon transformation by ras. Suppression of HMW TMs, in particular that of TM1 and TM2 occur very rapidly, before the morphological transformation is evident, suggesting that the loss of TM1 may be an early event during tumorigenesis. Therefore, the loss of TM1 expression may serve as a potential biomarker in cancer.

1.6 Cell migration

Cell migration is a central process in the development and maintenance of multicellular organisms, which involves rapid changes in the dynamics of actin filaments, together with the formation and disassembly of cell adhesion sites (Brakebusch, C and Fassler, R, 2003). Tissue formation during embryonic development, wound healing and immune responses all require the orchestrated movement of cells in a particular direction to a specific location. The migration process consists of four different steps, polarization, protrusion, traction and retraction. A polarized cell has distinct leading and trailing edges, which is a common feature of both fibroblastic and amoeboid motility. The leading edge points in the direction of movement and is driven by actin-polymerization-mediated protrusion. There are two main types of nucleators of actin polymerization: the Arp2/3 complex (actin-related proteins) and the formins mDia1 and mDia2. Cell polarity, which is essential for directed migration, is defined not only by actin-mediated protrusions but also by reorientation of the MTOC (microtubule-organizing center) towards the leading edge. Cdc42 regulates MTOC positioning through Par proteins and PKCζ (Jaffe and Hall, 2005).

Arp2/3 binds to the sides of existing actin filaments and promotes the extension of a new actin filament from its pointed end, forming a 70° angle with pre-existing filament (Weaver et al., 2003). Arp2/3 regulation is controlled by WAVE/Scar, WASP and N-WASP proteins. This complex is under the control of the small GTPase Rac. Unlike the Arp2/3 complex, formins bind to the barbed end of actin filaments and promote actin growth in a linear fashion (Watanabe and Higashida, 2004).

Actomyosin-based contraction is controlled by the small Rho GTPases Cdc42, Rac and RhoA (Jaffe and Hall, 2005). This contraction is provided the action of integrins in adhesions and tractional force is created at sites of adhesion by myosin II interactions with actin filaments. During migration, adhesions assemble at the leading edge and disassemble at the trailing edge. However, adhesions also disassemble at the front during protrusion and feed components to nascent adhesions at the leading edge in a process called adhesion turnover (Webb et al., 2004). Among the structural molecules, talin directly links integrins to actin and also activates them (Nayal et al., 2004). Rho GTPases are critical effectors in this process.

For cells to translocate forward, adhesions at the rear must disassemble and the trailing edge needs to retract. Several mechanisms promote disassembly; these include microtubule-dependent targeting of dynamin and subsequent endocytosis of some adhesion components (Ezratty et al., 2005); myosin-mediated contractility contributes through regulation of MLC phosphorylation by RhoA and Rac acting through Rho-kinase and PAK. Finally, dissolution of the adhesive contacts at the rear edge is also achieved through affinity

downregulation by phosphorylation by phosphatases, such as calcineurin, or simply by proteolysis of molecules such as talin by the proteases calpain (Franco et al., 2004).

1.6.1 Cell migration in cancer

Errors during this process have serious consequences, including mental retardation, vascular disease, tumor formation and metastasis. Cancer cells migrate as single cells or in small groups to spread from the initial site of tumor growth. They acquire an invasive phenotype characterized by both the loss of cell-cell interactions and increased cell motility. These cells are able to enter the blood or lymphatic vessels (intravasation) and to cross the vessel wall to exit the vasculature in distal organs where they can continue to proliferate forming a second tumor. Cancer cell migration is typically regulated by integrins, matrix-degrading enzymes, and cell-cell adhesion molecules. Several cytokines and growth factors have been shown to stimulate invasion and to be upregulated in a variety of tumor types. An understanding of the mechanism by which cells migrate may lead to the development of novel therapeutic strategies for controlling invasive tumor cell.

1.6.1.1 LPA in cancer cell migration

Lysophosphatidic acid (LPA) has numerous cellular effects including cell proliferation, cytoskeleton reorganization, cell adhesion and migration (Moolenaar 1999, Sengupta et al., 2004). LPA activates Rac1 to promote cell spreading, lamellipodium formation and cell migration in fibroblasts (van Leeuwen et al., 2003a). In pancreatic tumor cells, Erk activation is indicated to play a critical role for LPA-stimulated cell migration (Strähle et al. 2003). Further LPA-induced squamous cell carcinoma motility is mediated by EGF receptor transactivation (Gschwind et al., 2002). LPA can promote the expression/activation of various invasion-associated proteases such as metalloproteinases in ovarian cancer cells (Pustilnik et al., 1999; Fishman et al., 2001). Thus, these metalloproteinases cleave extracellular matrix molecules such as fibronectin, which greatly facilitates cancer cell migration.
Bian et al. (2006) found that the $G_{12/13}$ pathway was required for LPA-induced FAK autophosphorylation and efficient cell migration. Their studies showed that the $G_{12/13}$-RhoA-ROCK signaling pathway mediates LPA-induced FAK autophosphorylation and contributes to LPA-stimulated cell migration. They also showed that the disruption of the G_i-Ras-MEKK1 pathway led to significant reduction in LPA-stimulated cell migration.

1.6.1.2 PDGF in cancer cell migration

PDGF triggers rapid disassembly of focal adhesions and subsequent reassembly, the so called focal adhesion turnover, and continued adhesion assembly in concert with the spreading protrusion (Melton et al., 2007). Cell migration studies indicated that PDGF stimulated migration, but it had no effect on cell proliferation but PDGF-induced migration was inhibited by inhibitors of MEK/ERK, PI3K/Akt, EGFR and Src pathways in osteoblastic cells (Kim et al., 2007). Inhibition of protein kinase C and ERK also attenuated PDGF-stimulated mesangial cell migration (Harper et al., 2007). Cerutis et al. (2004) showed that LPA plus PDGF elicited complete wound filling in periodontal ligament fibroblasts (PDLFs).
Furthermore, Banerjee et al. (2006) identified PDGF as a key regulator of vascular smooth muscle cells motility induced by breast cancer cells. The PI3K signaling, but not the ERK signaling was strongly involved in the PDGF-stimulated glioma cell motility in T98G and U87-MG cells (Cattaneo et al., 2006). In vitro, PDGF-BB expression activates cell motility of isolated lymphatic endothelial cells, whereas in vivo, in murine fibrosarcoma cells PDGF induces tumor lymphangiogenesis, leading to enhanced metastasis in lymph nodes (Cao et al., 2004).

1.7 Aims of the thesis

Tenascin-C inhibits cell spreading on fibronectin by blocking the integrin $\alpha5\beta1$ co-receptor syndecan-4, which prevents focal adhesion and actin stress fiber formation (Huang et al., 2001; Orend, 2003). Studies by Ruiz et al. (2004), showed that the expression profile of glioma cells is profoundly altered upon growth on a fibronectin/tenascin-C substratum in comparison to fibronectin alone. This involved downregulation of actin stabilizing TM1 and up-regulation od EDNRA amongst many other molecules. Here, it should be addressed how tenascin-C prevents the formation of actin stress fibers and focal adhesions, two important components involved in cell spreading, in more detail. In particular, the mechanism of how inactivation of syndecan-4 by tenascin-C causes cell rounding on a fibronectin/tenascin-C substratum should be determined at the molecular level. Moreover, cytoplasmic signaling that leads to reduced expression of TM1 and inactivation of FAK should be examined in detail and it should be determined whether TM1 is a critical component of tenascin-C-induced cell rounding. In addition, it should be analyzed how tenascin-C affects cell migration. Cells in tissues do encounter signals from the ECM together with signals from growth factors. As an example, in the tenascin-C enriched neuronal stem cell nice, tenascin-C and PDGF receptor signaling is important for maintenance of the stem cell phenotype. Therefore, it should be determined how signaling from growth factors such as PDGF, LPA, endothelin and EGF affects cell adhesion and migration of cells on a fibronectin/tenascin-C substratum.

2 Results

2.1 Part A : Endothelin receptor type B counteracts tenascin-C-induced endothelin receptor type A-dependent focal adhesion and actin stress fiber disorganization (publication)

Publishede article : Cancer Res 2007; 67: (13), 6163-6173

Abstract

Tenascin-C, an extracellular matrix molecule of the tumor specific microenvironment, counteracts the tumor cell proliferation-suppressing effect of fibronectin by blocking the integrin α5β1/syndecan-4 complex. This causes cell rounding and stimulates tumor cell proliferation. Tenascin-C also stimulates endothelin receptor type A (EDNRA) expression. Here, we investigated whether signaling through endothelin receptors affects tenascin-C-induced cell rounding. We observed, that endothelin receptor type B (EDNRB) activation inhibited cell rounding by tenascin-C and induced spreading by restoring expression and function of FAK, paxillin, RhoA and tropomyosin-1 (TM1) via activation of EGFR, PLC, JNK and the PI3K pathway. In contrast to EDNRB, signaling through EDNRA induced cell rounding which correlated with FAK inhibition and TM1 and RhoA protein destabilization in the presence of tenascin-C. This occurred in a MEKdependent manner. Thus, tumorigenesis might be enhanced by tenascin-C involving EDNRA signaling. Inhibition of tenascin-C in combination with blocking both endothelin receptors could present a strategy for sensitization of cancer and endothelial cells towards anoikis.

Introduction

Cancer is a product of the tumor-host microenvironment, where mutual stimulation of tumor and stromal cells induce tumor formation and progression into malignant metastasizing cancers. The adhesion modulatory extracellular matrix (ECM) molecule tenascin-C is one factor in the tumor-specific microenvironment that is highly expressed in most solid tumors. Tenascin-C actions promote malignant transformation, uncontrolled proliferation, metastasis, angiogenesis and escape from tumor immunosurveillance. Tenascin-C acts very early during malignant transformation as well as throughout tumor progression through distinct effects on various cell types within a tumor (1). We and others have previously shown that tenascin-C inhibits the tumorigenesis-suppressing activity of integrin α5β1-mediated cell adhesion to fibronectin by blocking syndecan-4 (2, 3) and downstream RhoA (4) and FAK (2, 5, 6) activation. This in turn triggers tumor cell proliferation, presumably by overriding the G0 and G1/S cell cycle checkpoints (7). Moreover, tenascin-C can activate a variety of oncogenic signaling pathways such as EGFR (8, 9), ERK/MAP kinase and Wnt and can downregulate the tumor suppressor-like molecule tropomyosin-1 (TM1) (10).

Endothelins (ET1-3) and their G protein-coupled receptors EDNR subtypes A and B were originally found to be involved in the regulation of blood pressure (11). Endothelin receptor signaling plays an important role during embryonic development, and in physiological processes such as neurotransmission, renal function and regulation of cell proliferation (12). EDNRB signaling has a promigratory and proliferative effect on microvascular endothelial cells (13). Signaling through EDNRA also stimulated angiogenesis in particular by induction of vascular endothelial growth factor (14, 15). ET1 activates phospholipase-C β, thus increasing intracellular calcium ion levels and activation of PKC. It also activates PI3K, JNK, ERK/MAP kinase and EGF receptor (EGFR) signaling. Moreover, ET1-induced signaling leads to activation of FAK and paxillin (16). Since signaling by EDNRA and EDNRB plays an important role in endothelial cell proliferation and survival, blocking these receptors provides a promising approach in clinical treatment of systemic pulmonary hypertension and chronic heart failure (17). Inhibition of endothelin receptor signaling may also be useful in cancer therapy, since inhibition of EDNRB with a selective pentapeptidic antagonist (BQ788) inhibited growth of human melanoma cells in cell

culture and in the nude mouse (18). In advanced prostate cancer, treatment with an EDNRA-selective inhibitor (ABT-627) delayed disease progression in patients with hormone-refractory prostate cancer (19).

We showed that tenascin-C induces expression of EDNRA. This was accompanied by enhanced phosphorylation of ERK1/2 and induction of c-Fos (10), all downstream targets of EDNRA. Here, we investigated the possibility that EDNRA signaling contributes to the tenascin-C-induced cell phenotype. We show, that EDNRA signaling induced cell rounding in the presence of tenascin-C. This occurred by blocking FAK and paxillin activation, and inhibiting RhoA and TM1 protein stability in a MEK-dependent manner. In contrast to EDNRA, signaling by EDNRB counteracted cell rounding by tenascin-C and stimulated cell spreading on a fibronectin/tenascin-C (FN/TN-C) substratum in an EGFR-, PLC-, JNK- and PI3Kdependent, but MEK-independent manner. In the absence of syndecan-4 activation, EDNRB signaling restored focal adhesion assembly by activating FAK and paxillin and, reorganized the actin cytoskeletal by normalizing RhoA and TM1 expression.

Material and Methods

Construction and purification of his-tagged human tenascin-C

The cDNA encoding human tenascin-C (HxBL.pBS) encompassing all alternative fibronectin type III repeats (20) was subcloned into the pCEP-Pu vector (21). A C-terminal his-tag (6 additional his residues) was introduced by PCR with primer PhTN-C R-2 (CGGGATCCTAATGATGATGATGATGATGTGCCCGTTTGCGCCT). A 3-piece ligation of NotI/NdeI, a NdeI/6his -BamHI and a NotI/BamHI vector fragments gave rise to pCEP-huTNC-his. The construct was confirmed by restriction enzyme analysis, PCR and partial sequencing. After transfection of pCEP-huTNC-his into HEK293-EBNA1 cells (ATCC, CRL-10852), human tenascin-C expression and secretion were determined by immunoblotting with the monoclonal antibody B28.13 (22). For large-scale production of recombinant human tenascin-C, 293:pCEP-huTNC-his cells were grown to confluency in the presence of 2.5 µg/ml puromycin (Sigma, Bucks, Switzerland) in DMEM and 10% FCS. For collection of conditioned medium, cells were washed with PBS prior to growth in serum-free DMEM without puromycin for 2 days. Cells were recovered in DMEM supplemented with 10% FCS and 2.5 µg/ml puromycin for 1 day before transfer to serum-free medium. This cycle was repeated up to 8-times with no reduction in yield during prolonged culture.

Proteins from conditioned medium were ammonium sulfate precipitated and dialyzed against PBS/0.01%Tween-20 prior to chromatography on a gelatine-agarose column (Sigma, Bucks, Switzerland, G5384). The eluate was passed over a nickel column (Invitrogen, Carlsbad, CA USA, Pro Bond Resin no. R801-01) and bound tenascin-C was eluted with 300 mM imidazol (Sigma, Bucks, Switzerland), 250 mM Na phosphate pH 7.4, 450 mM NaCl, 0.01% Tween-20 and dialyzed against PBS/0.01% Tween-20. The purity and absence of contamination by fibronectin was determined by Gelcode staining and immunoblotting. The biological activity of recombinant human tenascin-C was compared to the chicken tenascin-C used previously (2). In cell adhesion assays, T98G cells were plated for different times onto pure tenascin-C, fibronectin or mixed fibronectin and fibronectin/FNIII13 substrata containing human or chicken tenascin-C. As with chicken tenascin-C (2), cells did not spread on FN/TN-C unless syndecan-4 was activated with FNIII13. To test whether addition of a his-tag at the C-terminus of tenascin-C interfered with heparin binding to the

fibrinogen globe, heparin binding of chicken and human tenascin-C were compared by ELISA. No differences were detected (data not shown).

Cell plating, inhibitor studies, preparation of cell lysates

Human T98G glioblastoma, J82 urinary bladder carcinoma and MDA-MB435 breast carcinoma cells (American Type Culture Collection) were grown in DMEM supplemented with antibiotics and 10% FCS (Sigma, Bucks, Switzerland). Cells were transferred into DMEM, 10% FCS 24 h before the experiment and serum-starved for 18 h. Cells were trypsinized and replated after inhibition of trypsin with 100 ng/ml Trypsin inhibitor (Sigma, Bucks, Switzerland) in serum-free DMEM onto 10-cm2 dishes (Falcon Becton Dickinson) coated with equimolar amounts of fibronectin and tenascin-C (1µg/cm2). Finally, 1% heat-inactivated BSA (Serva, Heidelberg, Germany) was used to block the uncoated surface prior to UV-sterilization for 15 min in a sterile bench. Since the inhibitors were dissolved in DMSO and DMSO interfered with cell spreading on fibronectin, cells were plated on the different substrata in serum-free medium 1h or 12h prior to incubation with 100 ng/ml EGF, 20 nM ET1, 20 nM ET3, conditioned medium (taken from 48h serum-free cultures of T98G cells), or inhibitor PKI166 (2 µM, Novartis, Basel, Switzerland), UO126 (25 µM, Calbiochem Germany, Darmstadt), wortmannin (20 µM), SP600125 (20 mM), U73122 (20 nM), BQ123 (100 nM) and BQ788 (100 nM) (Sigma, Bucks, Switzerland) for 4h followed by lysis in sample buffer (250 mM Tris-HCl pH 7,0, 10% SDS, 50 % glycerol, bromphenolblue, 100mM DTT).

Immunoblotting

Equal amounts of protein were separated in 8% self-made or 4-12% precast Bis-Tris-glycine gels (Invitrogen, Novex), transferred onto PVDF nylon membrane (Millipore Immobilon-P, Bedford, USA) and stained with 0.2% Ponceau-S (Sigma, Bucks, Switzerland) in 7.5% TCA for confirmation of equal protein loading prior to blocking the membrane in 10% horse serum (or 1% BSA), TBS, 1% Tween-20. For immunoblotting, the following mouse monoclonal antibodies against TM1-3 (TM311, Sigma, Bucks, Switzerland, 1:1000), vinculin (hVIN-1, Sigma, Bucks, Switzerland, 1:1000), α-tubulin (Ab-1, Oncogene, Dietikon Switzerland, 1:5000), RhoA (26C4, Santa Cruz Biotechnology Inc., Heidelberg, Germany, 1:1000), P-ERK1/2 (Cell Signaling, Allschwil, Switzerland, 1:1000), FAK and phospho-Y397-FAK (BD Biosciences, Allschwil, Switzerland, 1:1000), rabbit polyclonal antibodies, paxillin and

phospho-Y118-paxillin (Abcam, Cambridge, UK, 1:1000), and ERK1/2 (Cell Signaling, Allschwil, Switzerland, 1:1000), phospho-S910-FAK (Biosource, Lucerne, Switzerland, 1:1000) and secondary HRP-coupled antibodies (Amersham, UK) were used. Binding of antibodies was detected with ECL-plus (Amersham, UK).

Real-time reverse transcription PCR

Total RNA was isolated from three independent plates using the RNeasy Mini Kit (Qiagen, Basel, Switzerland) and RNAse-free DNAse set (Qiagen, Basel, Switzerland) following the manufacturer's instructions. From 0,5 –1 µg of total RNA single strand cDNA was generated by using the SuperScript III First-Strand Synthesis Super Mix (Invitrogen, Carlsbad, CA, USA) with random hexamers. Expression of the respective gene was detected by quantitative RT–PCR on an ABI Prism 7000 TaqMan using SYBR green PCR MasterMix (Applied Biosystems, Warrington, Cheshire, UK) with the following primers (5' to 3'): GAPDH, ATCTTCTTTTGCGTCGCCAG (forward) and AATCCGTTGACTCCGACCTTC (reverse), tenascin-C, TGCCCATATCTCAGGGCTAC (forward) and GATGCCATCCAGGAAACTGT (reverse), EDNRA, GCCATATTTTAGGACAGGTAAAATAACA (forward) and AACACACAAAAGGGCAGTACTTCTT (reverse) (10), EDNRB, TCACCTAAAGCAGAGACGGGAA (forward) and AGGACCAGGCAAAAGACGG (reverse), αTM (TPM1), GCACCGAAGATGAACTGGACAA (forward) and reverse CATCGGTGGCCTTTTTCTCTG (reverse), βTM (TPM2), CCAACAACTTGAAATCCCTGG (forward) and CTTTGGTGGAATACTTGTCCGC (reverse) and RhoA, GCAGGTAGAGTTGGCTTTATGG (forward) and CTTGTGTGCTCATCATTCCGA (reverse) (23). Primers were designed with the ABI Primer Express software (Applied Biosystems, Warrington, Cheshire, UK). Relative expression of the respective gene was determined after normalization to GAPDH and was calculated with the formula: relative expression level = 2^{-DDCT}.

Immunofluorescence

Cells were serum-starved prior to plating on fibronectin and FN/TN-C for the indicated time points in serum-free medium plus ET1 or ET3. Cells were fixed in 4% PFA and stained with the indicated primary and secondary FITC-labeled antibodies or with TRITC-labeled phalloidin (Sigma, Bucks, Switzerland). Images were captured using a NIKON Diaphot300 (NIKON Video Microscope with Openlab program,

Coventry, UK) with 40x and 100x objectives for immunofluorescence and a Leica LEITZ DMIL microscope (Wetzlar, Germany) with a 10x objective for phase-contrast.

Tissue Micro Arrays (TMA) and Immunohistochemistry

TMAs with 190 glioblastoma (GBM), and 158 gliomas WHO grade I-III have been constructed from archived paraffin blocs as described (24). Expression of tropomyosin and tenascin-C in gliomas grade I, II and IV was determined by immunohistochemistry with antibody TM311 (dilution 1:3`500) and B28.13 (22), respectively as described before (10). The staining was scored semi-quantitatively in a range of 0 to 3, independently by two researchers.

Results

Kinetics of EDNRA induction by tenascin-C

We previously described that a tenascin-C containing fibronectin substratum triggered EDNRA expression in T98G cells 12h after contact with the substratum, both at RNA and protein level (10). Here, we investigated the kinetics of EDNRA induction by tenascin-C with real time RT-PCR. EDNRA RNA levels did not vary between cells plated on fibronectin or FN/TN-C at 1h and 3h (**Fig. 1A**). However, a slight and 2.7-fold increase was observed after 5h and 19h, respectively in cells plated on FN/TN-C. We also determined expression of EDNRB and found that in contrast to EDNRA, RNA levels of EDNRB were low and did not change at any of the time points mentioned above (data not shown). Thus, in contrast to EDNRB, expression of EDNRA is strongly induced by tenascin-C at RNA and protein level.

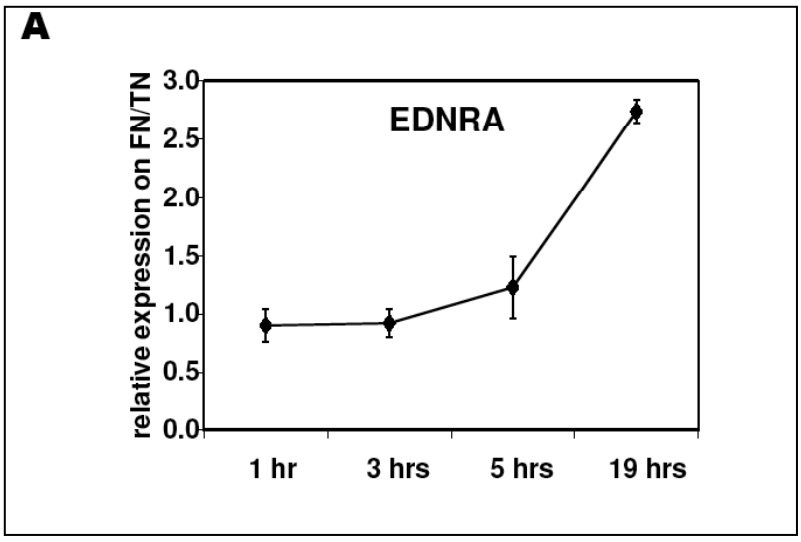
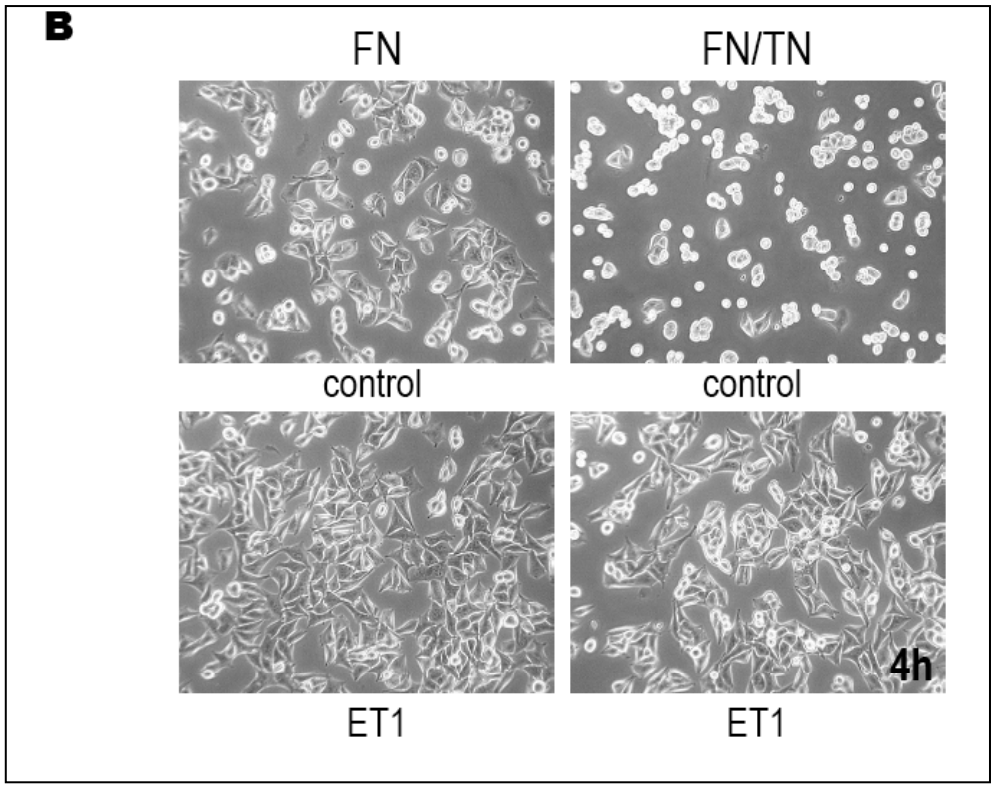


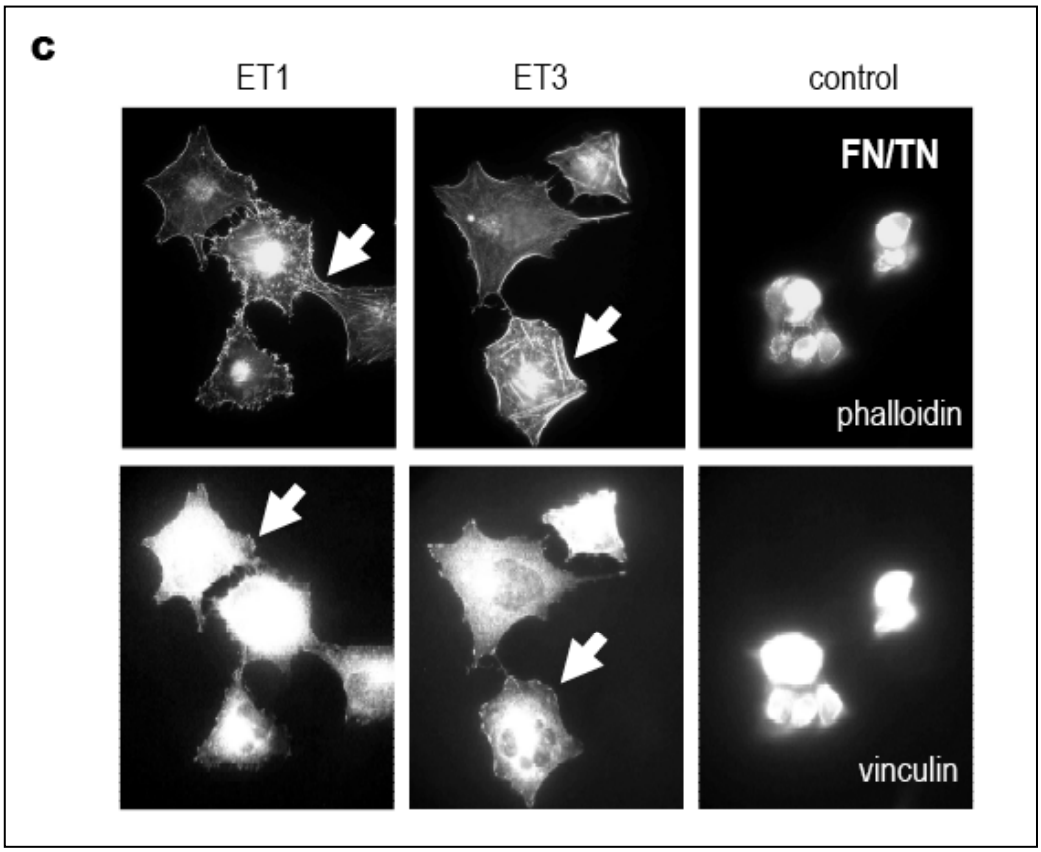
Fig. 1 Kinetics of EDNRA expression and restoration of cell adhesion by ET1 on FN/TN-C
(**A**) Expression of EDNRA was determined by real time-RT-PCR in T98G cells upon plating on fibronectin and FN/TN-C and after RNA preparation at the indicated time points. Expression of EDNRA on FN/TN-C is expressed as fold-difference in comparison to fibronectin alone. GAPDH expression was used for normalization. T98G cells were plated for 4h on fibronectin, and on FN/TN-C in the presence and absence of ET1 before documentation of cell adhesion

ET1 signaling restores cell adhesion on a fibronectin/tenascin-C substratum

To examine whether EDNR signaling affects tenascin-C-induced cell rounding, we determined cell spreading of T98G cells on fibronectin in the presence and absence of tenascin-C, upon stimulation with the EDNR ligand endothelin-1 (ET1) in serum-free medium. As previously observed, we confirmed that cells did not spread on FN/TN-C. But, stimulation with ET1 restored cell spreading on the FN/TN-C substratum (**Fig. 1B**). This was accompanied by actin stress fiber and focal adhesion formation 1h, 4h and 12h after plating of the cells (**Fig. 1C, Supplemental Fig. 1**).

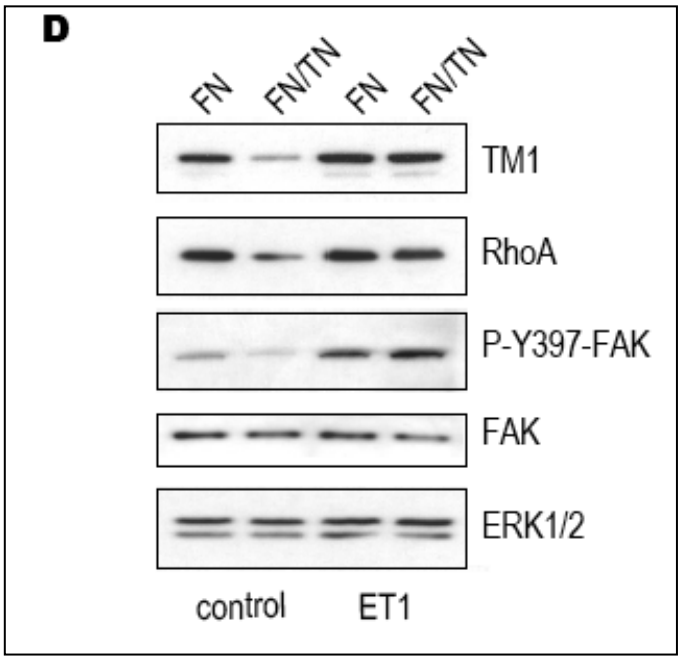


T98G cells were plated for 4h on fibronectin, and on FN/TN-C in the presence and absence of ET1 before documentation of cell adhesion (**B**). Control: serum-free medium.



T98G cells were plated for 4h on fibronectin, and on FN/TN-C in the presence and absence of ET1 before documentation of immunofluorescent staining for polymerized actin (arrow) with TRITC-phalloidin and for focal adhesions (arrow) with an anti-vinculin antibody (**C**). Control: serum-free medium.

To determine how ET1 inhibited tenascin-C-induced cell rounding, the expression and function of tenascin-C target molecules was assayed. T98G cells were grown for 4h on fibronectin or FN/TN-C in the presence or absence of ET1. Cell extracts were then analyzed by immunoblotting for FAK, active FAK (PY397-FAK), RhoA and TM1. Autophosphorylation of FAK at Y397, and expression of RhoA and TM1 were largely reduced in serum-free medium in the presence of tenascin-C. However, upon stimulation with ET-1, expression of P-Y397FAK, RhoA and TM1 were restored to levels observed on fibronectin alone. On fibronectin, ET1 also accelerated spreading and enhanced phosphorylation of FAK (**Fig. 1D**).



T98G cells were plated for 4h on fibronectin, and on FN/TN-C in the presence and absence of ET1 before lysis and immunoblotting for FAK, RhoA, TM1 and ERK1/2 (**D**)

ET1 inhibits tenascin-C signaling through activation of EDNRB

T98G cells express two ET1 receptors, EDNRA (**Fig. 1A**) and EDNRB (25). To examine EDNR-specific effects we analyzed cell adhesion on FN/TN-C in the presence of specific inhibitors for EDNRA (BQ123) and EDNRB (BQ788) upon stimulation with ET1. T98G cells were plated for 1h or 12h on fibronectin or FN/TN-C before addition of ET1 and the respective EDNR inhibitor. Cells were then analyzed for adhesion followed by lysis and immunoblotting. As shown in **figure 2A**, inhibition of EDNRB with BQ788 caused cell rounding, thus blocking ET1-induced cell spreading on FN/TN-C 5h (data not shown) and 16h after plating. Inhibition of EDNRB by BQ788 also blocked restoration of autophosphorylation of FAK and reexpression of RhoA and TM1 on FN/TN-C at 5h and 16h (**Fig. 2B**). In contrast, the EDNRA specific inhibitor BQ123 did not block restoration of cell spreading by ET-1. Inhibition of EDNRA through BQ123 did also not affect FAK phosphorylation and RhoA and TM1 re-expression induced by ET1.

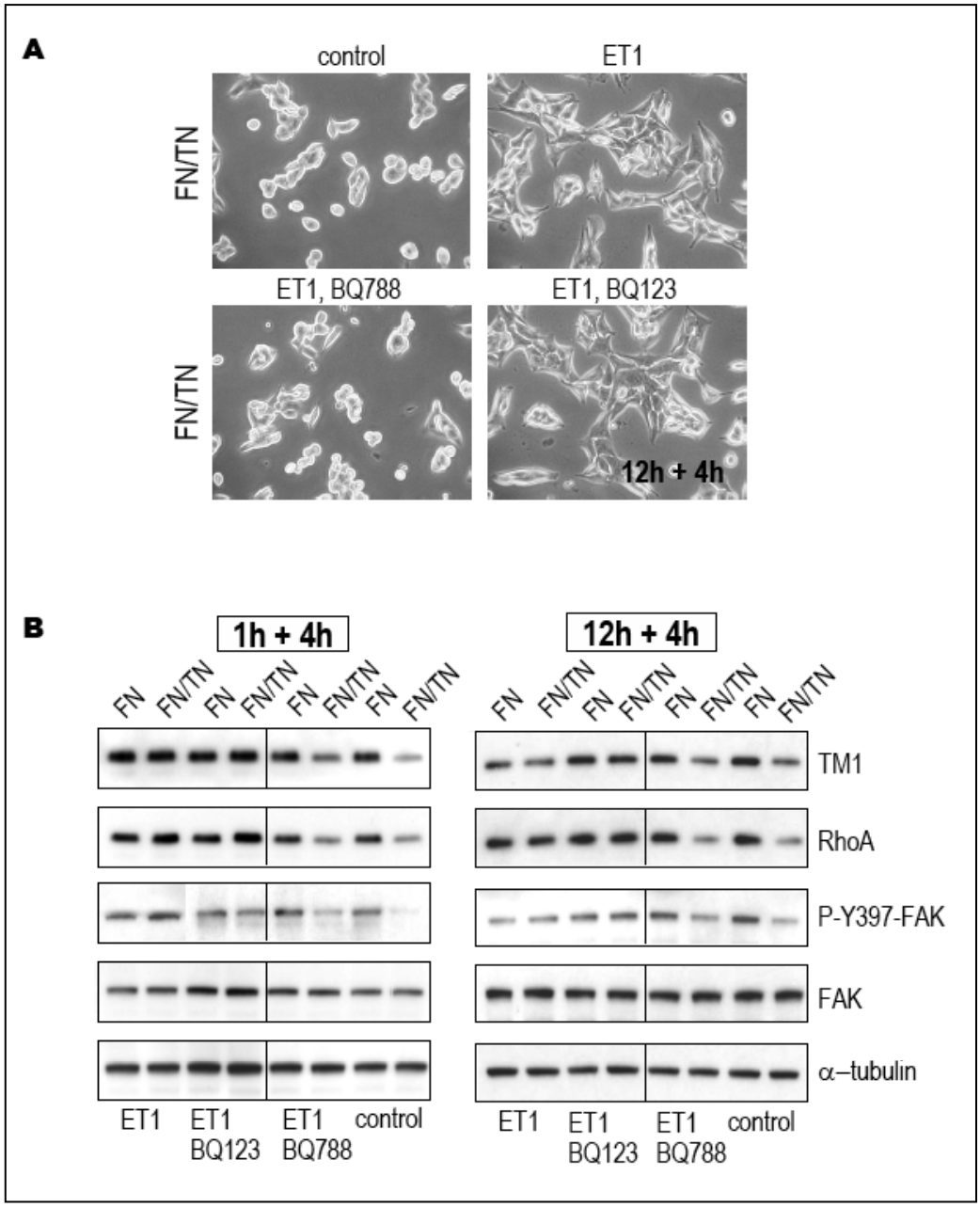
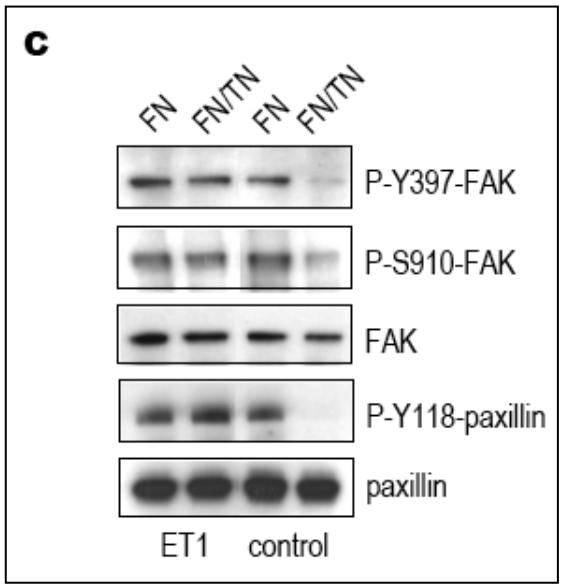


Fig. 2 EDNRB activation inhibits cell rounding on FN/TN-C
T98G cells were plated on fibronectin (not shown) and FN/TN-C in serum-free medium for 1h (**B, C**) and 12h (**A, B**) before addition of ET1 in the absence and presence of the EDNRA and EDNRB inhibitors BQ123 and BQ788, respectively for another 4h. (**A**) Documentation of cell adhesion. (**B, C**) immunoblotting for the indicated molecules.
Control: serum-free medium plus DMSO.

To confirm that signaling specifically through EDNRB inhibits tenascin-C-induced cell rounding, T98G cells were stimulated with ET3, an EDNRB ligand that has several fold higher affinity for EDNRB than for EDNRA (11). Subsequently, cell adhesion was determined prior to lysis and immunoblotting. ET3 also stimulated cell spreading, and formation of focal adhesions and actin stress fibers in the presence of tenascin-C (**Fig. 1C**). Moreover, spreading was EDNRB-dependent since it was blocked with BQ788 (**Supplemental Fig. 2A**). As for ET1, activation of EDNRB with ET3 restored TM1 and RhoA expression and autophosphorylation of FAK in the presence of tenascin-C (**Supplemental Fig. 2B**).

ET1 restores cell spreading by mediating focal adhesion assembly

EDNRB signaling leads to cell-matrix adhesion and appearance of numerous focal contacts even in the presence of tenascin-C (**Fig. 1C**). To investigate in more detail how EDNRB signaling could enhance focal adhesion assembly, we examined whether FAK was phosphorylated at S910, a site that is involved in binding of FAK to paxillin (26, 27). Whereas P-S910-FAK levels were low on FN/TN-C, phosphorylation of FAK at S910 returned to levels as on fibronectin upon stimulation with ET1 (**Fig. 2C**). Next, we examined whether FAK was functional in phosphorylating paxillin at Y118, one of its cognate phosphorylation sites (28), after stimulation with ET1. In cells plated on FN/TN-C in the absence of ET1, phosphorylation at Y118 in paxillin was abolished (**Fig. 2C**). In contrast, cells plated on FN/TN-C in the presence of ET1 showed high levels of phosphorylated paxillin (**Fig. 2C**). Altogether, these data show that ET1 signaling through EDNRB restored FAK and paxillin activities in the presence of tenascin-C.



T98G cells were plated for 4h on fibronectin, and on FN/TN-C in the presence and absence of ET1 before lysis and immunoblotting for FAK, RhoA, TM1 and ERK1/2 (C). Control: serum-free medium.

Activation of EDNRB induces cell spreading in an EGFR-, PLC-, PI3K- and JNK-dependent manner

We next investigated by which pathway EDNRB inhibits tenascin-C-mediated cell rounding. Since EDNR signaling activates EGFR, we addressed whether EDNRB-induced cell spreading on FN/TN-C involves EGFR function. Cells were plated on FN/TN-C for 5h together with ET1 and the inhibitor PKI166, which prevents autophosphorylation of EGFR (29). Cell adhesion was documented prior to lysis and immunoblotting. Inhibition of EGFR by PKI166 completely blocked cell spreading by ET1/EDNRB (**Fig. 3A**).

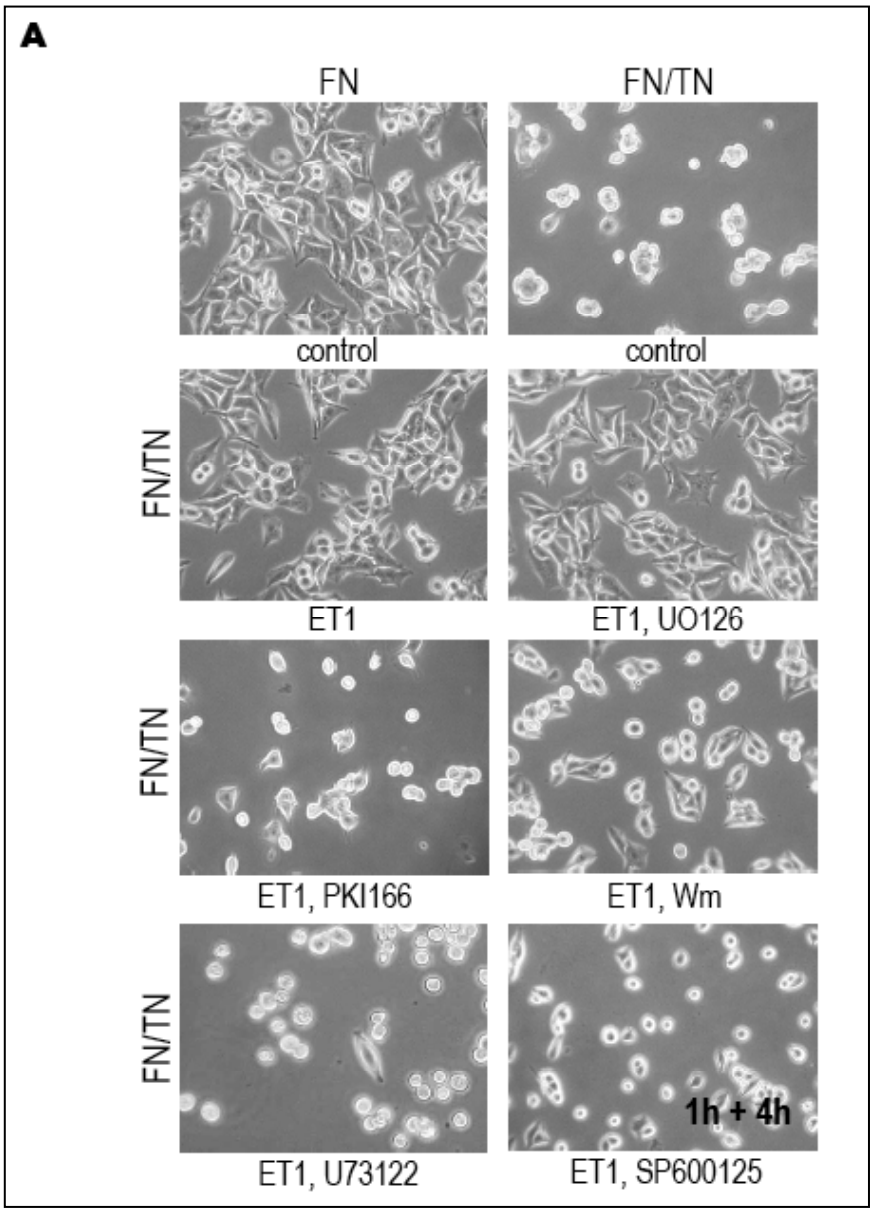
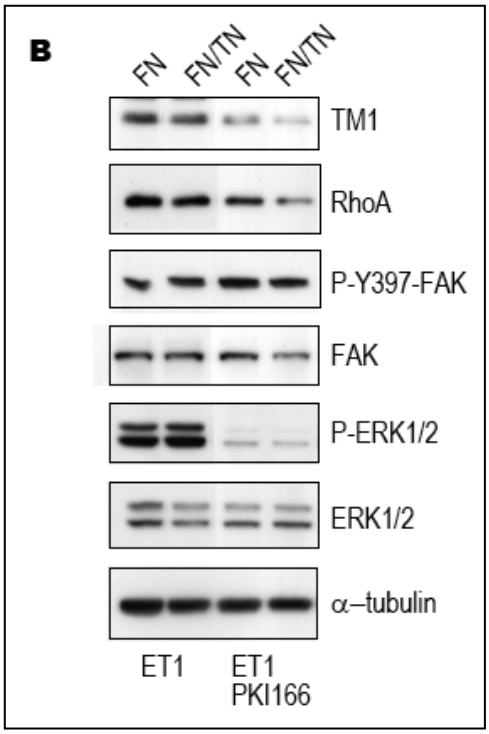


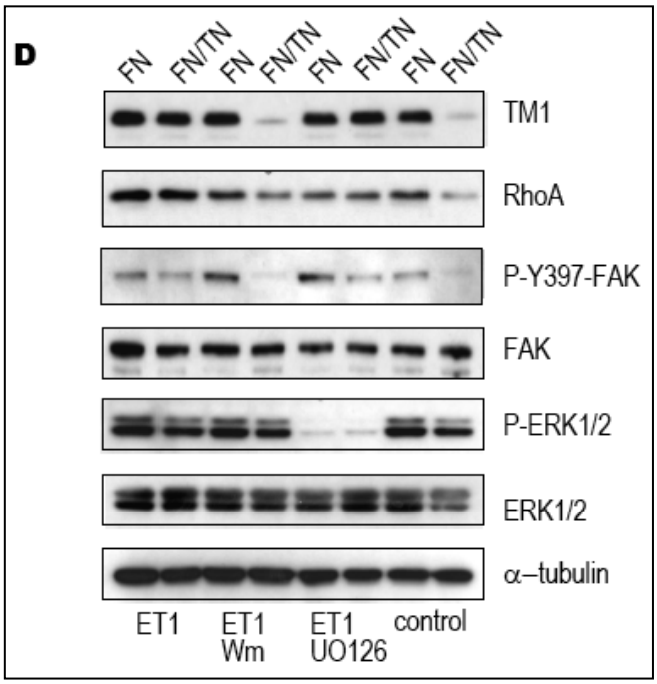
Fig. 3 EDNRB activation induces cell spreading dependent on EGFR, PLC, PI3K and JNK
T98G cells were plated on fibronectin or FN/TN-C for 1h in serum-free medium before addition of ET1 and DMSO in the presence or absence of the respective inhibitors. (**A)** documentation of cell adhesion. Control see Fig. 2.

Moreover, as shown in **Figure 3B**, PKI166 efficiently blocked ERK1/2 phosphorylation and ET1-induced TM1 and RhoA expression in the presence of tenascin-C. However, ET1-induced autophosphorylation of FAK was not affected by PKI166 (**Fig. 3B**). Thus, ET1 restores TM1 and RhoA protein expression in the presence of tenascin-C through activation of EGFR. In contrast, activation of FAK by EDNRB is independent of EGFR signaling, but FAK activation alone is not sufficient to mediate cell spreading.



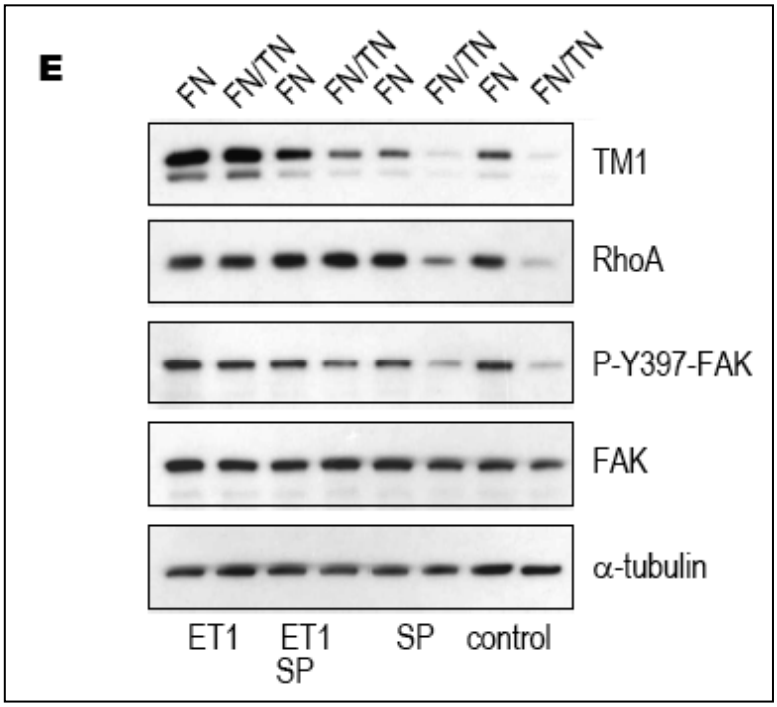
T98G cells were plated on fibronectin or FN/TN-C for 1h in serum-free medium before addition of ET1 and DMSO in the presence or absence of the respective inhibitor. (**B**) immunoblotting. Control see Fig. 2.

ET1-treated cells were allowed to adhere on FN/TN-C in the presence of inhibitors for PLC, MEK, PI3K and JNK. Inhibition of PLC with U73122 blocked ET1-induced cell spreading on FN/TN-C (**Fig. 3A**), which correlated with low levels of P-FAK, TM1 and RhoA (**Fig. 3C**). Examining MEK and PI3K downstream of EGFR and PLC, revealed that the MEK inhibitor UO126 did not affect ET1-induced cell spreading on the tenascin-C substratum. This was in contrast to the PI3K inhibitor wortmannin which diminished cell spreading (**Fig. 3A**). Cell rounding by wortmannin correlated with largely reduced RhoA and TM1 levels and lack of FAK autophosphorylation in the presence of ET1 on FN/TN-C (**Fig. 3D**). These data suggest that EDNRB-induced cell spreading on FN/TN-C depends on EGFR, PLC and PI3K, but does not involve the MEK pathway.



T98G cells were plated on fibronectin or FN/TN-C for 1h in serum-free medium before addition of ET1 and DMSO in the presence or absence of the respective inhibitors. (**D**) immunoblotting. Control see Fig. 2.

Next, we examined whether c-Jun aminoterminal kinase JNK, a downstream effector of EDNR and EGFR signaling, is involved in cell spreading on FN/TN-C upon activation of EDNRB. Inhibition of JNK with SP600125 not only prevented ET1-induced cell spreading (**Fig. 3A**), but also blocked FAK autophosphorylation and TM1 reexpression in the presence of tenascin-C, which was in contrast to RhoA, the expression of which was not altered upon JNK inhibition (**Fig. 3E**). Altogether, these data suggest that EDNRB activation counteracts tenascin-C-induced cell rounding through EGFR and downstream PLC, JNK and PI3K, and that all three molecules FAK, RhoA and TM1 need to be active in order to allow cell spreading on FN/TN-C.



T98G cells were plated on fibronectin or FN/TN-C for 1h in serum-free medium before addition of ET1 and DMSO in the presence or absence of the respective inhibitors.
(**E**) immunoblotting. Control see Fig. 2.

Inhibition of EDNRA induces cell spreading in the presence of tenascin-C

Since plating cells on a tenascin-C-containing substratum leads to increased expression of EDNRA, we asked whether EDNRA signaling could induce cell rounding in cells plated on FN/TN-C. We determined cell adhesion in the presence of both EDNR inhibitors at a time point when EDNRA expression was induced by tenascin-C. T98G cells were plated in serum free medium for 12h before addition of ET1 and the respective inhibitor for an additional 30 minutes (**Supplemental Fig. 3**) or 4h (**Fig. 4**). We observed that whereas cells were round 12h after plating on FN/TN-C, they spread with ET1 in the presence of both inhibitors (**Fig. 4A**). Since inhibition of EDNRB by BQ788 prevented cell spreading (**Fig. 2A**), restoration of cell spreading under these conditions was due to inhibition of EDNRA. Already 30 minutes upon addition of exogenous ET1, T98G cells were spread on FN/TN-C (**Supplemental Fig. 3A**). Thus, inhibition of EDNRA rapidly overcomes cell rounding on FN/TN-C. Simultaneous inhibition of both EDNRs in the absence of exogenously

added ET1 also induced cell spreading suggesting that EDNRA is active in the absence of exogenously provided ET1 (**Fig. 4A, Supplemental Fig. 3A**). EDNRA is presumably activated in T98G cells on FN/TN-C by secreted endothelins ((25), M. Kammerer and G. Orend, data not shown). To proof this possibility, cells on FN/TN-C were stimulated with conditioned medium derived from 48h serumfree cultures of T98G cells. Cells remained round on the FN/TN-C substratum with conditioned medium, but spread under these conditions upon inhibition of EDNRA with BQ123 (**Fig. 4A**).

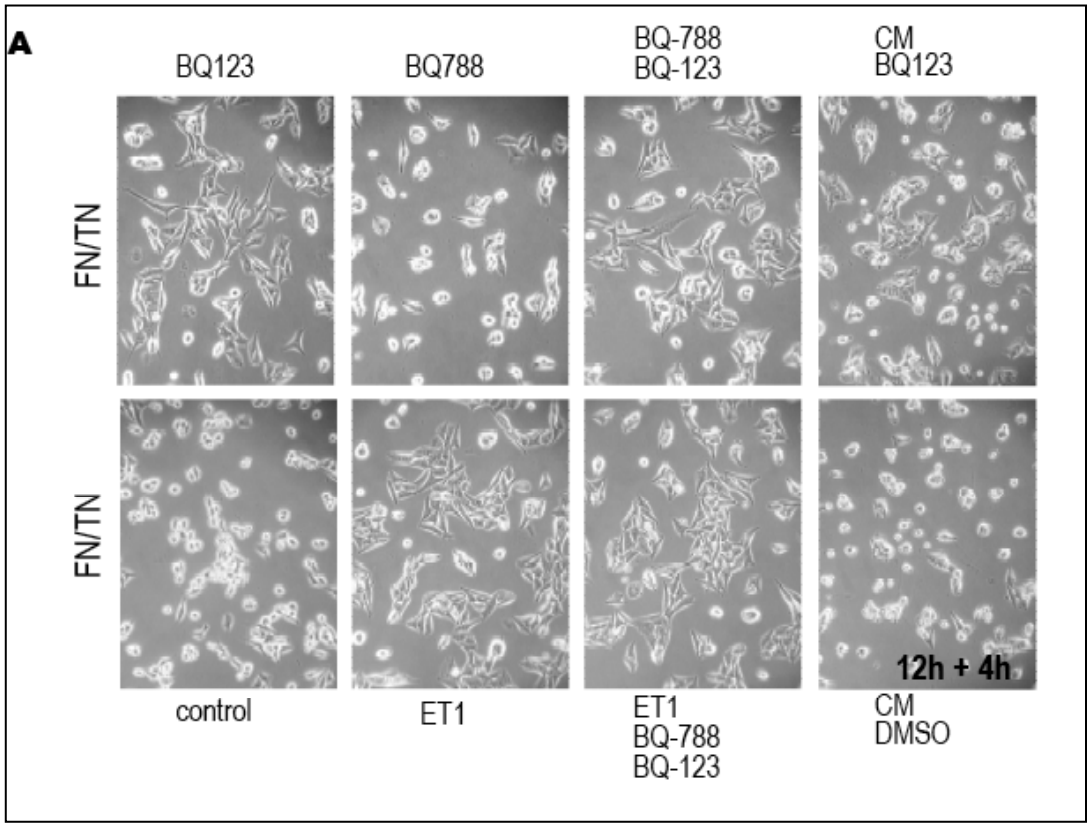
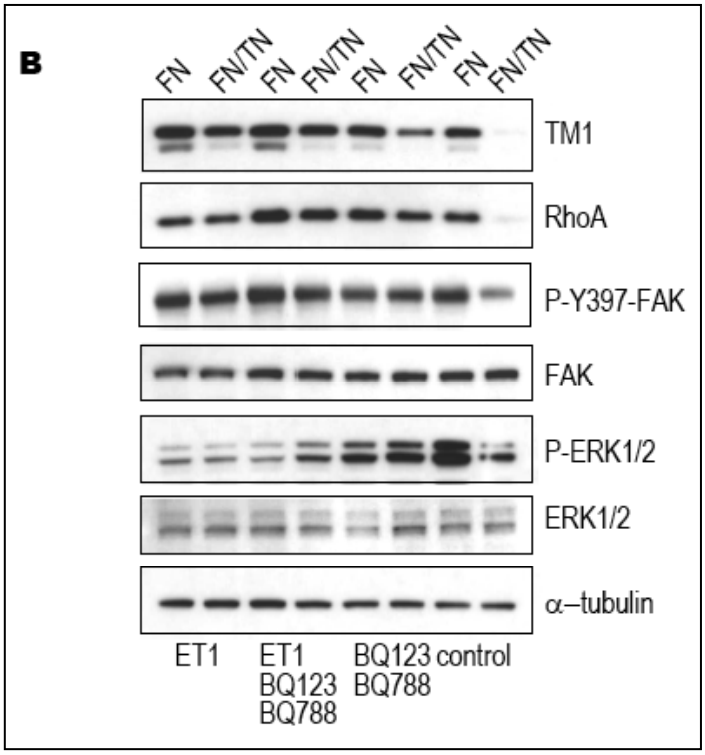


Fig. 4 EDNRA-induced cell rounding on FN/TN-C is MEK-dependent
T98G cells were plated for 12h (**A**) on fibronectin or FN/TN-C in serum-free medium before addition of conditioned medium (CM) from 48h serum-free cultures, ET1, DMSO, BQ123, BQ788 and UO126. (**A**) documentation of cell adhesion.

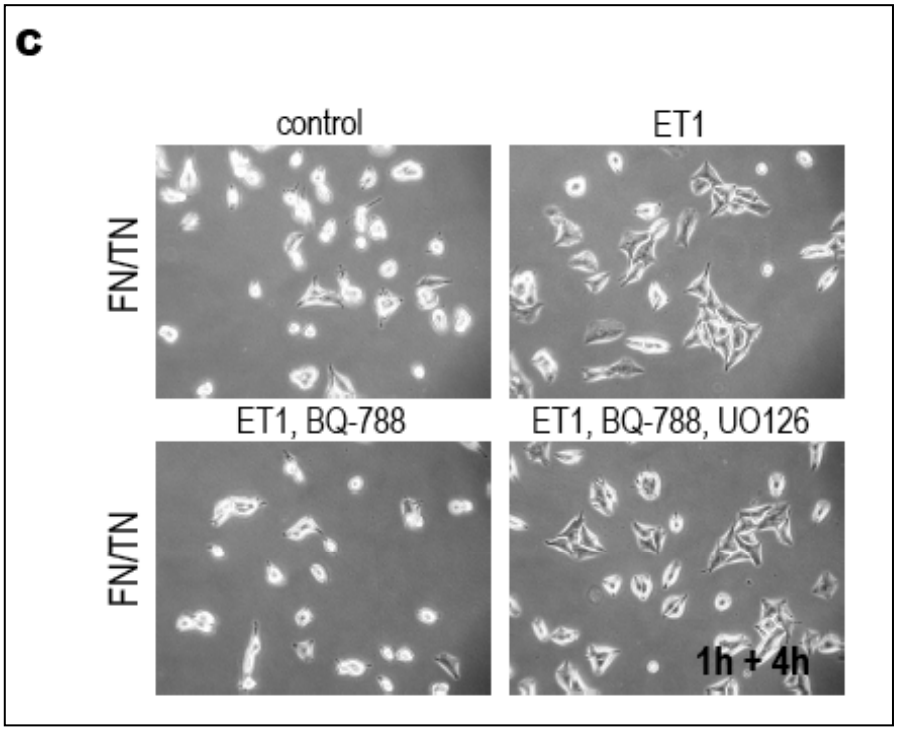
Immunoblotting revealed that FAK autophosphorylation and expression of RhoA and TM1 were restored at 30 minutes (**Supplemental Fig. 3B**) and 4h (**Fig. 4B)** upon blocking of EDNRA. In addition, inhibition of both endothelin receptors enhanced ERK1/2 phosphorylation on FN/TN-C which was in contrast to the control with very low P-ERK1/2 levels. To examine whether activation of EDNRA by tenascin-C causes rounding in MDA-MB435 breast carcinoma and J82 urinary bladder carcinoma cells that express EDNRA as determined by real time RT-PCR (M. Kammerer and G. Orend, unpublished), we determined adhesion of these cells on fibronectin and FN/TN-C upon treatment with BQ123. As for T98G, these cells did not spread on FN/TN-C and, inhibition of EDNRA with BQ123 restored cell spreading on FN/TN-C (**Supplemental Fig. 4A**). Thus, activation of EDNRA mediates rounding of all three cell lines by tenascin-C.



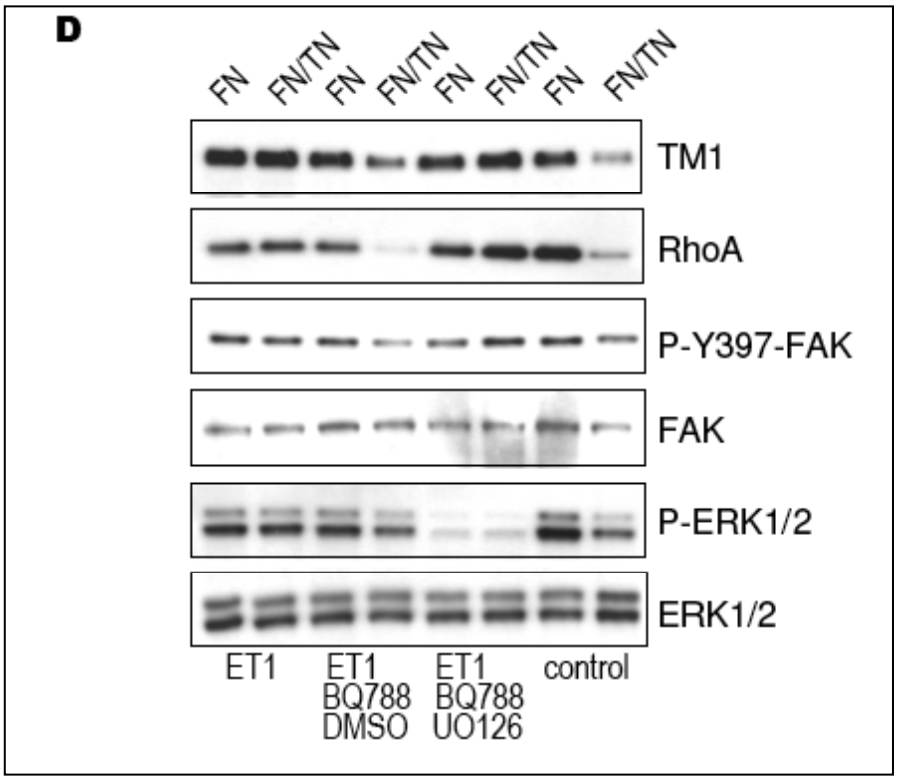
T98G cells were plated for 12h (**B**) on fibronectin or FN/TN-C in serum-free medium before immunoblotting for the indicated molecules (B).

EDNRA-specific cell rounding by tenascin-C is MEK-dependent

We wanted to know whether the MAP kinase pathway, that blocks expression of TM1 (30, 31), is involved in EDNRA-stimulated inhibition of TM1 expression by tenascin-C. Therefore, cells plated onto FN/TN-C were stimulated with ET1 in the presence of the EDNRB inhibitor BQ788, which allows to determine signaling in response to EDNRA activation. Inhibition of the MEK pathway by UO126 restored cell spreading in combination with BQ788 (**Fig. 4C**). Thus, MEK is downstream of EDNRA. UO126 also normalized TM1 and RhoA expression and FAK autophosphorylation on FN/TN-C to levels as on fibronectin upon treatment with ET1 and BQ788 (**Fig. 4D**). Thus, activation of MEK through EDNRA contributes to tenascin-C-induced cell rounding on fibronectin by blocking FAK activation and RhoA and TM1 expression.



T98G cells were plated for 1h (**C**) on fibronectin or FN/TN-C in serum-free medium before addition of ET1, DMSO, BQ788 and UO126. (**C**) documentation of cell adhesion.



T98G cells were plated for 1h (**D**), on fibronectin or FN/TN-C in serum-free medium before addition of ET1, DMSO, BQ788 and UO126. (**D**) immunoblotting for the indicated molecules.

Regulation of TM1 and RhoA RNA and protein levels on FN/TN-C

To determine whether tenascin-C affects RNA levels of αTM (coding for TM2 and 3), βTM (coding for TM1) and RhoA, a real time RT-PCR experiment was performed on RNA prepared from cells that were grown in the presence or absence of tenascin-C for different time points. In T98G cells RNA levels of αTM started to drop at 5h and remained about 40-50% below those on fibronectin after 19h on FN/TN-C (**Fig. 5A**). A similar observation was made for J82 cells (**Supplemental Fig. 5B**). This was in contrast to earlier time points (1h and 3h) where no substratum-specific differences in αTM expression were observed in T98G cells (**Fig. 5A**). Similarly, βTM and RhoA RNA levels were not downregulated on FN/TN-C in comparison to fibronectin at any time point (**Fig. 5B, C**). These results show that RNA levels of αTM, βTM and RhoA are not reduced in the presence of tenascin-C at early time points.

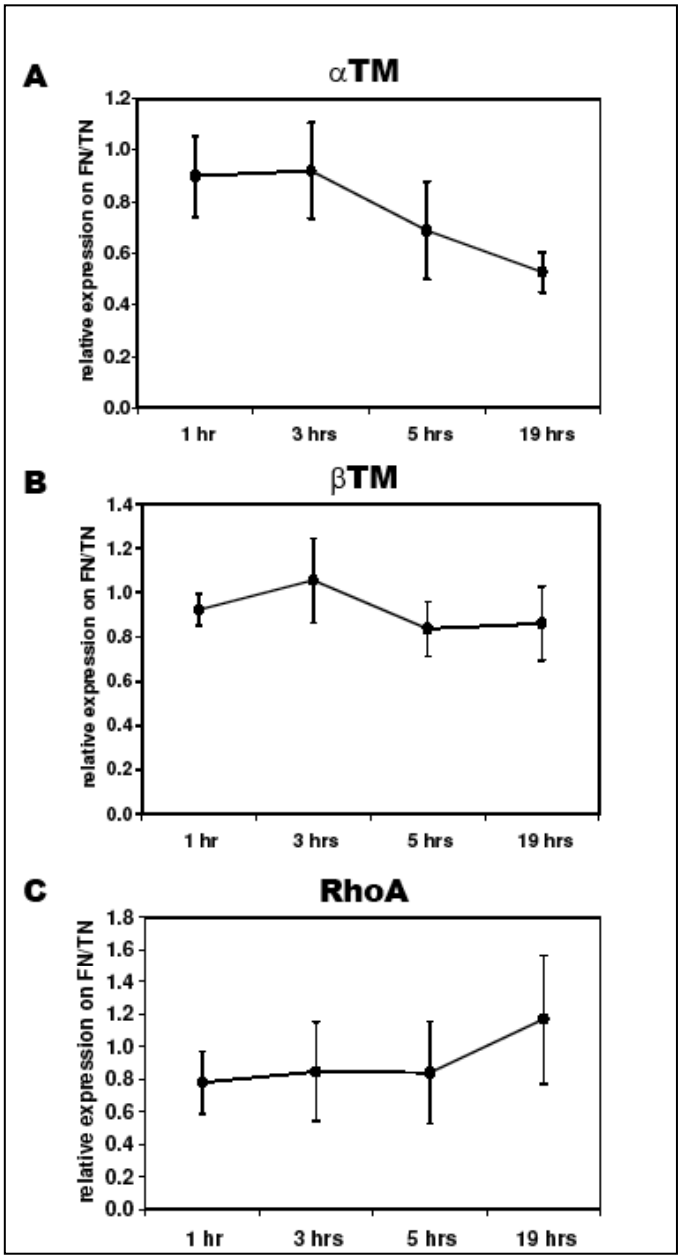
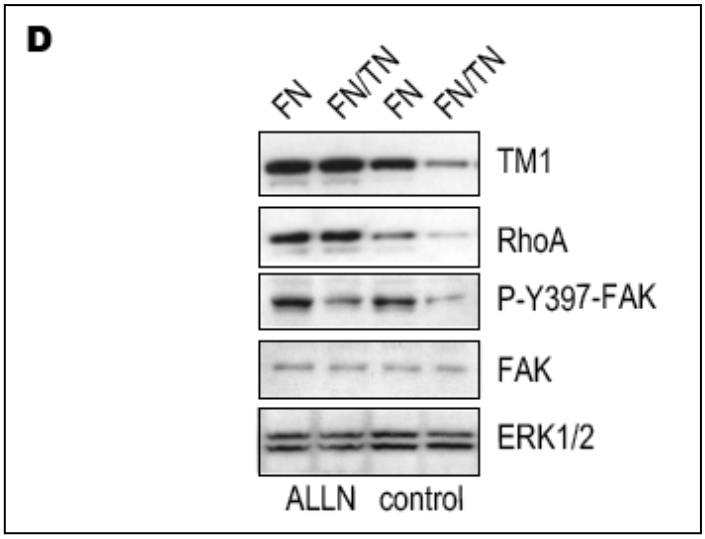


Fig. 5 Regulation of αTM, TM1 and RhoA RNA and protein levels on FN/TN-C
T98G cells were plated on fibronectin or FN/TN-C for the indicated time points (**A - C**) in serum-free medium (**A – C**), before RNA extraction and real time RT-PCR (**A – C**).

Next, we examined TM1 and RhoA protein stability on FN/TN-C and observed that TM1 and RhoA protein levels were restored on FN/TN-C to levels as observed on fibronectin upon treatment with the proteasome inhibitor ALLN (**Fig. 5D**). In contrast, phosphorylation of FAK was not restored with ALLN and this observation correlated with the inability of cells to spread on FN/TN-C under these conditions (**Table 1).**

Since αTM levels dropped significantly after 19h and, BQ123 restored TM1 expression on FN/TNC, we examined whether EDNRA signaling contributed to reduced αTM RNA levels in T98G and J82 cells. But inhibition of EDNRA by BQ123 only slightly increased αTM levels on FN/TN-C in both cell lines (**Supplemental Fig. 5**). In summary, our data suggest, that enhanced proteolysis of TM1 and RhoA is the major mechanism by which EDNRA negatively regulates expression of RhoA and TM1 on FN/TN-C.



T98G cells were plated on fibronectin or FN/TN-C for 5h (**D**) in EGF and ALLN (**D**) before lysis and immunoblotting (**D**).

Table 1 Summary of cellular signaling in response to tenascin-C upon modulation of EDNRA and EDNRB activation

Treatment on FN/TN-C	Inhibition	Active EDNR	Spreading	P-Y397-FAK	RhoA	TM1
no GF		A	0	0	0	0
ET3		B	1	1	1	1
ET3, BQ788	EDNRB	(A)	0	0	0	0
ET1		(A) B	1	1	1	1
ET1, BQ788	EDNRB	A	0	0	0	0
ET1, PKI166	EGFR	(A) B	0	**1**	0	0
ET1, U73122	PLC	(A) B	0	0	0	0
ET1, Wm	PI3K	(A) B	0	0	0	0
ET1, SP600125	JNK	(A) B	0	0	**1**	0
ET1, UO126	MEK	(A) B	1	1	1	1
ET1, BQ788, UO126	EDNRB, MEK	A	1	1	1	1
ET1, BQ123	EDNRA	B	1	1	1	1
ET1, BQ123, BQ788	EDNRA, EDNRB	no	1	1	1	1
BQ123, BQ788	EDNRA, EDNRB	no	1	1	1	1

Cell spreading and downstream events presented in this study are summarized. The presence (1) or absence (0) of cell spreading (4th column), on FN/TN-C upon stimulation with ET1 or ET3 is indicated as deduced from immunofluorescent staining with TRITC-phalloidin and an anti-vinculin antibody or by phase contrast microscopy. Data are shown on the effect of the inhibitors BQ788 (EDNRB), BQ123 (EDNRA), PKI166 (EGFR), U73122 (PLCβ/γ), Wm (PI3K), UO126 (MEK) and SP600125 (JNK). The absence (0) or presence (1) of phosphorylated FAK (P-Y397-FAK), and RhoA and TM1 protein on FN/TN-C is depicted. Relative activity of EDNRA and EDNRB is shown in the second column.

Expression of tenascin-C and EDNRA in cancer tissue

Next, we compared gene expression levels of tenascin-C and EDNRA in 80 GBM and four non-tumoral brain tissue (GeneChips U133 Plus, Affymetrix; manuscript in preparation) by SPIN analysis (sorting points into neighborhoods) (32) and by systematically evaluating gene expression changes using the ONCOMINE 3.1 platform (33). Tenascin-C and EDNRA mRNA expression revealed some correlation in GBM (pearson correlation 0.47) and in astrocytoma with high expression in gliomas and low expression in non-tumoral brain tissue (**Fig. 6A, Supplemental Table 1**). This is compatible with the notion that tenascin-C might have some role in

EDNRA regulation in GBM. Moreover, the majority of GBM exhibited also a high expression of EDNRB (**Fig. 6A, Supplemental Table 1**) with EDNRA and EDNRB showing a negative correlation (pearson correlation – 0.25) (**Fig. 6A**). On protein level, as determined by immunohistochemistry on TMAs, tropomyosin expression displayed different staining patterns. Tumor tissue was homogenously positive or negative for TM1-3, while staining of aberrant blood vessels was prominent in most tumors (**Fig. 6B**). There was neither a correlation of TM1-3 protein expression with tumor grade (Fisher exact test, p=0.09) nor with tenascin-C protein expression (Fisher exact test, p=0.09) in GBM. These observations suggest that other factors than tenascin-C influence tropomyosin expression in gliomas under steady-state conditions in a cell type specific manner.

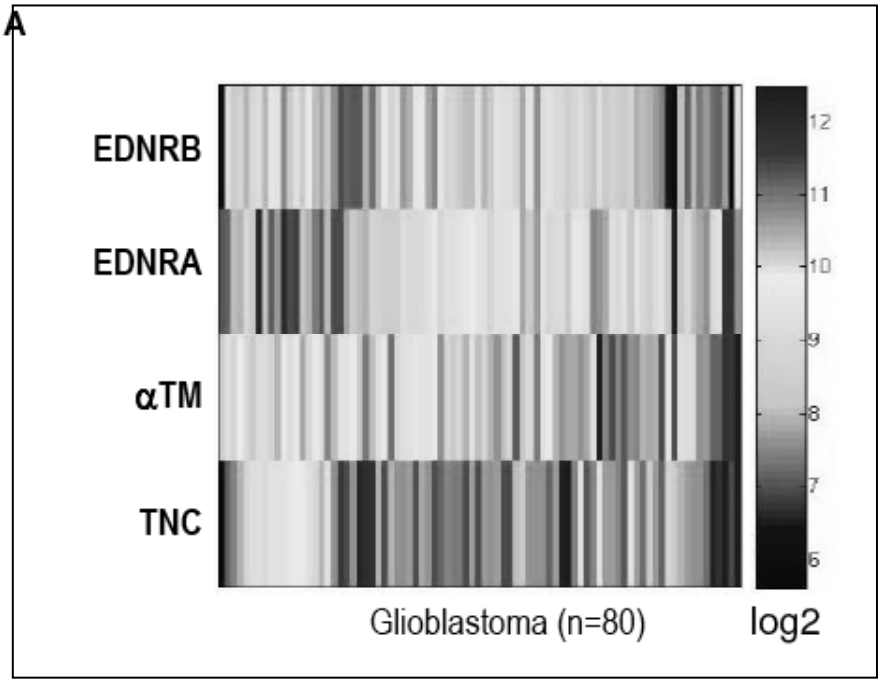


Fig. 6 Expression of tenascin-C, EDNR and tropomyosin in gliomas
(**A**) Gene expression of tenascin-C and potential mediators as ordered by SPIN analysis for 80 GBM. The ordering of points is iteratively permutated in search of a linear ordering. Expression is represented in a colour scale (log2) as determined on GeneChip 133PLUS2.

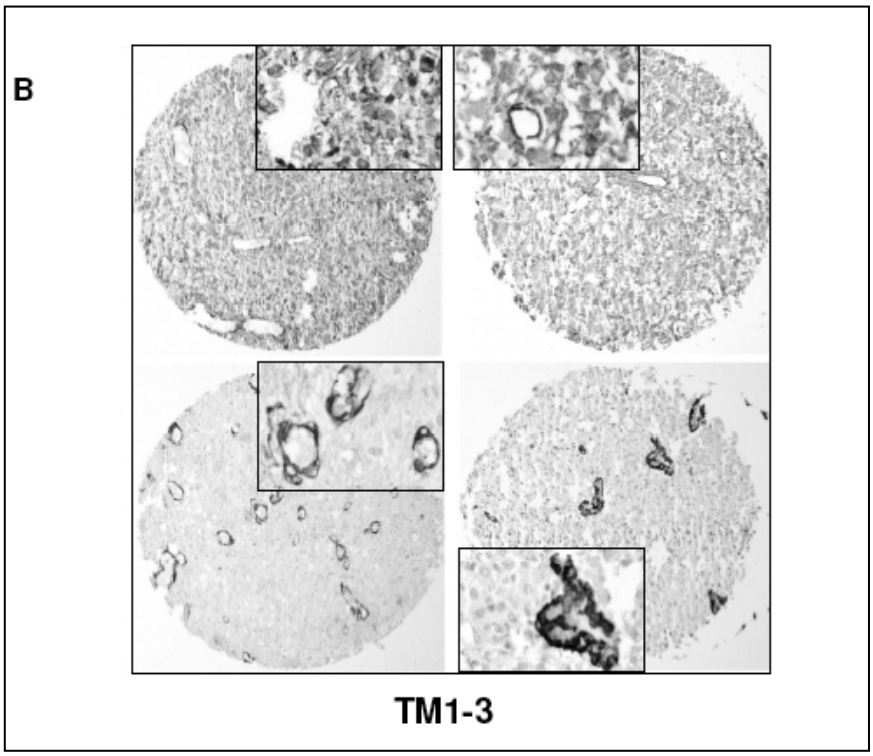


Fig. 6 Expression of tenascin-C, EDNR and tropomyosin in gliomas
(B) Four representative examples of immunohistochemical staining for TM1-3 of a glioma TMA are shown.

Searching the Oncomine database for a potential linked expression of tenascin-C and EDNRA in other human cancers revealed a high tenascin-C expression and increased EDNRA expression in malignant pancreatic ductal carcinoma, seminoma, bladder carcinoma, breast carcinoma and in metastatic ovarian carcinoma (**Supplemental Fig. 6, Supplemental Table 1**). On the contrary, a lowered tenascin-C expression in metastatic prostate and a subset of colon carcinoma correlated with reduced EDNRA expression. These observations are compatible with a potential regulation of EDNRA by tenascin-C in these cancers. EDNRB and αTM were also increased in astrocytoma, ovarian carcinoma, breast carcinoma, seminoma and pancreatic ductal carcinoma (**Supplemental Table 1**) which also matches with a potential regulation of αTM by EDNRB in these cancers.

A

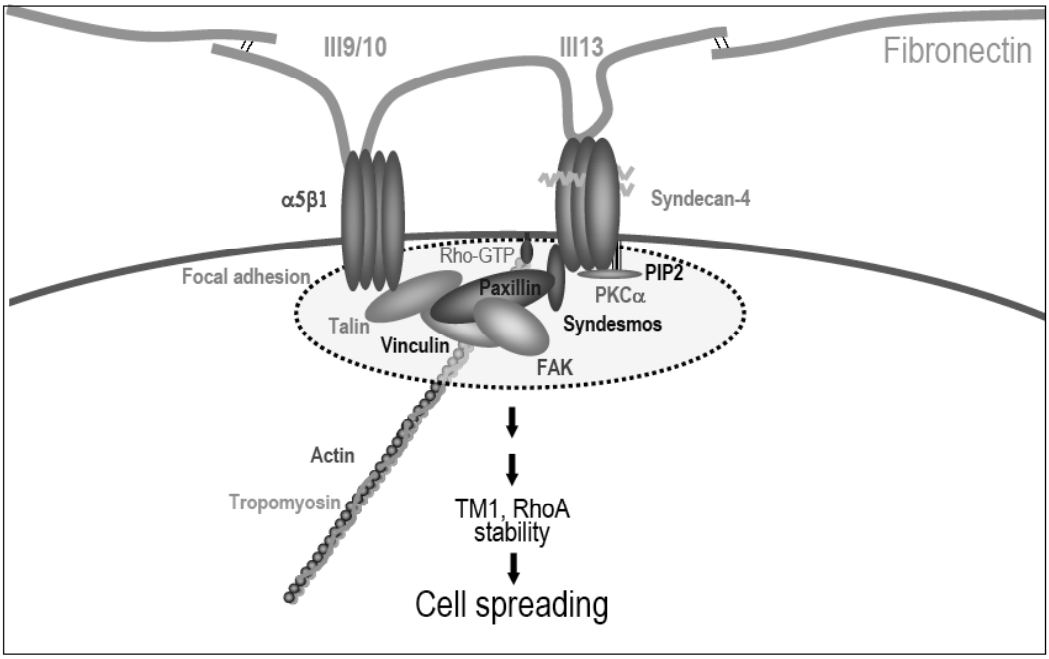


Fig. 7 Tenascin-C induces EDNRA signaling that is counteracted by EDNRB
(**A**) Upon activation of integrin α5β1 and syndecan-4 by fibronectin, cells activate FAK and RhoA upstream of TM1 which leads to actin polymerization and filament stabilization and subsequent cell spreading.

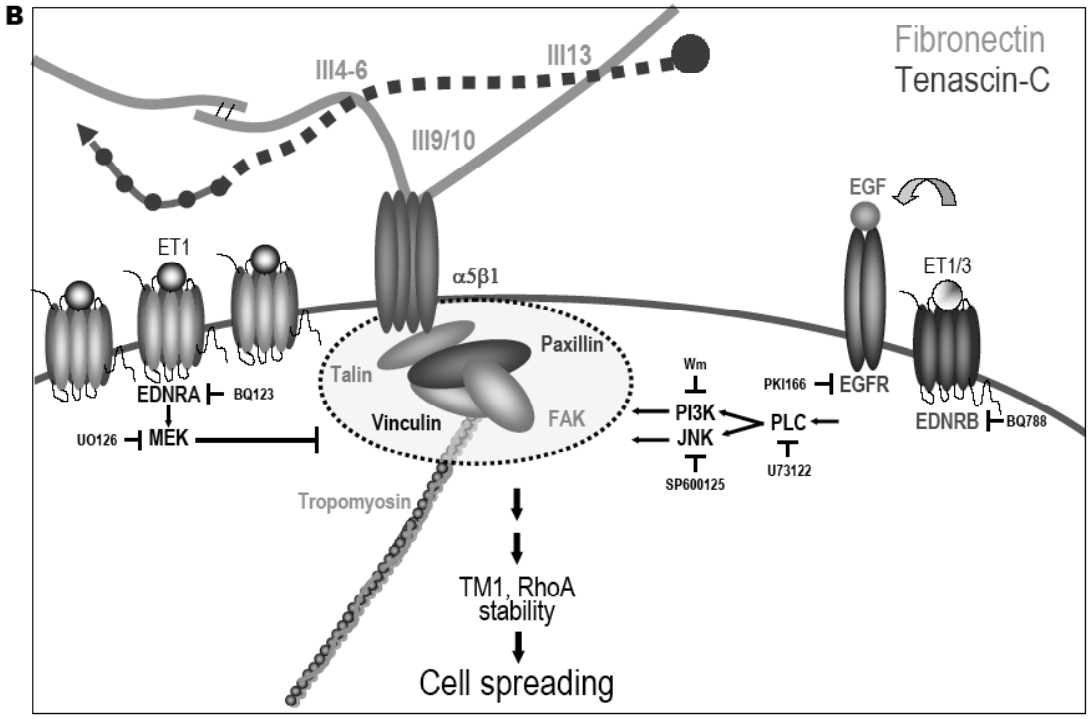


Fig. 7 Tenascin-C induces EDNRA signaling that is counteracted by EDNRB

(**B**) Tenascin-C induces EDNRA expression and EDNRA-induced MEK-dependent signaling causes enhanced proteolysis of TM1 and RhoA. This leads to cell rounding on a FN/TN-C substratum. In contrast to EDNRA, signaling by EDNRB (induced by ET1 or ET3) restores FAK and paxillin phosphorylation and downstream stabilization of TM1 and RhoA and, subsequently leads to cell spreading on FN/TN-C. This involves EGFR, PLC, PI3K, JNK, and other not yet identified downstream signaling molecules. EDNRB signaling might activate EGFR indirectly, presumably due to enhanced maturation of pro-EGF (40). It is remarkable that EDNRB-induced cell spreading on FN/TN-C occurs independent on activation of syndecan-4 by fibronectin.

Discussion

Previously, we and others showed that the function of FAK (2, 5), RhoA (4) and TM1 (10) is compromised in cells grown on FN/TN-C. This occurred in a syndecan-4 dependent manner since activation of syndecan-4 restored cell spreading and FAK activation in the presence of tenascin-C (2, 5) (**Fig. 7A**). Here we observed, that tenascin-C does not only interfere with RhoA activation (4), but also with RhoA protein stability. Moreover, we showed a complex repression of TM1 by tenascin-C. Although TM1 protein levels were reduced by tenascin-C, RNA levels were not lowered by tenascin-C at any time point up to 19h. In contrast, TM1 protein stability is largely reduced by tenascin-C, since inhibition of the proteasome with ALLN restored TM1 protein levels on FN/TN-C. After 19h, RNA levels of αTM coding for TM2 and 3, that are less expressed in T98G cells, significantly dropped. Reduced αTM levels might contribute to reduced TM1 expression, since TM1 forms heterodimers with TM2 and 3.

Our results show that inhibition of FAK, RhoA and TM1 is responsible for cell rounding by tenascin-C. To override the cell spreading block by tenascin-C, the function of all three molecules FAK, RhoA and TM1 needs to be restored (**Fig. 7B, Table 1**). This can be accomplished by activation of EDNRB, which induces cell spreading on FN/TN-C presumably due to FAK and paxillin activation and RhoA and TM1 protein stabilization. EDNRB-induced cell spreading on FN/TN-C was specific since it could be blocked with BQ788, a specific inhibitor of EDNRB (18) that is used in treatment of pulmonary hypertension (17). Restoration of expression and function of FAK, RhoA and TM1 by EDNRB signaling involved EGFR, PLC, PI3K and JNK, since specific inhibitors for these enzymes blocked EDNRB-specific cell spreading and reexpression of the tenascin-C target molecules.

We observed that activation of paxillin, an important component of integrin (28), syndecan-4 (34) and ET1 (11) signaling plays a key role in EDNRB-induced cell spreading on FN/TN-C, since EDNRB signaling induced phosphorylation of paxillin at Y118, one of its cognate phosphorylation sites for FAK (28) in the presence of tenascin-C. Whereas, phosphorylation of FAK at S910 and of paxillin at Y118 was absent or very low in the presence of tenascin-C in serum-free medium, EDNRB signaling restored phosphorylation in FAK and paxillin. We conclude, that

phosphorylation of FAK at S910 is involved in restoration of cell spreading on FN/TN-C presumably by phosphorylating paxillin. Localization of paxillin into focal adhesions is dependent on syndecan-4, which involves binding of syndesmos to syndecan-4 and paxillin (34). Tenascin-C impairs syndecan-4 activation by fibronectin (2), and prevents localization of paxillin into focal adhesions (5). Now, we showed that EDNRB activation restores focal adhesion formation on the FN/TN-C substratum. This suggests, that EDNRB signaling restores paxillin function on a FN/TN-C substratum through bypassing the requirement for syndecan-4 for the recruitment of paxillin into focal adhesions (**Fig. 7B**).

Tenascin-C stimulated EDNRA expression (10). By using the clinically relevant EDNRA-specific inhibitor BQ123 (17) we observed that inhibition of EDNRA restored cell spreading, which suggests that signaling through EDNRA mediated cell rounding on FN/TN-C. Similar to T98G glioblastoma cells, activation of EDNRA in MDA-MB435 breast carcinoma and J82 urinary bladder carcinoma cells also supported cell rounding by tenascin-C that could be blocked with BQ123. Whereas endogenously expressed ET1 activated EDNRA, exogenously added ET1 at a high concentration of 20 nM also activated EDNRB. Thus, upon activation of both receptors, signaling by EDNRB dominated over signaling by EDNRA in T98G, and in MDA-MB435 and J82 cells. Different affinities of EDNRA and EDNRB for the ligand, as has been reported by others (35), can explain our observation that activation of EDNRB as well as inhibition of EDNRA restored cell spreading on FN/TN-C.

Since activation of EDNRA (our data) and MEK downregulates TM1 expression (30, 31, 36), we examined whether EDNRA-associated cell rounding on FN/TN-C was dependent on MAP kinase signaling. Indeed, inhibition of MEK with UO126 blocked EDNRA-specific cell rounding on FN/TN-C and caused restoration of FAK phosphorylation and stabilization of RhoA and TM1. Our data link TM1 and RhoA protein instability to EDNRA (**Table 1**).

Tenascin-C induced EDNRA expression later than 5h after plating, suggesting that EDNRA signaling is important for tenascin-C-induced cell rounding on fibronectin at later time points. This could present a mechanism to enhance or maintain tenascin-C-induced cell rounding. Whether this involves syndecan-4 is unknown. A potential link of EDNRA to syndecan-4 in causing cell rounding by tenascin-C is supported by

our observation that ligation of syndecan-4 is sufficient to restore cell spreading on Fibronectin and to attenuate tumor cell proliferation in the presence of tenascin-C (2). Moreover, activation of EDNRA did not induce cell rounding in cells that were spread on fibronectin, where syndecan-4 is engaged as coreceptor in integrin signaling. We speculate that in situations where syndecan-4 is not working as coreceptor for integrin signaling, syndecan-4 might acquire other functions as e.g. promoting EDNRA signaling resulting in cell rounding in the presence of tenascin-C.

Tenascin-C and endothelin receptors are instrumental in neural crest cell (NCC) migration during embryonic development, since NCC migration was blocked in chicken embryos that were treated with tenascin-C antisense morpholinos (37). The homozygous knock-outs of EDNRA and EDNRB are embryonic lethal (38) and EDNRA and EDNRB signaling is crucial in NCC migration (39). Our data provide a functional link between tenascin-C, EDNRA and NCC migration. Tenascin-C may promote NCC migration along tenascin-C-rich cues by stimulating EDNRA signaling.

Our results suggest, that responses towards tenascin-C largely depend on the growth factor receptor status of a cell. EDNRA supports cell rounding by tenascin-C, through concomittant repression of RhoA and TM1. Our report is the first to link EDNRA with MEK signaling and cell rounding on a FN/TN-C substratum and enhanced proteolysis of RhoA and TM1. Moreover, similar expression of tenascin-C and EDNRA in 8 human cancers is compatible with tenascin-C playing a role in regulation of EDNRA *in vivo*. In contrast to EDNRA, we found that EDNRB blocks cell rounding by tenascin-C through activation of FAK and normalizing expression of RhoA and TM1. We linked EDNRB, EGFR, PLC, PI3K and JNK to cell spreading in the presence of tenascin-C. Moreover, EDNRB signaling appears to antagonize signaling through EDNRA. A potential interdependence of the two signaling pathways has been reported; inhibition of EDNRA with BQ123 significantly enhanced EDNRB-induced proliferation of endothelial cells (13). An increased EDNRB and αTM expression found in 5 human cancers supports the possibility that EDNRB signaling could be involved in αTM regulation in a steady-state situation. More detailed information is required to unravel potential interrelations between tenascin-C, EDNRA, EDNRB and tropomyosin in invasive human cancer tissue.

In summary, we presented a detailed mechanism by which tenascin-C causes cell rounding through EDNRA, and how cells modulate the anti-adhesive properties of tenascin-C through EDNRB and presumably other signaling. This is the first study to reveal that cell responses towards tenascin-C are modulated by growth factor signaling. More information is required about signaling pathways that support or counteract tenascin-C actions *in vivo* to use this knowledge for prediction of tumor malignancy.

Acknowledgement

We are grateful to Harold Erickson and Pia Wülfing for providing human tenascin-C cDNA and breast cancer material, respectively, Marie-France Hamou and ES Yong for technical assistance, Eytan Domany for sharing the SPIN software and Francois Lehembre and Matthias Chiquet for critically reading the manuscript. This work was supported by grants from the Swiss National Science Foundation 3100A0-102145/1 (GO), the Novartis Stiftung für Medizinisch Biologische Forschung (GO), Association for International Cancer Research (GO), the Swiss Cancer League OCS-01419-08-2003 (GO), Münster University Hospital grant IMFGÖ120415 (MG) and the National Medical Research Council, Singapore (GWY). Work from A.D. was part of her bachelor thesis in biotechnology at the University of Mannheim, Germany.

Abbreviations

ET1/3, endothelin-1/3, EDNR, endothelin receptor, EGFR, epidermal growth factor receptor, ERK, extracellular-signal regulated kinase, FAK, focal adhesion kinase, FCS, fetal calf serum, FN, fibronectin, FNIII13, fibronectin type III repeat 13, GAPDH, glycerol aldehyde dehydrogenase, GBM, glioblastoma multiforme, JNK, c-Jun aminoterminal kinase, MAPK, mitogen activated protein kinase, MEK, MAP kinase/ERK kinase, PLC, phospholipase, PI3K, phosphoinositol-3-kinase, RT-PCR, reverse transcription polymerase chain reaction, SPIN, sorting points into neighborhoods, TN-C, tenascin-C, TMA, tissue microarray, Wm, wortmannin.

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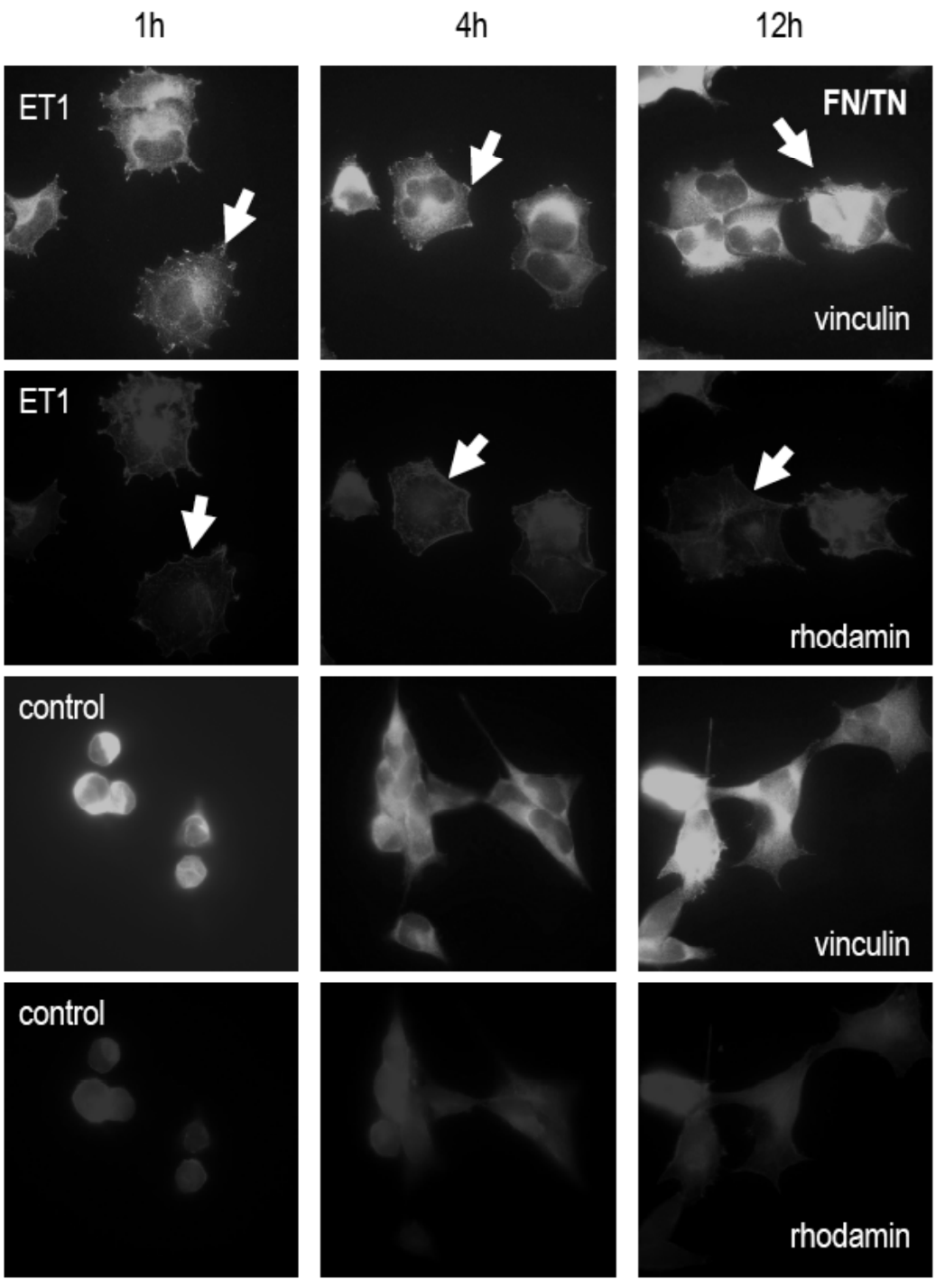
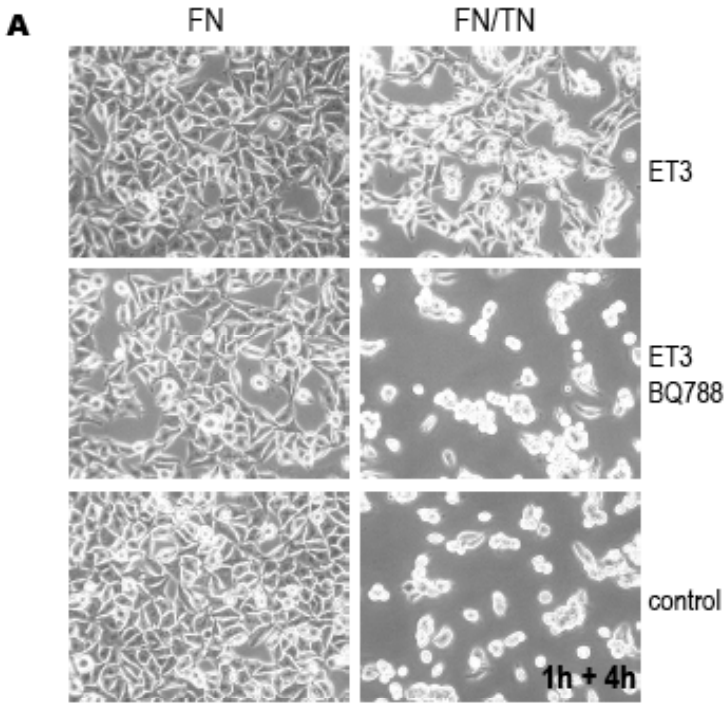
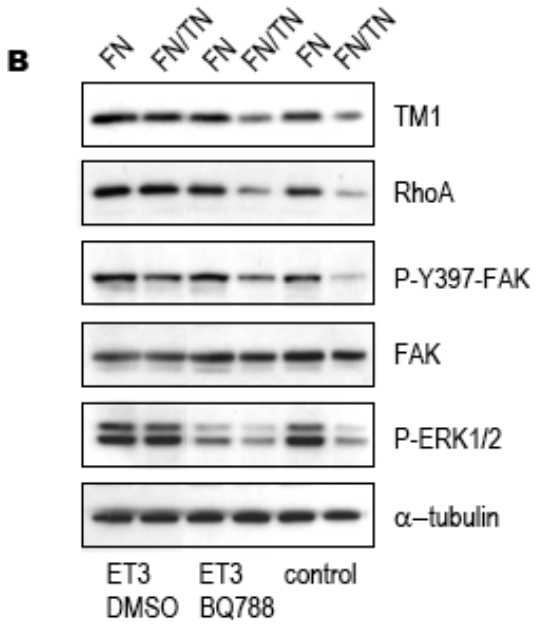
Supplemental Data

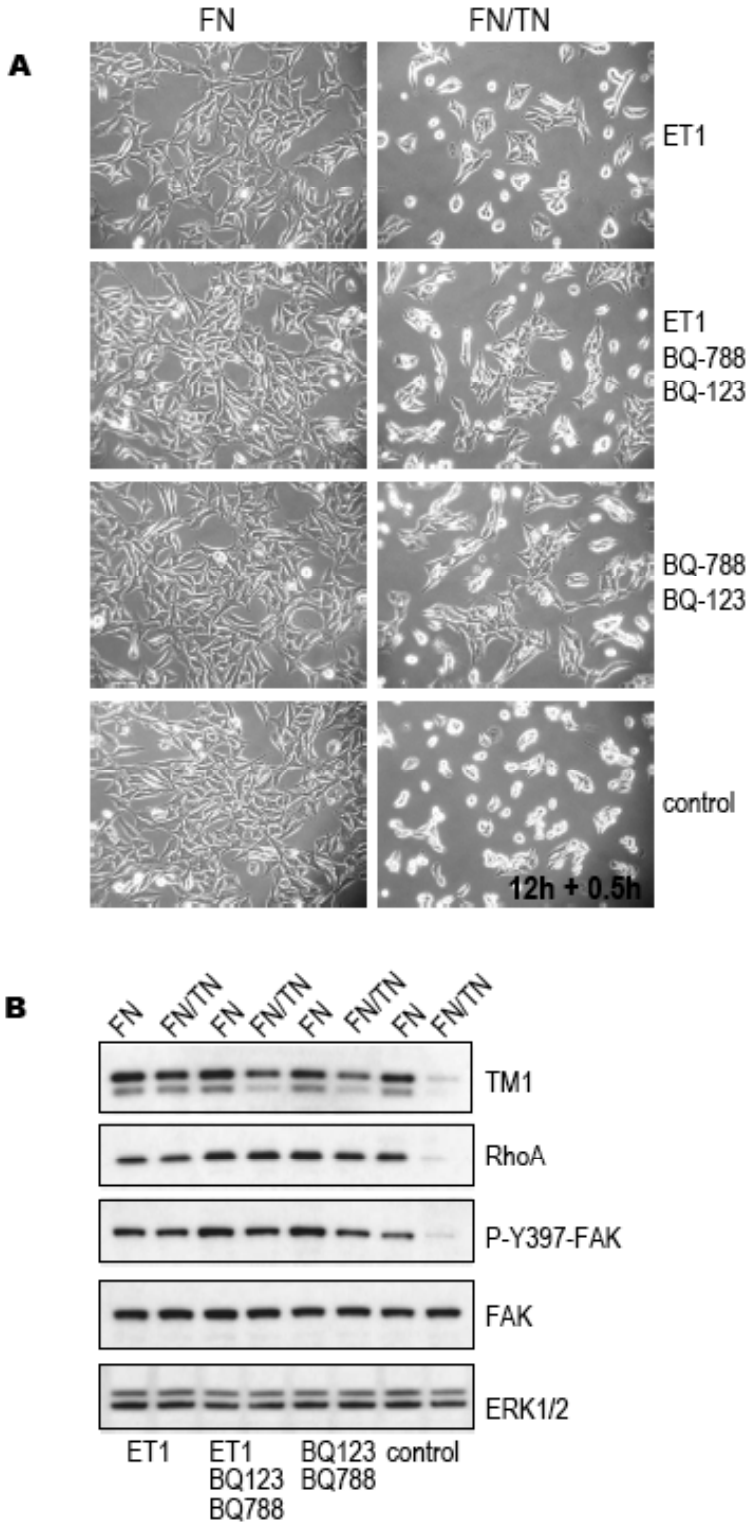


Supplemental Fig. 1 Kinetics of restored actin stress fiber and focal adhesion formation by ET1
T98G cells were plated for the indicated time points on FN/TN-C in the absence and presence of ET1 before immunofluorescent staining with TRITC-phalloidin or an anti-vinculin antibody.



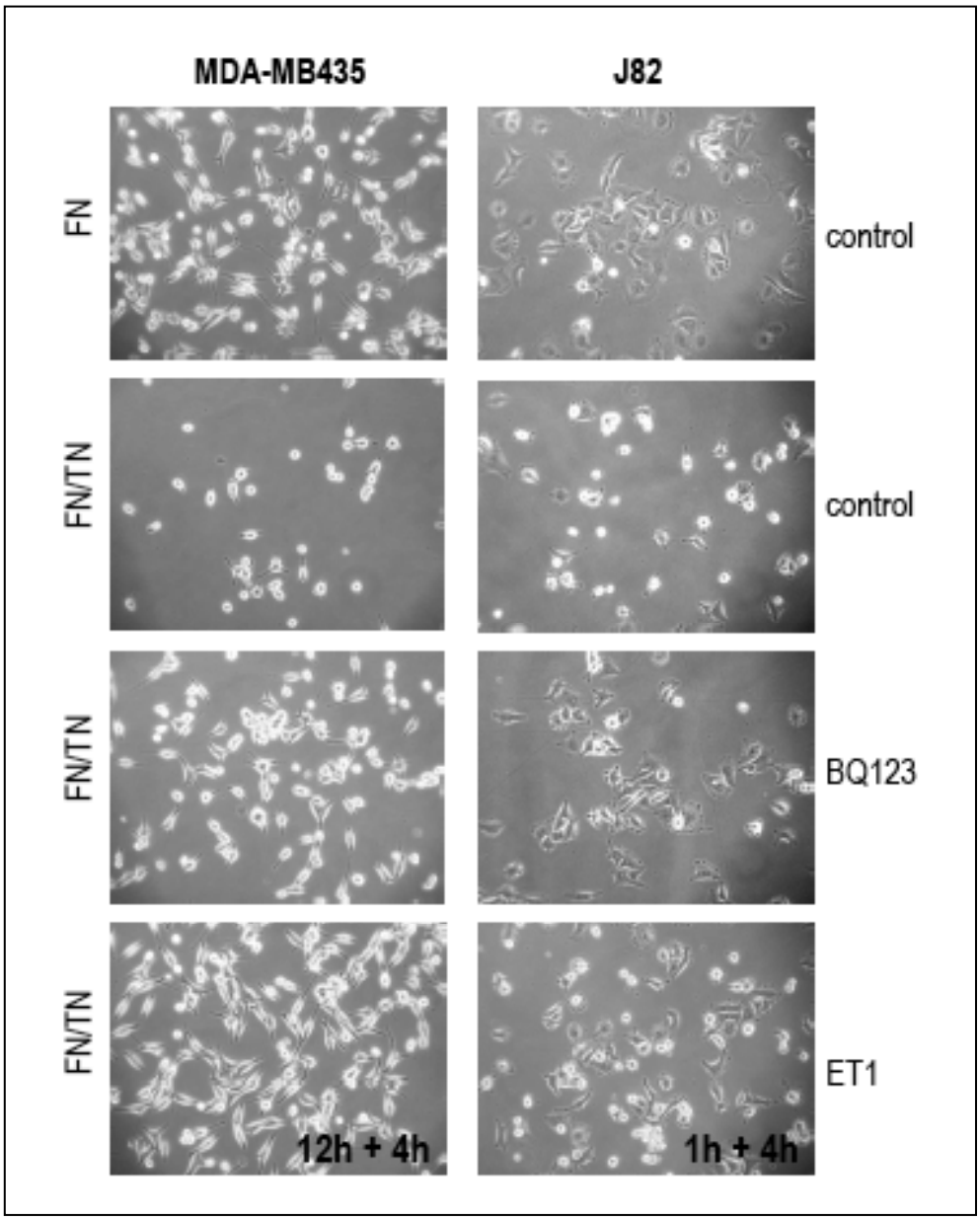


Supplemental Fig. 2 Inhibition of EDNRB blocks ET3-induced cell spreading on FN/TN-C
T98G cells were plated on fibronectin and FN/TN-C in serum-free medium for 1h before addition of ET3 in the absence and presence of the EDNRB inhibitor BQ788 or DMSO for another 4h. (**A**) Documentation of cell adhesion, (**B**) immunoblotting for the indicated molecules.

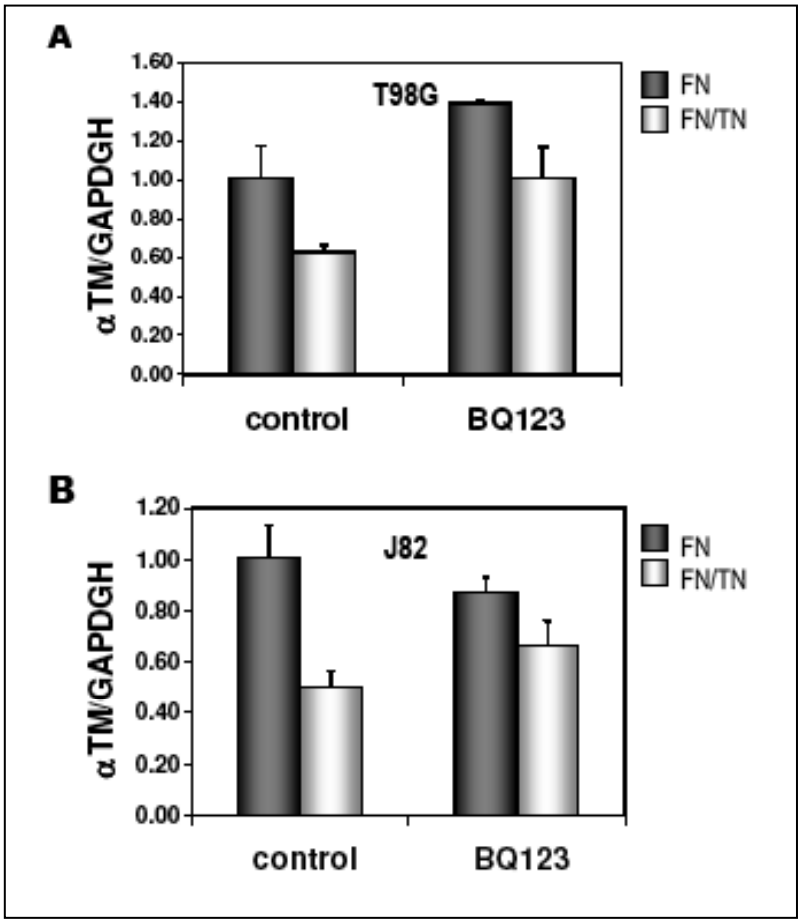


Supplemental Fig. 3 Rapid induction of cell spreading on FN/TN-C upon inhibition of EDNRA

T98G cells were plated for 12h on FN/TN-C in serum-free medium before addition of ET1 and the inhibitors BQ123 and BQ788 for 30 minutes. As control, cells were plated in serum-free medium plus DMSO. (**A**) Documentation of cell adhesion, (**B**) immunoblotting for the indicated molecules.



Supplemental Fig. 4 Induction of cell spreading on FN/TN-C in breast and urinary bladder carcinoma cells upon inhibition of EDNRA
Human breast carcinoma MDA-MB435 and J82 urinary bladder carcinoma cells were plated on FN/TN-C in serum-free medium before addition of ET1, DMSO and BQ123 before documentation of cell adhesion.

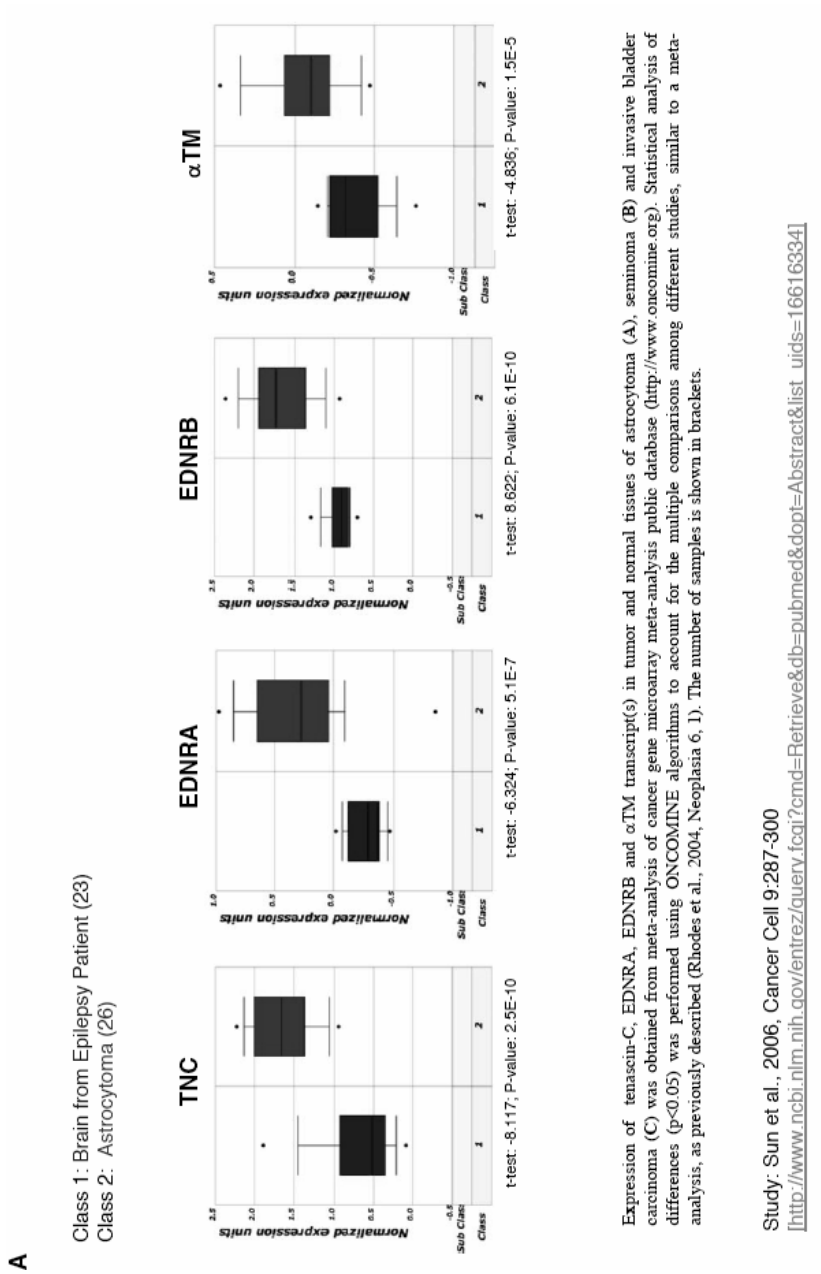


Supplemental Fig. 5 Effect of EDNRA inhibition on αTM repression
T98G (**A**) and J82 cells (**B**) were plated for 12h and 1h, respectively on fibronectin and FN/TN-C before addition of ET1, BQ123 or DMSO (control) for 4h followed by RNA extraction and real time RT-PCR.

Supplemental Table 1 Expression of tenascin-C, EDNRA, EDNRB and TPM1 in human cancers

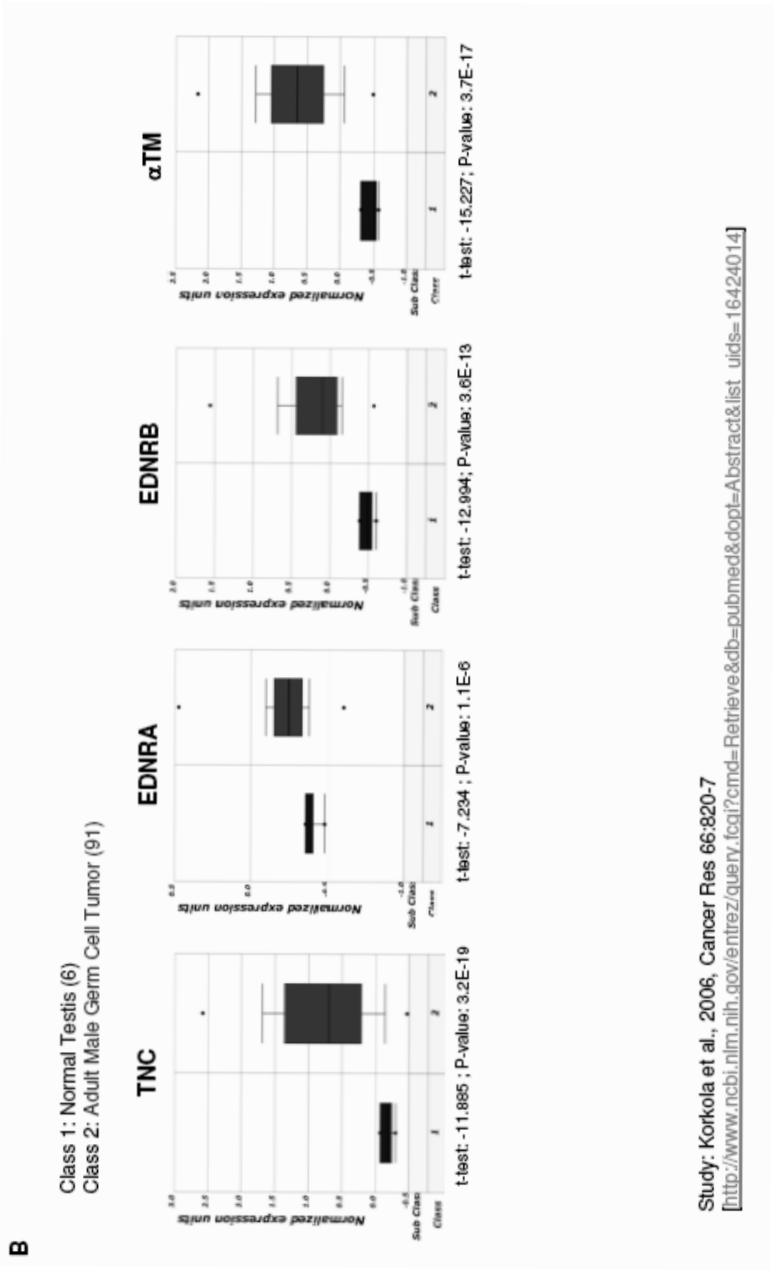
Organ	Material	TNC	EDNRA	EDNRB	αTM	Reference
Brain	Astrocytoma (26) vs brain epilepsy patient (23)	1 [a]	1	1	1	Sun et al., 2006, Cancer Cell 9, 287
Brain	GBM (77) vs brain epilepsy patient (23)	1	1	1	-1	Sun et al., 2006, Cancer Cell 9, 287
Brain	Glioma grade IV (76) vs grade III (24)	1	1	-1	1	Phillips et al., 2006, Cancer Cell 9, 157
Ovary	Ovarian Carcinoma metastasis (37) vs primary carcinoma (86)	1	1	1	1	Bittner 2005, Nat Biotechnol 23, 183
Bladder	Bladder superficial transitional cell carcinoma with carcinoma in situ (CIS) (13) vs no CIS (14)	1	1	1	1	Dyrskjot et al., 2004, Cancer Res 64, 4040
Breast	Grade 1 (6) vs normal (7)	1	1	1	1	Yip & Götte [b]
Breast	Grade 3 (7) vs normal (7)	0	1	1	1	Yip & Götte [b]
Testis	Seminoma (91) vs normal (6)	1	1	1	1	Korkola et al., 2006, Cancer Res 66, 820
Pancreas	Pancreatic ductal carcinoma grade V (6) vs grade III (10)	1	1	nd	nd	Ishikawa et al., 2005, Cancer Sci 96, 387
Colon	Axin2 methylation positive (10) vs negative (30)	-1	-1	-1	nd [c]	Koinuma et al., 2006, Oncogene 25, 139
Prostate	Prostate carcinoma with metastasis (20) vs no metastasis (59)	-1	-1	-1	-1	Dhanasekaran et al., 2001, Nature 412, 822
Prostate	Carcinoma (16) vs benign hyperplasia (9)	-1	-1	nd	nd	Luo et al., 2001, Cancer Res 61, 4683
Prostate	Adenocarcinoma with metastasis (5) vs normal (8)	-1	-1	nd	-1	Vanaja et al., 2003, Cancer Res 63, 3877

Relative expression (0, equal; 1, increase and –1, decrease) of tenascin-C, EDNRA, EDNRB and αTM transcript(s) in tumor and normal tissues was obtained from meta-analysis of cancer gene microarray meta-analysis public database (http://www.oncomine.org). Statistical analysis of differences ($p<0.05$) was performed using ONCOMINE algorithms to account for the multiple comparisons among different studies, similar to a meta-analysis, as previously described (Rhodes et al., 2004, Neoplasia 6, 1).The number of samples is shown in brackets. (b) For real time RTPCR, breast cancer specimen (n=13) of grade 1 and grade 3 breast tumors were obtained from patients operated between 2002 and 2003 at the Department of Gynecology, University of Münster, Germany (Wülfing et al., 2005, Int J Oncol 26, 951). Tumor samples were snap frozen immediately after surgery and stored at –80° C until RNA extraction. Non-neoplastic breast tissue samples (n = 7) were obtained from patients undergoing reductive mammoplasty. (c) nd, not statistical significant or no data available. vs, versus.



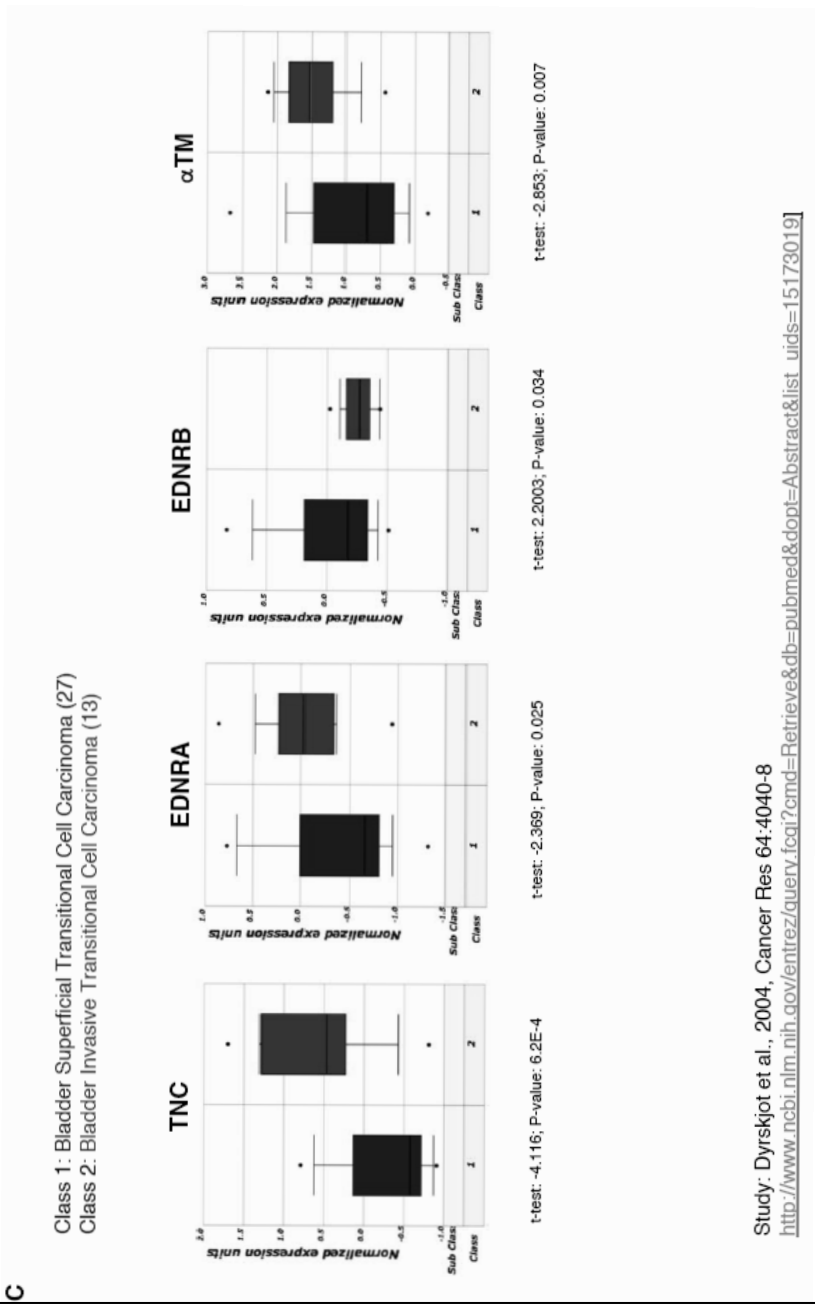
Supplemental Fig. 6 Expression of tenascin-C, EDNRA, EDNRB and αTM in human cancer tissue

Expression of tenascin-C, EDNRA, EDNRB and αTM transcript(s) in tumor and normal tissues of astrocytoma (**A**) was obtained from meta-analysis of cancer gene microarray meta-analysis public database (http://www.oncomine.org). Statistical analysis of differences ($p<0.05$) was performed using ONCOMINE algorithms to account for the multiple comparisons among different studies, similar to a meta-analysis, as previously described (33).The number of samples is shown in brackets.



Supplemental Fig. 6 Expression of tenascin-C, EDNRA, EDNRB and αTM in human cancer tissue

Expression of tenascin-C, EDNRA, EDNRB and αTM transcript(s) in tumor and normal tissues of seminoma (**B**) was obtained from meta-analysis of cancer gene microarray meta-analysis public database (http://www.oncomine.org). Statistical analysis of differences ($p<0.05$) was performed using ONCOMINE algorithms to account for the multiple comparisons among different studies, similar to a meta-analysis, as previously described (33).The number of samples is shown in brackets.



Supplemental Fig. 6 Expression of tenascin-C, EDNRA, EDNRB and αTM in human cancer tissue

Expression of tenascin-C, EDNRA, EDNRB and αTM transcript(s) in tumor and normal tissues of invasive bladder carcinoma (**C**) was obtained from meta-analysis of cancer gene microarray meta-analysis public database (http://www.oncomine.org). Statistical analysis of differences ($p<0.05$) was performed using ONCOMINE algorithms to account for the multiple comparisons among different studies, similar to a meta-analysis, as previously described (33).The number of samples is shown in brackets.

2.2 Part B : Promotion of a motile phenotype by the microenvironmental factors tenascin-C and LPA/PDGF

Manuscript in preparation.

Promotion of a motile phenotype by the microenvironmental factors tenascin-C and LPA/PDGF

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Abstract

Expression of tenascin-C, a prominent component of the tumor microenvironment and the neuronal stem cell niche, correlates with a poor survival prognosis for patients with gliomas and regulates oligodendrocyte progenitor maintenance, respectively. But the role of tenascin-C in these tissues and compartments is largely unknown at the mechanistic level. Here, we show that cooperative signaling by LPA/PDGF induces glioma cell spreading and migration on a mixed fibronectin/tenascin-C substratum. This effect depends on PI3K, ROCK and MEK, but is independent of syndecan-4, PLC and JNK. By using functional knock-down approaches, we identified paxillin and tropomyosin-1 as targets of tenascin-C downstream of syndecan-4, that are critical for LPA/PDGF-induced cell adhesion and migration under these conditions. Ectopic expression of paxillin and syndecan-4 binding syndesmos also bypassed syndecan-4 requirement for cell spreading. In summary, the microenvironmental factors tenascin-C, LPA and PDGF stimulate cell spreading and migration, and thereby may contribute to glioma progression and neuronal stem cell niche identity.

Introduction

The microenvironment is instrumental for stem cell niche identity and cancer progression. Growth factors including EGF and PDGF regulate oligodendrocyte progenitor (OP) maintenance in the neuronal stem cell niche (fFrench-Constant; Goldman and Sim, 2005; Goldman, 2003). The extracellular matrix molecule tenascin-C is another component of this compartment. Tenascin-C is required for proper cell responses of OPs toward PDGF (Gibney and McDermott, 2007), which suggests a synergistic effect of tenascin-C and PDGF. Tenascin-C is also expressed in the stroma of most solid tumors, where its high expression correlates with tumor progression for some cancers including gliomas (Chiquet-Ehrismann and Chiquet, 2003). Tenascin-C appears to play a role in tumor progression through stimulation of tumor cell proliferation, invasion, angiogenesis and suppression of the immune system (Orend and Chiquet-Ehrismann, 2003). But the underlying mechanisms are poorly understood. Tenascin-C competes with syndecan-4 binding to fibronectin, thus blocking cell adhesion to fibronectin. This translates into enhanced proliferation of glioma cells (Huang et al., 2001), presumably through activation of Wnt (Ruiz et al., 2004) and endothelin receptor type A (EDNRA) signaling (Lange et al., 2007). Tenascin-C promotes invasion through activation of HGF signaling (DeWever et al., 2004). We showed that activation of EDNRA signaling by tenascin-C inhibits FAK and blocks actin stress fiber formation through downregulation of RhoA and tropomyosin-1 (TM1) (Lange et al., 2007).

The proteoglycan syndecan-4 is a cofactor for integrin $\alpha 5\beta 1$ in cell spreading on fibronectin (Huang et al., 2001). Syndesmos, an adaptor protein that binds both syndecan-4 and paxillin (Denhez et al., 2003), may provide a molecular bridge between $\alpha 5\beta 1$ integrin and syndecan-4 in focal adhesions. Paxillin and FAK are other adaptor proteins that integrate signals from different membrane receptors including those from integrins and the PDGF receptor (Turner et al., 2005; Horwitz et al. 2005; Schlaepfer et al., 2004; Mitra et al., 2005).

The small GTPase RhoA and its downstream targets RhoKinase (ROCK) and myosin light chain (MLC) are important molecules in actin polymerisation, cell contractility and migration. In addition, actin filaments are stabilised by tropomyosins. Expression of tropomyosin-1 (TM1) is often downregulated in cancer (Martin and

Leder, 2001; Bhattacharya et al., 1990; Raval et al., 2003; Pawlak et al., 2004) and is repressed on a fibronectin/tenascin-C substratum (FN/TN) by a mechanism that involves reduced TM1 heterodimer stabilization due to reduced RNA levels of TM2 and TM3 by tenascin-C.

LPA and PDGF signaling is frequently deregulated in cancer and is highly elevated in gliomas. A high expression of both factors correlates with advanced cancers presumably by promoting survival, proliferation, migration and invasion (Stähle et al., 2003; Gschwind et al., 2002; Banerjee et al., 2002; Cao et al., 2004). An enhanced migration is a prerequisite for invasion and metastasis, thus presumably promoting tumor progression.

Here, we wanted to know how tenascin-C, LPA and PDGF affect adhesion and migration of glioma cells. We show that LPA/PDGF blocks cell rounding by tenascin-C and promotes cell migration. Details of the underlying mechanism will be presented.

Materials and Methods

Cell plating, inhibitor studies, preparation of cell lysates

Human T98G glioblastoma and MEFs were grown in DMEM supplemented with antibiotics and 10% FCS (Sigma, Bucks, Switzerland). Cells were transferred into DMEM, 10% FCS 24 h before the experiment and serum-starved for 18 h. Cells were trypsinized and replated after inhibition of trypsin with 100 ng/ml trypsin inhibitor (Sigma, Bucks, Switzerland) in serum free DMEM onto dishes (Falcon Becton Dickinson) coated with equimolar amounts of fibronectin and tenascin-C (1µg/cm^2). Recombinant his-tagged human tenascin-C, FNIII13 and serum-derived horse fibronectin was purified as described (Huang et al., 2001; Lange et al., 2007). Finally, 1% heat-inactivated BSA (Serva, Heidelberg, Germany) was used to block the uncoated surface prior to UV-sterilization for 15 minutes in a sterile bench. Since the inhibitors were dissolved in DMSO and DMSO interfered with cell spreading on fibronectin, cells were plated on the different substrata in serum free medium 1 h prior to incubation with 100 ng/ml EGF, 1µM LPA and 80 ng/ml PDGF-BB (Sigma, Switzerland), or inhibitor UO126 (25 µM, Calbiochem Germany, Darmstadt), Wortmannin (20 µM), SP600125 (20 mM) and U73122 (20 nM), respectively (Sigma, Bucks, Switzerland) for 4 h followed by lysis in sample buffer (250 mM Tris-HCl pH 7,0, 10% SDS, 50 % glycerol, bromphenolblue, 100mM DTT).

Immunoblotting

Equal amounts of protein were separated in 8% self-made or 4-12% precast Bis-Tris-glycine gels (Invitrogen, Novex), transferred onto PVDF nylon membrane (Millipore Immobilon-P, Bedford, USA) and stained with 0.2% Ponceau-S (Sigma, Bucks, Switzerland) in 7.5% TCA for confirmation of equal protein loading prior to blocking the membrane in 10% horse serum (or 1% BSA), TBS and 1% Tween-20. For immunoblotting, the following mouse monoclonal antibodies against TM1-3 (TM311, Sigma, Bucks, Switzerland, 1:1000), vinculin (hVIN-1, Sigma, Bucks, Switzerland, 1:1000), a-tubulin (Ab-1, Oncogene, Dietikon Switzerland, 1:5000), RhoA (26C4, Santa Cruz Biotechnology, Heidelberg, Germany, 1:1000), P-ERK1/2 (Cell Signaling, Allschwil, Switzerland, 1:1000), FAK and phospho-Y397-FAK (BD Biosciences, Allschwil, Switzerland, 1:1000), MLC (Santa Cruz Biotechnology, Heidelberg,

Germany, 1:1000); rabbit polyclonal antibodies, paxillin and phospho-Y118-paxillin (Abcam, Cambridge, UK, 1:1000), and ERK1/2 (Cell Signaling, Allschwil, Switzerland, 1:1000), phospho-S910-FAK (Biosource, Lucerne, Switzerland, 1:1000), phospho-S19-MLC (Santa Cruz Biotechnology, Heidelberg, Germany, 1:1000) and secondary HRP-coupled antibodies (Amersham, UK) were used. Binding of antibodies was detected with ECL-plus (Amersham, UK).

Immunofluorescence

Cells were serum-starved prior to plating on fibronectin and FN/TN for the indicated time points in serum free medium supplemented with the indicated growth factors. Cells were fixed in 4% PFA and stained with the indicated primary and secondary FITC-labeled antibodies or with TRITC-labeled phalloidin (Sigma, Bucks, Switzerland). Images were captured using a NIKON Diaphot300 (NIKON Video Microscope with Openlab program, Coventry, UK) at 400- and 1000-fold magnification (immunofluorescence) and with a Leica LEITZ DMIL microscope (Wetzlar, Germany) at a 100-fold magnification for phase-contrast pictures.

RhoA activation assay

T98G cells (2 x 10^3) were serum starved for 18 h prior to plating onto fibronectin or FN/TN in 6-well dishes (10 cm^2/well, Falcon) for 15 and 30 minutes or 4 h in serum free or LPA/PDGF-supplemented medium, washed with 3 ml ice-cold PBS prior to lysis in 70 µl Cell ice-cold lysis buffer per well (GLISA, Cytoskeleton, Lucerne, Switzerland). Lysates were adsorbed to rhotekin-bound plates (GLISA) with equalized protein amount (0.4-2.0 mg/ml) in the presence of binding buffer for 30 minutes at 4°C. After washing, the plate was incubated with antigen-presenting buffer prior to incubation with an anti-RhoA primary antibody for 45 minutes. After incubation with a HRP-labelled secondary antibody, bound RhoA antibody was determined by measuring OD 490 nm. Positive and negative controls were provided by the manufacturer.

Cell migration assay

Glass cover slips (10 mm^2, medite Medizintechnik AG, Nunningen, Switzerland) were precoated with poly-L-lysin (0,01 %, mol wt 70000-150000, Sigma) for 1 h prior to successive coating with fibronectin, tenascin-C and BSA. Serum starved cells (1 - 2 x 10^3 cells) were plated onto the cover slips for 4 h in serum free medium. Cells on the cover slip were transferred into FN/TN coated 6-well dishes (10 cm^2, Falcon) for 12 h, 24 h and 48 h in serum free medium, or in medium supplemented with EGF, LPA, PDGF and LPA/PDGF, respectively. Pictures were taken from three randomly chosen areas from the cover slips and cells were counted that had migrated into the 6-well dish.

Quantitative and semiquantitative RTPCR

Total RNA was isolated from three independent plates using the RNeasy Mini Kit (Qiagen, Basel, Switzerland) and RNAse-free DNAse set (Qiagen, Basel, Switzerland) following the manufacturer's instructions. From 0,5 –1 µg of total RNA single strand cDNA was generated by using the SuperScript III First-Strand Synthesis Super Mix (Invitrogen, Carlsbad, CA, USA) with random hexamers. Expression of the respective gene was detected by quantitative RTPCR on an ABI Prism 7000 TaqMan using SYBR green PCR MasterMix (Applied Biosystems, Warrington, Cheshire, UK) with the following primers (**Supplemental Table 1**). Primers were designed with the ABI Primer Express software (Applied Biosystems, Warrington, Cheshire, UK). Relative expression of the respective gene was determined after normalization to GAPDH and was calculated with the formula: relative expression level = 2^{-DDCT}.

Supplemental Table 1 Primers used for quantitative SYBR green RTPCR

Name	Forward	Reverse
murine syndecan-4	GTATCCATGTCCAGCACTG	ATGCGTAGAACTCATTGGTG
murine syndecan-1	CACTTCTGTCATCAAAGAGGTTGT	CTGTCCAAAAGGCTCTGAGAAG
murine syndecan-2	CCCAAAGTGGAAACCATGAC	GCAAAGAGAAAGCCGATCAC
murine paxillin	CTACAGCTTCCCCAACAAGC	CCCCCAAGGGAGTGTTATTT
αTM	GAGCGTCTGGCAACAGCTTT	TCTTTTTGGGCTCGACTCTCA
βTM	TCAGACCGCAAATATGAAGAGGT	TCCTTCCAGGATCACCAGCTT
γTM	GAGGTGGCTCGTAAGTTGGTG	CTCTGTGCGTTCCAAGTCTCC
RhoA	GCAGGTAGAGTTGGCTTTATGG	CTTGTGTGCTCATCATTCCGA
GAPDH	TCCTCTGACTTCAACAGCGACA	CGTTGAGGGCAATGCCA
chicken syndesmos	AACTTCCTGGCCAACTCCTT	GCGTATTCATTGCCTGCTCT

Generation of gene knock-down and overexpressors

T98G cells were stably transfected with shRNA constructs specific for aTM together with a pCIneo plasmid as described (Huang et al., 2001) (**Supplemental Table 2**) (SuperArray). Cells were subcloned and downregulation of aTM was determined by immunoblotting and real time RTPCR, respectively. Paxillin was transiently downregulated with siRNA oligo nucleotides (**Supplemental Table 2**). A plasmid with the chicken syndesmos sequence was cotransfected together with a pCIneo plasmid and G418 restistant cells were selected. Expression of the transgene was determined by RTPCR.

Supplemental Table 2 Oligos used for knocking down gene expression

sh-human αTM oligos	5`-CTGAGGATTCAGACCGCAAAT-3’
	5`-AAGCACATCGCTGAGGATTCA-3’
sh-control	Scrambled artificial sequence which does not match any human or mouse gene, not further disclosed by the manufacturer.
si-Luciferase (control)	Sense : CTTACGCTGAGTACTTCGAdTdT
	Antisense : TCGAAGTACTCAGCGTAAGdTdG
si-murine paxillin oligos	Sense : GAGCCCTCACCTACCGTCAdTdG
	Antisense : TGACGGTAGGTGAGGGCTCdTdG

Acknowledgement

We like to thank Frank Echtermeyer (University of Münster, Germany), Richard Hynes (Massachusetts Institute of Technology, Cambridge, MA, USA) and Paul Goetinck (Massachusetts General Hospital, Harvard Medical School, Charlestown, MA, USA) for MEF S4-/- cells, and constructs for FNIII13 and syndesmos, respectively. This work was supported by funding from the Swiss National Science Foundation, The Novartis Foundation for Biomedical Research and the Association for International Cancer Research granted to Gertraud Orend.

Results

Cooperative signaling by LPA and PDGF induces cell spreading on a fibronectin/tenascin-C substratum

We examined cell adhesion on FN/TN in the presence of different growth factors and observed that cells remained as round in the presence of LPA, PDGF-BB (PDGF) and EGF during 4 h after plating as in the absence of growth factors (**Fig. 1A**). This was in contrast to LPA/PDGF, where 78 % of cells spread on FN/TN in comparison to fibronectin (**Fig. 1B**). To understand how LPA/PDGF induced cell spreading on FN/TN, we used immunostaining and observed that cells formed vinculin containing focal adhesions and exhibited polymerised actin upon stimulation with LPA/PDGF, which was in contrast to serum free medium (**Fig. 1C**) and LPA and PDGF alone (data not shown), where cells were round and lacked these structures. By immunoblotting we determined expression of RhoA, FAK, paxillin and TM1. LPA/PDGF induced phosphorylation of FAK, paxillin and ERK1/2 and restored expression of TM1 and RhoA. This was in contrast to LPA or PDGF alone, where expression of these molecules remained significantly lower on FN/TN than on fibronectin (**Fig. 1D**).

Fig. 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**), counting spread cells in triplicates (**B)**.

Fig. 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 immunostaining for vinculin and polymerised actin (**C**), lysis and immunoblotting for the indicated molecules (**D**) and RNA extraction followed by real time RTPCR for αTM (**E**). Expression was normalized to GAPDH. Spread cells of three randomly chosen fields at a magnification of 200-fold were counted.

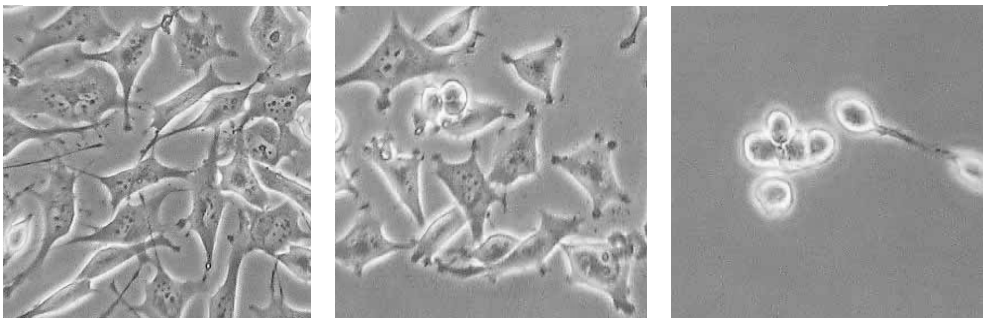
Since tenascin-C inhibits expression of TM1 by downregulating αTM expression, which subsequently reduces TM1 heterodimer protein stability (Lange et al., 2007), here, we examined how LPA/PDGF restored TM1 expression. By using real time RTPCR, we found that LPA/PDGF strongly increased low αTM expression, which exceeded levels on FN/TN in serum free medium (**Fig. 1E**). This was in contrast to βTM, γTM and RhoA RNA levels, that were not affected 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 levels of RhoA and TM1.

Syndecan-4 is upstream of RhoA, FAK and TM1, but is not required for LPA/PDGF-induced cell spreading on FN/TN

So far we showed that RhoA, FAK and TM1 are targets of tenascin-C, the inhibition of which is linked to cell rounding by tenascin-C and, that LPA/PDGF blocks cell rounding on FN/TN through restoring expression and function of these molecules. Since tenascin-C-induced cell rounding could be abolished by activation of syndecan-4 (Huang et al., 2001; Orend, 2005; Midwood et al., 2004), here we wanted to know whether activation of syndecan-4 had an effect on RhoA, FAK and TM1. First, we confirmed that activation of syndecan-4 with FNIII13 restored spreading of T98G cells on a FN/TN substratum (**Fig. 2A**). This correlated with restored expression of RhoA, P-FAK and TM1 (**Fig. 2B**). By real time RTPCR we observed that FNIII13 increased αTM levels (**Fig. 2C**), which demonstrates that syndecan-4 regulates RhoA and FAK upstream of TM1 at αTM expression level, thus enhancing TM1 heterodimer protein expression.

A **T98G**



FN FN/TN/III13 FN/TN

QuickTime™ and a
TIFF (LZW) decompressor
are needed to see this picture.

Fig. 2 Role of syndecan-4 in LPA/PDGF-induced cell spreading on FN/TN
T98G (**A – C**) were plated on the indicated substrata for 4 h in 1% FCS (**A, B**), serum free medium (**C**) before documentation of cell adhesion (**A**), lysis and immunoblotting (**B**) and RNA extraction and real time RTPCR for αTM (**C**).

By using wildtype MEFs and MEFs from syndecan-4 knock-out mice, we analysed whether the activation status of syndecan-4 is linked to cell spreading and expression of RhoA, P-FAK and TM1 expression in another cell system. Whereas FNIII13 restored cell spreading in wildtype MEFs to 55% of that of cells on fibronectin, FNIII13 failed to do so in MEF S4-/- (**Supplemental Fig. 1A, B**), which confirms that inhibition of syndecan-4 is the major mechanism of tenascin-C-induced cell rounding at an early time point of adhesion (Huang et al., 2001; , Midwood et al., 2004). Similar to T98G cells, RhoA and P-FAK levels increased in wildtype MEFs in the presence of FNIII13 on FN/TN in comparison to its absence (**Supplemental Fig. 1C**). In MEFs, expression of TM1-3 was downregulated on FN/TN, but was restored to normal levels with FNIII13. This was different to MEF S4-/-, where TM1-3 expression was independent on the substratum (**Supplemental Fig. 1C**). In MEF S4-/- cells RhoA, P-FAK and P-paxillin levels remained very low on all substrata including the FNIII13 supplemented FN/TN substratum. Collectively, these results suggest that syndecan-4 is required for activation of FAK and paxillin and for stable expression of RhoA and TM1, thus promoting cell spreading.

To investigate whether LPA/PDGF-induced cell spreading on FN/TN is syndecan-4 dependent, we analysed spreading of MEF S4-/- upon stimulation with LPA/PDGF. Whereas MEF S4-/- were round on FN/TN in the absence of growth factors, 58% of cells spread upon stimulation with LPA/PDGF in comparison to fibronectin (**Fig. 2D, E)**. These data revealed, that signaling by LPA/PDGF is suffcient to induce spreading on FN/TN in the absence of syndecan-4, which suggests that LPA/PDGF can bypass syndecan-4 requirement for cell spreading. As determined by immunoblotting, stimulation by LPA/PDGF strongly induced phosphorylation of paxillin and ERK in MEF S4-/- (**Fig. 2F**) as well as in wildtype MEFs on FN/TN (data not shown). In summary, we showed that syndecan-4 regulates RhoA and FAK upstream of αTM expression. Moreover, signaling by LPA/PDGF appears to bypass syndecan-4 requirement for cell spreading on FN/TN. This correlates with activation of paxillin, which points at paxillin as an important downstream target of LPA/PDGF-induced cell spreading on FN/TN.

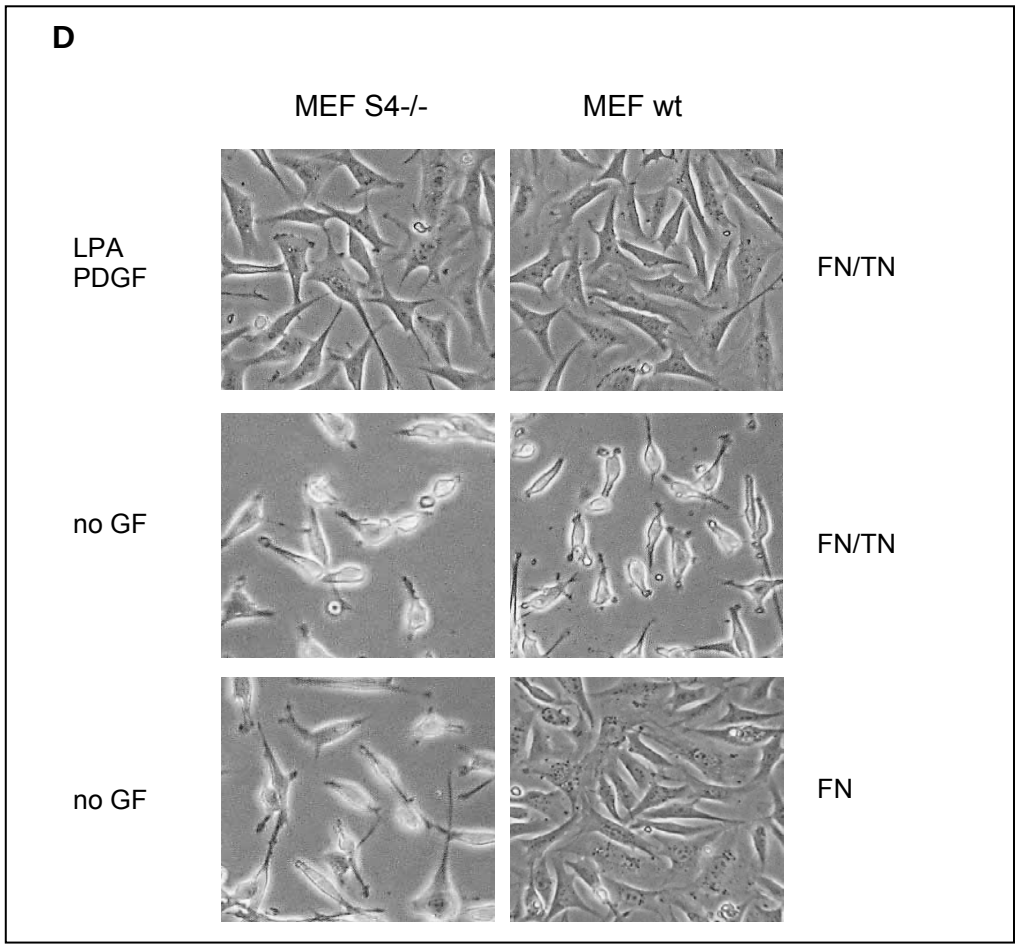


Fig. 2 Role of syndecan-4 in LPA/PDGF-induced cell spreading on FN/TN
MEF wt and MEF S4-/- cells (**D**) were plated on the indicated substrata for 4 h in serum free medium or upon stimulation with LPA/PDGF before documentation of cell adhesion.

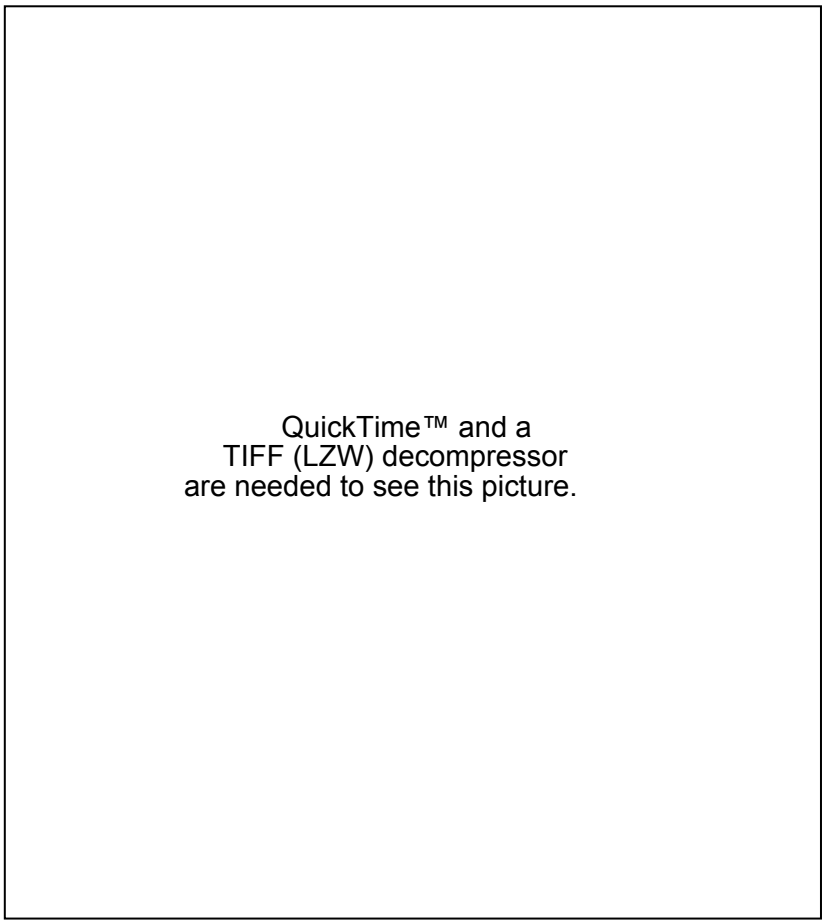


Fig. 2 Role of syndecan-4 in LPA/PDGF-induced cell spreading on FN/TN
MEF wt and MEF S4-/- cells (**D – F**) were plated on the indicated substrata for 5 h in serum free medium in presence or absence of LPA/PDGF (**F**) before documentation of lysis and immunoblotting (**F**)

Activation of paxillin through syndesmos bypasses syndecan-4 requirement for cell spreading

To address the question whether syndecan-4 independent LPA/PDGF-induced cell spreading requires paxillin, we determined cell spreading in MEF S4-/- upon knocking down paxillin with siRNA oligonucleotides. By real time RTPCR (data not shown) and immunoblotting we confirmed efficient depletion of paxillin from the cells (**Fig. 3A**). Cells hardly attached and spread on fibronectin (data not shown) nor on FN/TN in the presence of LPA/PDGF (**Fig. 3B**), which demonstrates that paxillin is critically involved in LPA/PDGF-linked bypassing of syndecan-4 requirement for cell spreading.

Fig. 3 Effect of paxillin and syndesmos on cell adhesion signaling upon plating on FN/TN
Knock-down of paxillin by siRNA in MEF S4-/- (**A**) was determined by documentation of cell shape (**B**).

Next, we examined whether high expression of syndesmos, a paxillin binding linker between integrin $\alpha5\beta1$ and syndecan-4 may overcome cell rounding by tenascin-C. Therefore, T98G cells ectopically expressing chicken syndesmos were generated and expression of the construct was confirmed by semiquantitative RTPCR (**Fig. 3C**). T98G:syndesmos cells spread on FN/TN to a similar extent as on fibronectin, which was different to tenascin-C, where cells remained round (**Fig. 3D**). Moreover,

T98G:syndesmos cells formed vinculin containing focal adhesions and actin filaments on FN/TN in serum free medium, which were absent from the parental cells

(**Fig. 3E**). To address the mechanism of spreading on FN/TN upon ectopic expression of syndesmos, we determined expression of the tenascin-C targets TM1, RhoA, FAK and paxillin by immunoblotting. In contrast to tenascin-C, where phosphorylation or expression of these molecules was very low, levels of TM1, RhoA, P-FAK and P-paxillin were as high on FN/TN as on fibronectin upon ectopic expression of syndesmos (**Fig. 3F**). Together, these data suggest that syndesmos plays an important role in cell spreading on FN/TN, presumably by recruiting paxillin, FAK and other signaling molecules to the plasma membrane independent on syndecan-4, which initiates focal adhesion and actin stress fiber formation. Moreover, our data suggest that syndesmos is involved in stabilisation of RhoA and TM1.

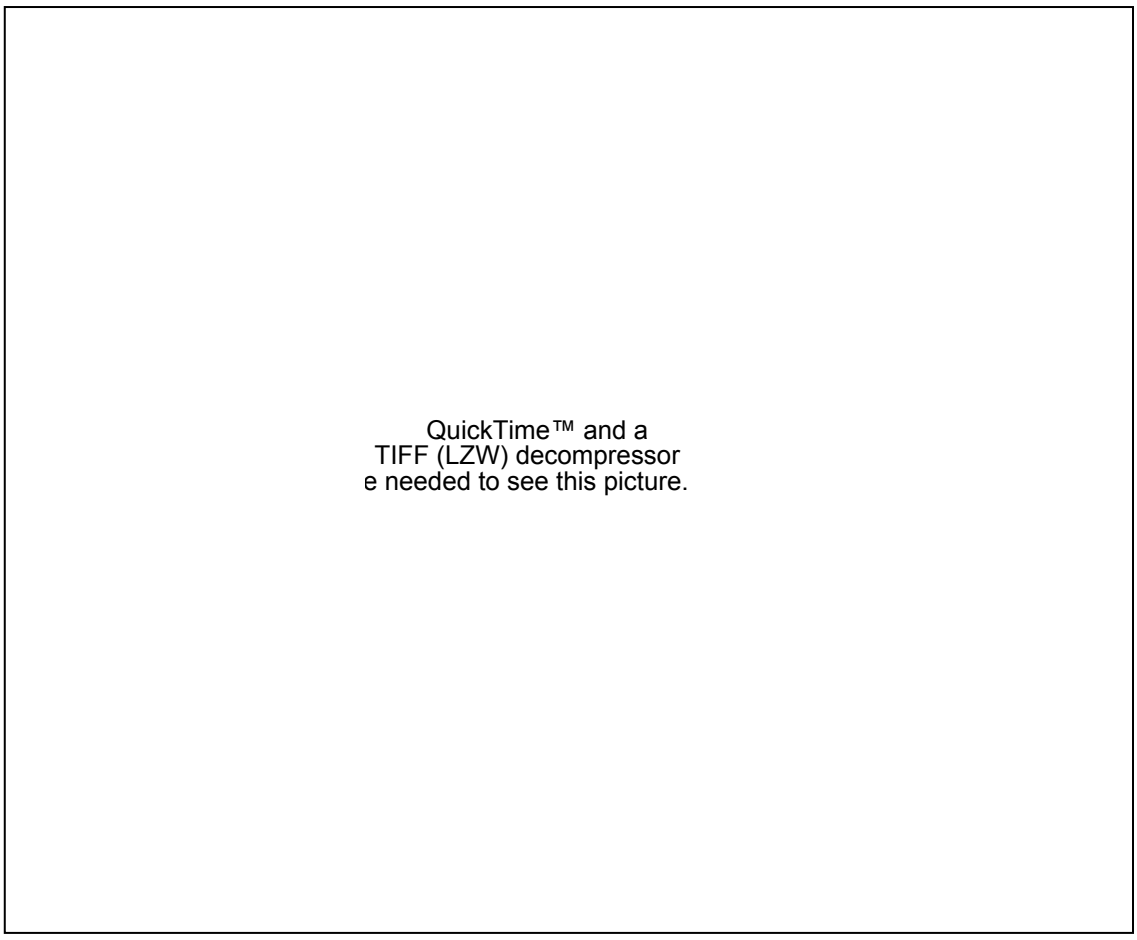


Fig. 3 Effect of paxillin and syndesmos on cell adhesion signaling upon plating on FN/TN
Ectopic expression of syndesmos in T98G cells (**C**) was determined by immunoblotting and RTPCR. Documentation of the presence of actin stress fibers and focal adhesions by immunofluorescence (**E**), and expression of tenascin-C target molecules by immunoblotting (**F**).

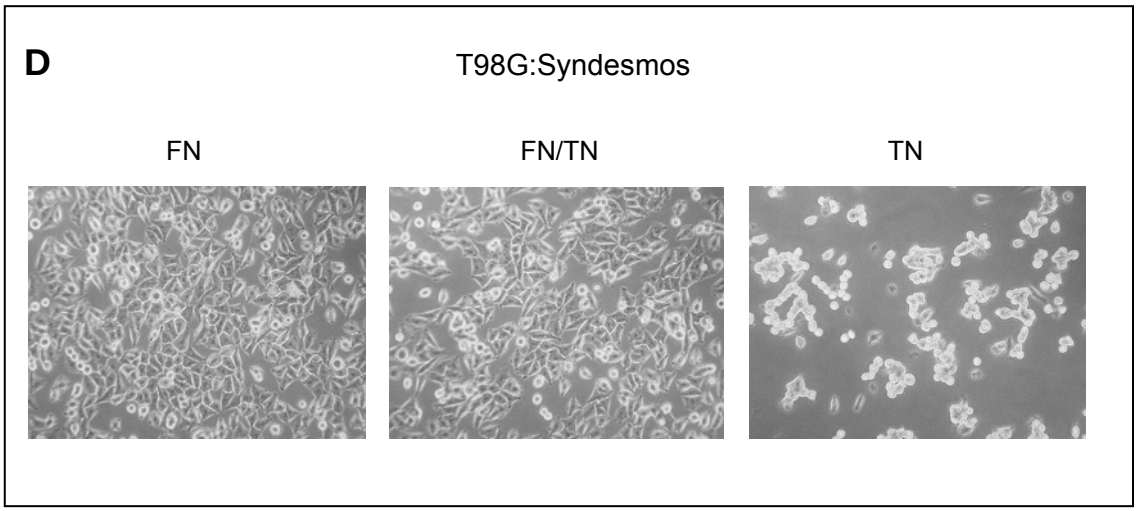


Fig. 3 Effect of paxillin and syndesmos on cell adhesion signaling upon plating on FN/TN
Ectopic expression of syndesmos in T98G cells was determined by documentation of cell shape (**D**).

Expression of tropomyosin-1 is critical for LPA/PDGF-induced cell spreading on FN/TN

Tenascin-C downregulates TM1 and, ectopic expression of TM1 restored cell spreading on FN/TN (Ruiz et al., 2004), suggesting that low TM1 levels are critical for tenascin-C-induced cell rounding. Here, we confirmed that elevated TM1 expression in T98G:TM1D10 cells promotes cell spreading on FN/TN, which is documented by formation of actin stress fibers and microtubules (**Supplemental Fig. 2**). A similar observation was made for REF52 cells, that were engineered to ectopically express TM1. In contrast to parental cells that remained round, REF:TM1 cells spread with actin stress fibers and focal adhesions on FN/TN (data not shown), confirming that repression of TM1 is a general mechanism of tenascin-C induced cell rounding. We wanted to know whether ectopically expressed TM1 had an effect on RhoA expression and phosphorylation of FAK. As shown in **Fig. 4A**, levels of RhoA and of phosphorylated FAK were restored in T98G:TM1D10 cells on FN/TN to levels as on fibronectin. These data suggest that TM1 is also upstream of FAK and RhoA, and supports our notion, that high and low P-FAK, RhoA and TM1 levels together, are critical for cell spreading and cell rounding, respectively (Lange et al., 2007).

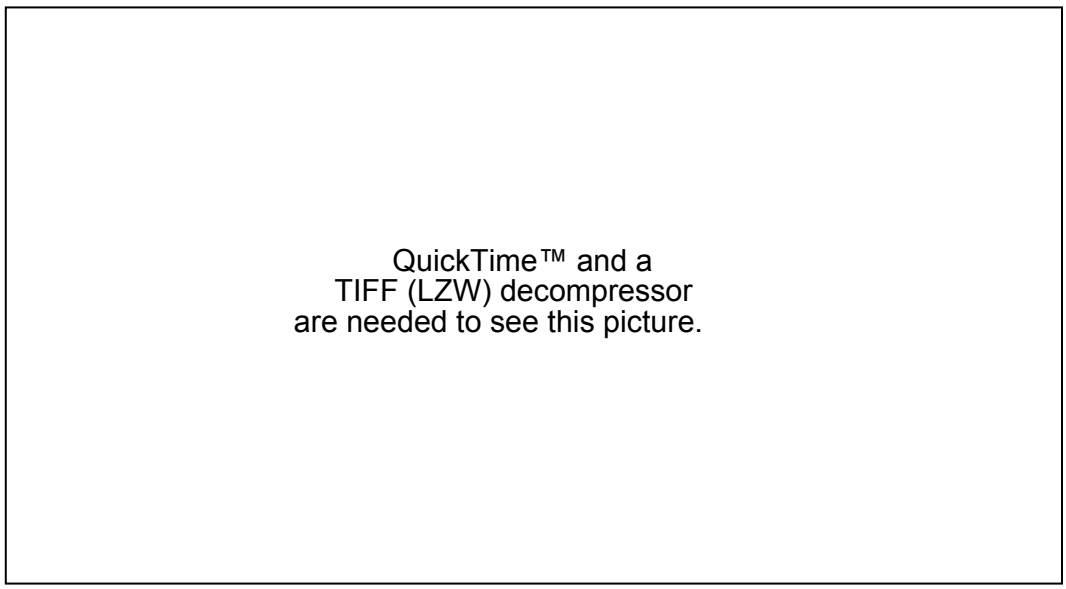


Fig. 4 Effect of TM1 levels on LPA/PDGF-induced cell spreading on FN/TN
T98G cells with ectopic expression of βTM (T98G:TM1D10) (**A**) and knock-down of αTM (**B**) were plated for 4 h in medium supplemented with 1% FCS (**A**), 10% FCS on plastic (**B**) or on fibronectin in the absence or presence of tenascin-C (**A**), before lysis and immunoblotting (**B**).

To examine whether high levels of TM1 are required for LPA/PDGF-induced cell spreading, we generated cells stably suppressing αTM by shRNA and plated cell clones with 2 different shRNAs on FN/TN together with LPA/PDGF. Cells with aTM knocked down exhibited lowered TM1-3 expression and, thus mimicked TM1 heterodimer downregulation by tenascin-C (Lange et al., 2007) (**Fig. 4B**). These cells hardly attached and did not spread on fibronectin in serum free medium (**Fig. 4C**), which suggests that TM1-3 are required for cell spreading on fibronectin. In addition, the analysed αTM knock down cells did not spread on FN/TN together with LPA/PDGF, which was in contrast to 80% of sh control cells that were triggered to spread on FN/TN upon stimulation with LPA/PDGF in comparison to cells on fibronectin in serum free medium (**Fig. 4C, D**). This result suggests that high TM1 levels are critical for LPA/PDGF-stimulated spreading on FN/TN.

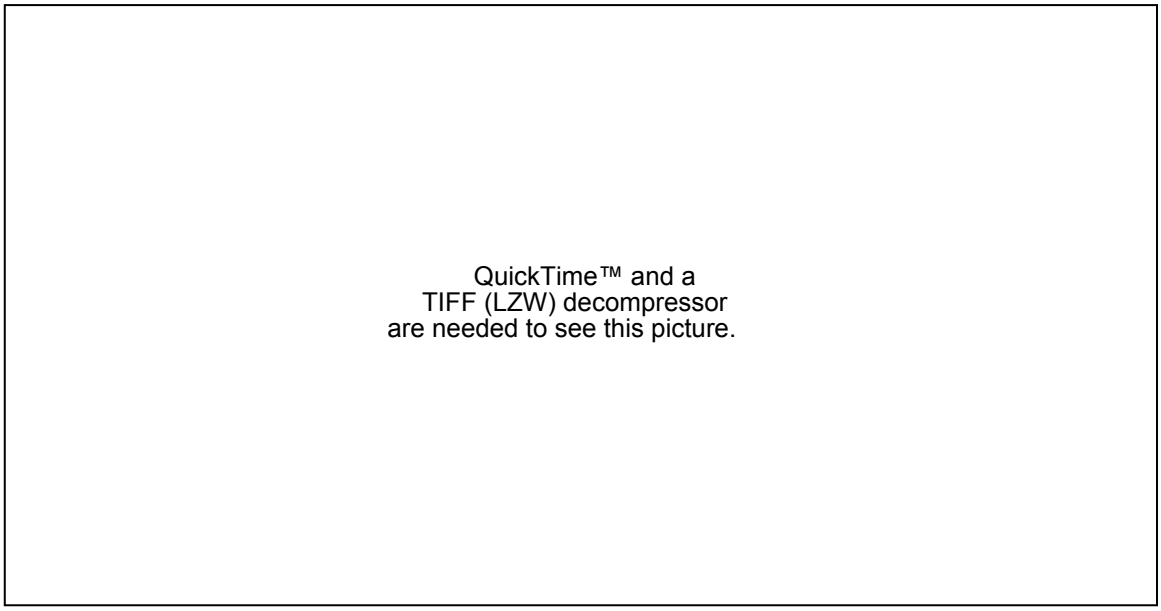


Fig. 4 Effect of TM1 levels on LPA/PDGF-induced cell spreading on FN/TN
T98G cells with knock-down of αTM (**B - E**) were plated for 4 h in medium supplemented with LPA/PDGF (**C**) on fibronectin in the absence or presence of tenascin-C (**C**), before immunofluorescence for polymerized actin (**D**).

Next, we analysed expression of RhoA, P-FAK and P-paxillin in the αTM knock-down cells. LPA/PDGF elevated TM1 expression in the shaTM3D2 cells on both substrata (**Fig. 4E**) in comparison to serum free medium. This suggests that signaling by LPA/PDGF counteracts the knock-down of αTM. LPA/PDGF-associated increased TM1 levels correlated with enhanced levels of RhoA and P-FAK even in the αTM knock down cells. In contrast to the sh-control cells, in which LPA/PDGF triggered high phosphorylation of paxillin, P-paxillin was undetectable in all shαTM cells (**Fig. 4E** and data not shown). This observation points again to paxillin as an important downstream effector of LPA/PDGF-induced spreading on FN/TN. Together, our data suggest that TM1 expression is tightly linked to cell shape on FN/TN. Spread cells exhibited high TM1 levels, which was in contrast to round cells that showed low TM1 expression on FN/TN. Whereas ectopic expression of TM1 restored expression and function of RhoA, FAK and paxillin, forced down-regulation of TM1 blocked cell spreading by LPA/PDGF through targeting RhoA, FAK and paxillin. This observation

also suggests that TM1 levels, RhoA expression and FAK activation are tightly linked and interdependent.

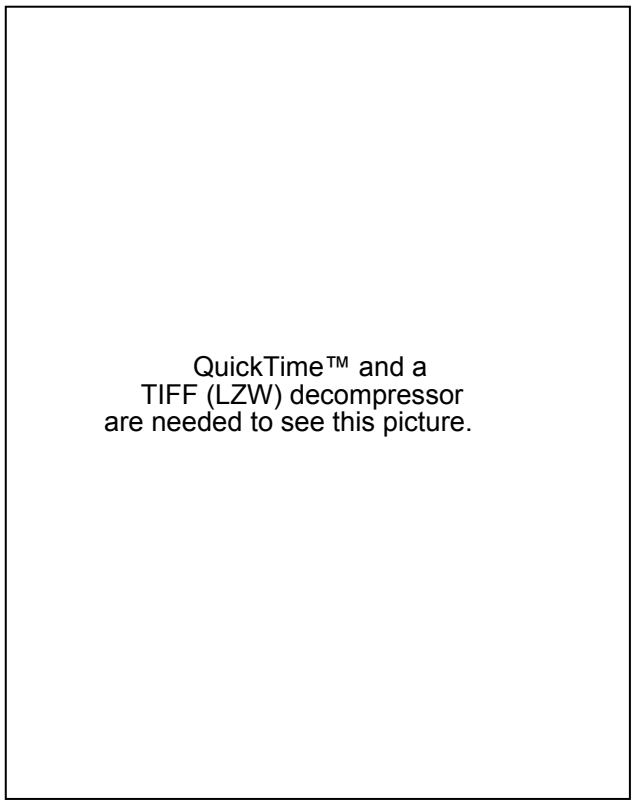


Fig. 4 Effect of TM1 levels on LPA/PDGF-induced cell spreading on FN/TN
T98G cells with knock-down of αTM were plated for 4 h in medium supplemented with LPA/PDGF (**E**) on plastic on fibronectin in the absence or presence of tenascin-C, before lysis and immunoblotting.

LPA/PDGF-specific expression of RhoA, P-FAK and TM1 is PI3K and MEK-dependent

By using chemical inhibitors we examined which pathway downstream of the receptors for LPA and PDGF is responsible for cell spreading on FN/TN. Cells were treated with inhibitors for PI3K (wortmannin), MEK (UO126), PLCγ (U73122), JNK (SP600125) and ROCK (Y27632) and, cell spreading and expression of RhoA, FAK and TM1 was analysed by immunoblotting. Inhibition of PI3K (**Fig. 5A**) and MEK (**Fig. 5B**) is documented by a lack of phosphorylation of downstream Akt and ERK1/2, respectively. We found, that whereas wortmannin did not affect spreading on fibronectin at this concentration (data not shown), it counteracted cell spreading induced by LPA/PDGF on FN/TN (**Fig. 5C**). This was in contrast to the other inhibitors that did not interfere with LPA/PDGF-induced cell spreading (**Supplemental Fig. 3A**). Upon inhibition of PI3K by wortmannin, levels of phosphorylated FAK and paxillin, and of RhoA and TM1 were very low or absent in cells plated onto FN/TN together with LPA/PDGF (**Fig. 5D**). Upon inhibition of MEK

by UO126, cells were elongated in the presence of LPA/PDGF (**Fig. 5C**). But, UO126 did not only lower expression of TM1, it also reduced phosphorylation of FAK and

paxillin and expression of RhoA on both substrata (**Fig. 5E**). Expression of these molecules apparently remained high enough to allow cell spreading on FN/TN (**Fig. 5C**). Inhibition of PLCg and JNK did not compromise high levels of RhoA, TM1 and phosphorylated FAK by LPA/PDGF (**Supplemental Fig. 3B, C**).

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Fig. 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 for another 4 h in serum free medium or together with LPA/PDGF. (**A-B**) immunoblotting for the indicated molecules.

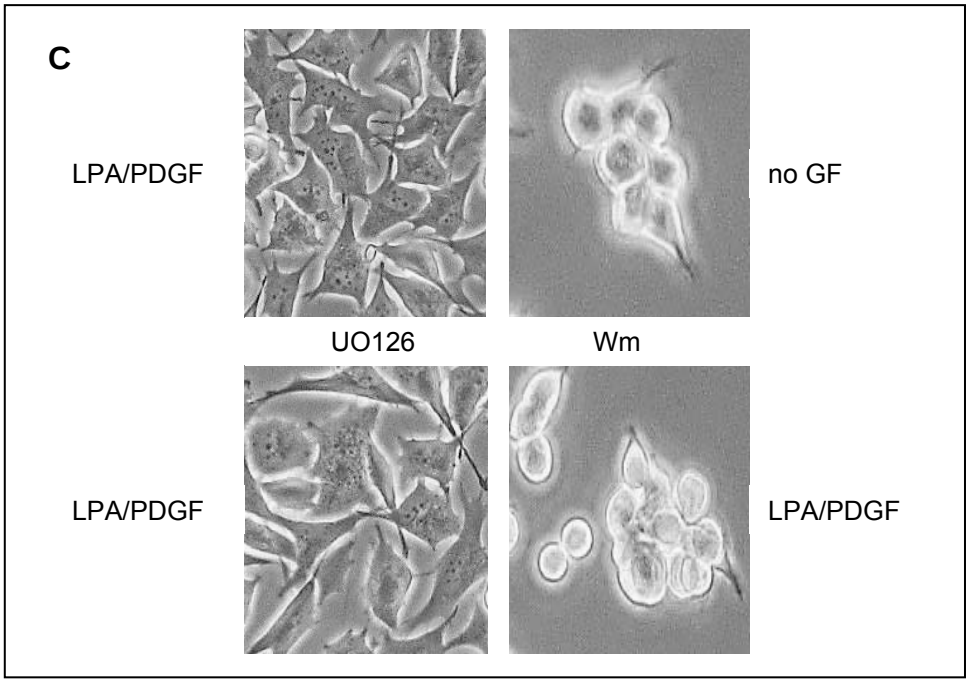

Fig. 5 LPA/PDGF-induced adhesion signaling is PI3K and MEK-dependent
Cells were plated onto FN/TN for 1 h before addition of wortmannin (Wm) or UO126 for another 4 h in serum free medium or together with LPA/PDGF. (**C**) documentation of cell shape.

Since LPA/PDGF increased RhoA levels on FN/TN, we wanted to know whether LPA/PDGF had an influence on RhoA activation. Therefore, we measured GTP-loading of RhoA in T98G cells on fibronectin or FN/TN in serum free medium. By ELISA for rhotekin-bound RhoA we observed that RhoA activation was 73, 70 and 90% of that on fibronectin at 15 and 30 minutes and 4 h after plating, in serum free medium, respectively. Stimulation by LPA/PDGF insignificantly elevated rhotekin-bound RhoA over that without growth factors (**Supplemental Fig. 4A**). These data suggest that activation of RhoA is independent on matrix adhesion in these cells.

Next, we addressed the possibility that ROCK, a kinase involved in actin polymerisation downstream of RhoA is affected by tenascin-C and/or LPA/PDGF signaling. Upon plating together with the ROCK inhibitor Y27632, cells still spread on FN/TN. They exhibited protrusions resembling filopodia (**Supplemental Fig. 4B**). This change in cell shape supports inhibition of ROCK by Y27632. High expression of RhoA, phosphorylated FAK and TM1 remained unchanged on FN/TN together with LPA/PDGF upon ROCK inhibition. Whereas MLC, a downstream target of ROCK, was not phosphorylated on FN/TN in serum free medium, MLC got phosphorylated with LPA/PDGF on FN/TN. This was even enhanced by Y27632 leading to as high P-MLC levels on FN/TN as on fibronectin in serum free medium. We conclude, that signaling by LPA/PDGF restored MLC phosphorylation by a ROCK-independent mechanism.

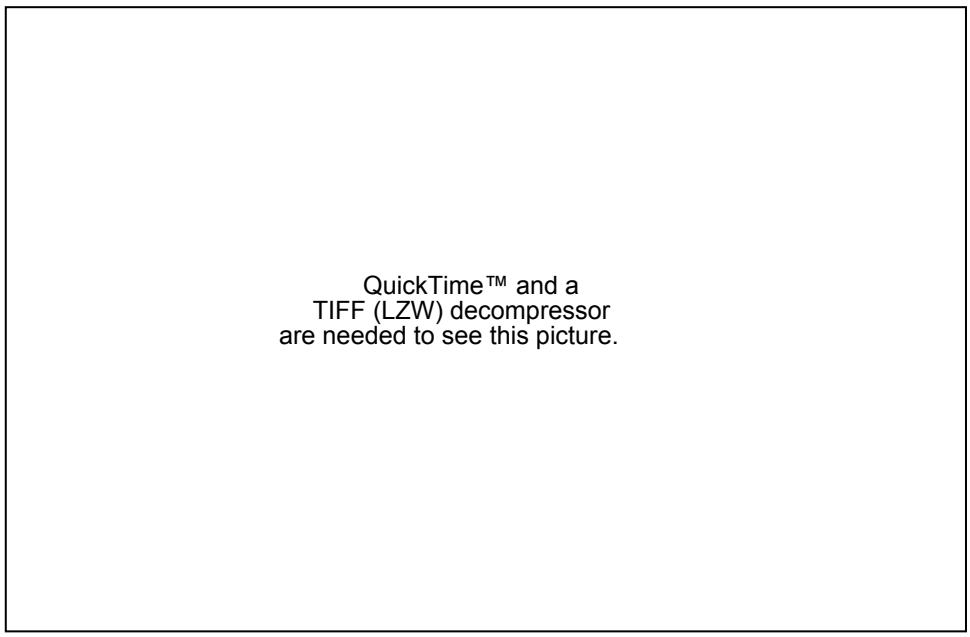

Fig. 5 LPA/PDGF-induced adhesion signaling is PI3K and MEK-dependent
Cells were plated onto the two substrata for 1 h before addition of wortmannin (Wm) or UO126 for another 4 h in serum free medium or together with LPA/PDGF. (**D, E**) immunoblotting for the indicated molecules,.

Tenascin-C promotes glioma cell migration upon LPA/PDGF stimulation in a ROCK and MEK dependent manner

So far we showed that LPA/PDGF induces cell spreading on FN/TN. Given, that an anti-adhesive tenascin-C substratum can promote cell migration (Zagzag et al., 2002; 1992; Tamaoki et al. 2005; Nishio et al., 2005), we asked whether LPA/PDGF affects cell migration on FN/TN. Migration of T98G glioblastoma cells was monitored on FN/TN upon stimulation with EGF, LPA and PDGF. In contrast to immobile cells in the absence of growth factors (**Fig. 6A**), LPA and PDGF alone (data not shown), LPA/PDGF stimulated cell migration after 24 h, which was even more enhanced after 48 h (**Fig. 6A, D**). 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 but, with a rounded cell shape, which was different to an elongated shape upon stimulation with LPA/PDGF. Interestingly, EGF completely blocked migration that occurred at 48 h in serum free medium (**Fig. 6B**). These data show that LPA/PDGF triggered cell migration on the FN/TN substratum. Moreover, we revealed that cell

migration on a tenascin-C substratum can be enhanced or blocked depending on the co-stimulus from growth factors.

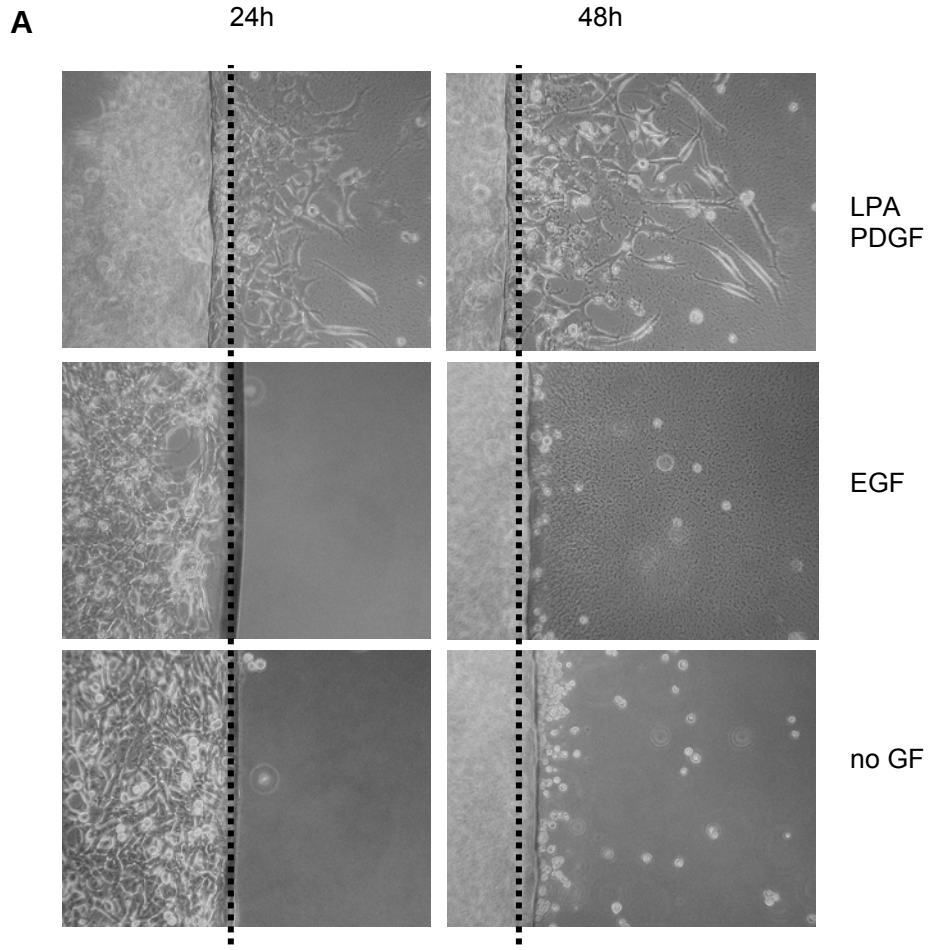

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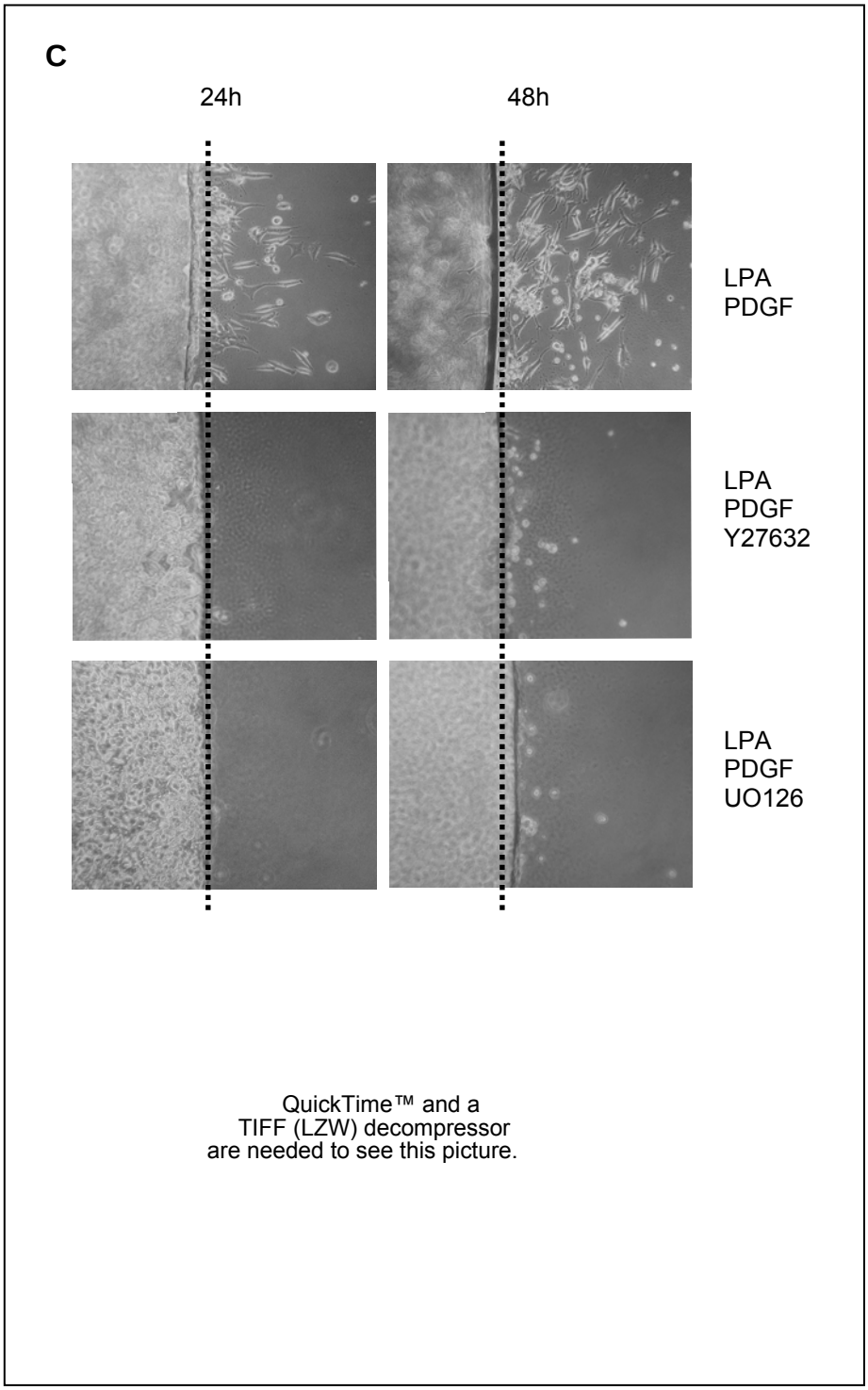
Fig. 6 Induction of cell migration by tenascin-C upon stimulation with LPA/PDGF
Cell migration from a glass cover slip coated with PLL, fibronectin and tenascin-C onto a FN/TN-coated plastic surface is documented for T98G in the presence of the indicated supplements. Cell morphology (**A**) and numbers of migrated cells (**B**) is shown. Cells having passed the rim (margin highlighted) from three randomly chosen areas were counted in triplicates.

Next, we asked whether LPA/PDGF-induced migration was affected by an altered expression and function of syndesmos and TM1. Therefore, migration of the indicated cells on a FN/TN substratum was determined in the absence or presence of LPA, PDGF (data not shown) and LPA/PDGF (**Supplemental Fig. 5**). Whereas all cells did not migrate in the absence of growth factors, control T98G:pCIneo and T98G:sh control cells migrated very well on FN/TN upon stimulation with LPA/PDGF in a time-dependent manner (**Supplemental Fig. 5**). In contrast to the control cells, T98G:syndesmos, T98G:TM1D10 and T98G:shαTM3D2 cells, did at most only marginally move in the presence of LPA/PDGF during 48 h. These data emphasize that tenascin-C and LPA/PDGF collaborate in cell migration. Moreover, strong adhesion through ectopic expression of syndesmos or βTM, as well as reduced adhesion upon suppression of TM1-3 was not compatible with LPA/PDGF-stimulated cell migration on FN/TN.

To address which signaling downstream of LPA/PDGF triggers cell migration on FN/TN, we analysed cell migration upon inhibition of ROCK and MEK with Y27632 and UO126, respectively. Although inhibition of ROCK did not affect cell spreading after 4 h (**Supplemental Fig. 4A**), it largely compromised LPA/PDGF stimulated cell migration on FN/TN at all time points (**Fig. 6C, D**). Similarly, cells did not migrate on FN/TN in the presence of LPA/PDGF upon inhibition of MEK with UO126 (**Fig. 6C, D**). In conclusion, LPA/PDGF promoted cell migration on FN/TN in a ROCK and MEK dependent manner.



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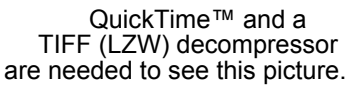

Fig. 7 Cooperative signaling by LPA/PDGF bypasses syndecan-4 requirement for cell spreading

(**A**) Upon adhesion to fibronectin, simultaneous activation of integrin $\alpha5\beta1$ and syndecan-4 leads to the establishment of focal adhesions. FAK is one of the first effectors to become phosphorylated and RhoA is trageted to the membrane where it is involved in actin polymerisation. Our data confirm that RhoA is an important downstream effector of syndecan-4 (Saoncella et al., 1999). Syndesmos might bridge syndecan-4 to integrins through its binding to syndecan-4 and to paxillin (Bass and Humphries, 2001). Tenascin-C competes with binding of syndecan-4 to FNIII13 within the 2nd heparin binding site of fibronectin, thus causing cell rounding on FN/TN, which is due to a lack of signaling from syndecan-4 (Huang et al. 2001). This prevents activation of FAK and focal adhesion formation. In addition, on a FN/TN substratum actin polymerisation and actin filament stabilisation is impaired by enhanced proteolytic degradation of RhoA and TM1-3 (Lange et al., 2007).

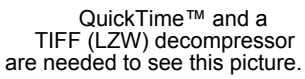

Fig. 7 Cooperative signaling by LPA/PDGF bypasses syndecan-4 requirement for cell spreading
(**B**) Cooperative signaling by LPA and PDGF bypasses syndecan-4 requirement for cell spreading on FN/TN by activating FAK and paxillin, restoring phosphorylation of MLC and expression of RhoA and TM1. This involves PI3K and MEK, since spreading was blocked by specific inhibitors. Thus, actin polymerisation, filament stabilisation and contractility is induced by LPA/PDGF signaling and thus triggers cell spreading on FN/TN. Upon stimulation of the receptors for LPA and PDGF, spreading on FN/TN triggers enhanced migration on this substratum. This effect is independent of syndecan-4, since it occurs in the absence of syndecan-4 expression. Our data confirm that syndesmos plays a crucial role as linker between syndecan-4 and integrin $\alpha 5\beta 1$, since ectopic expression of syndesmos restored cell spreading on FN/TN. Knock down of paxillin and αTM prevented LPA/PDGF-induced cell spreading on FN/TN. Ectopic expression of TM1 restored cell spreading on FN/TN presumably through a feedback mechanism that leads to FAK activation and RhoA stabilisation on FN/TN. Our data showed that signaling from EGF sustained cell rounding by tenascin-C, whereas LPA/PDGF signaling induced cell spreading and triggered cell migration. Adhesion or release from a tenascin-C enriched microenvironment has implications in tumor progression as well as in stem cell niche identity. Inhibition or downregulation and activation or overexpression is highlighted in red and blue, respectively.

Discussion

Cell adhesion to an ECM substratum can be modulated by growth factors. This applies in particular to cell rounding by tenascin-C and provides a means to adjust the adhesive properties of the microenvironment within a tissue according to the physiological status. A substratum of tenascin-C alone or in combination with fibronectin is anti-adhesive for most cells. Tenascin-C competes with syndecan-4 binding to fibronectin, thus preventing activation of syndecan-4 and causing cell rounding. Here, we proved that inhibition of syndecan-4 is the major mechanism of tenascin-C-induced cell rounding at an early time point of adhesion, since MEFs that lacked syndecan-4 did not spread on FN/TN upon activation with the syndecan-4 ligand FNIII13 4 h after plating. This was opposite to cells expressing syndecan-4, which did spread on FN/TN in the presence of FNIII13 (**Supplemental Table 3**).

In addition to activation of syndecan-4, here we showed that LPA/PDGF induces spreading on FN/TN, which was in contrast to LPA or PDGF alone, where cells remained round. We wanted to know whether LPA/PDGF-induced spreading was syndecan-4 dependent and found that cells with no syndecan-4 expression still spread on FN/TN upon stimulation with LPA/PDGF. We examined the possibility that elevated levels of syndecan-1 and –2 potentially compensated for the lack of syndecan-4, but expression of these syndecans as determined by real time RTPCR was not increased in MEF S4-/- cells over that in wildtype MEFs (data not shown). Our data support the possibility that signaling from LPA/PDGF bypasses the requirement of syndecan-4 for cell spreading on FN/TN. We analysed the underlying mechanism and demonstrate that RhoA, FAK, paxillin and TM1 are critical targets of tenascin-C. We showed that expression of RhoA and TM1 as well as phosphorylation of FAK and paxillin were very low on FN/TN in the absence of growth factors. But, upon stimulation with LPA/PDGF, expression and phosphorylation of these molecules was reverted to high levels as on fibronectin (**Fig. 7A, Supplemental Table 3**).

Next, we asked whether restored expression and activation of these molecules was necessary for LPA/PDGF-induced cell spreading on FN/TN. First, we addressed a potential role of RhoA. Whereas the levels of RhoA significantly dropped on FN/TN,

activation of RhoA as determined by the amount of GTP-loading, was not lowered up to 4 h. Apparently, activation of RhoA was independent on cell shape, and even very low RhoA levels were sufficient for actin polymerisation in these cells. Inhibition of ROCK, a downstream target of RhoA did not interfere with LPA/PDGF-induced spreading but rather enhanced spreading, which was also reflected by an increased MLC phosphorylation. This result points to a ROCK-independent mechanism of LPA/PDGF-induced MLC phosphorylation. It is possible that the observed enhanced ERK1/2 activation by these growth factors bypassed ROCK requirement for actin polymerization (Ridley et al.,2001). In contrast to RhoA and ROCK, knockdown of paxillin abolished spreading by LPA/PDGF (**Fig. 7B**), which points at paxillin as a major critical effector of tenascin-C-induced cell rounding.

Since syndesmos binds paxillin and thus may recruit syndecan-4 into focal adhesions (Bass and Humphries, 2001), we wondered whether ectopic expression of syndesmos had an effect on cell rounding by tenascin-C. We found that ectopically expressed syndesmos restored cell spreading on FN/TN, but not on tenascin-C. Again, cell shape correlated with expression and activation of FAK, paxillin, RhoA and TM1 (**Supplemental Table 3**). Spread T98G:syndesmos cells exhibited a high phosphorylation of FAK and paxillin and high expression of RhoA and TM1 on FN/TN. We conclude, that syndesmos may provide a platform for recruitment of paxillin and other adaptor proteins into focal adhesions, thus promoting cell spreading. High syndesmos levels may bypass syndecan-4 requirement for cell spreading on FN/TN as has been shown for other adhesion conditions.

Whereas TM1 levels were very low on FN/TN in the absence of growth factors, they were as high on this substratum as on fibronectin upon stimulation with LPA/PDGF. We confirmed that a forced expression of TM1 restored cell spreading (Ruiz et al., 2004), and found that this correlated with restored RhoA levels and FAK autophosphorylation. This result suggests a positive feedback mechanism by which TM1 sustains RhoA expression, actin filament stabilisation and FAK activation. That tropomyosin levels are critical was shown by knocking down expression of αTM,

which interfered with LPA/PDGF-induced cell spreading on FN/TN. By real time RTPCR we confirmed that TM1 expression is predominantly regulated at protein

stability level, since expression of βTM, that encodes TM1, was not altered (Lange, data not shown). In contrast to βTM, expression of αTM, which codes for TM2 and TM3, was regulated by cell adhesion and growth factors. Expression of αTM was low on FN/TN and was restored to levels as on fibronectin with FNIII13 as well as with LPA/PDGF. High αTM levels apparently promoted stabilisation of TM1 heterodimers.

We addressed the question which signaling events induced by LPA/PDGF were responsible for a restored expression and function of tenascin-C target molecules. By using chemical inhibitors, we identified PI3K and MEK as downstream signaling molecules, that are essential for LPA/PDGF-induced spreading on FN/TN. This was different to PLCγ and JNK, inhibition of which did not block LPA/PDGF-induced spreading (**Supplemental Table 3**). Although PLCγ is important in syndecan-4 mediated spreading, its activation does not appear to be required in the presence of LPA/PDGF. We observed that spreading induced by LPA/PDGF was different to that induced by EDNRB. Whereas EDNRB-induced spreading was dependent on PLCγ and JNK, but independent on MEK (Lange et al., 2007), here we demonstrated an opposite mechanism for LPA/PDGF-induced spreading, that was independent of PLCγ and JNK, but dependent on MEK. Spreading in both conditions was PI3K dependent. We conclude, that at least two separate mechanisms exist that overcome tenascin-C induced cell rounding, which could be relevant in human cancers.

We wanted to know whether LPA/PDGF signaling potentially contributes to tumor progression in a tenascin-C context. Since the acquisition of a motile phenotype is a prerequisite for invasion and metastasis, we examined cell migration in the presence of tenascin-C and LPA/PDGF, and observed that combined signaling by both growth factors strongly induced cell migration on FN/TN. We identified a mechanism by which tenascin-C collaborates with LPA/PDGF signaling and thus stimulates cell migration. This mechanism includes activation of FAK, paxillin and ERK1/2. Moreover, LPA/PDGF signaling also induced actin remodeling by restoring expression of RhoA and TM1 and phosphorylation of MLC. Together, LPA/PDGF

stimulated actin polymerisation, filament stabilisation and contractility on FN/TN, which links to enhanced cell migration. Our data suggest that inhibition of syndecan-4 by tenascin-C as well as remodeling of the actin cytoskeleton by LPA/PDGF signaling is important for cell migration on FN/TN (**Fig. 7B**). This mechanism may

contribute to tumor progression and may explain a malignant aspect of tenascin-C in cancer.

Here, we showed that tenascin-C collaborates with LPA/PDGF signaling. This collaborative signaling might also be relevant for the understanding of the role of tenascin-C in the neuronal stem cell niche, where maintenance of the oligodendrocyte precursor status and cell responses toward PDGF were impaired in the absence of tenascin-C (fFrench-Constant). Since we observed that syndecan-4 synergises with LPA/PDGF-mediated spreading on FN/TN, it will be interesting to see whether OP responsiveness toward PDGF involves syndecan-4 in a tenascin-C context. Our data also suggest that the adhesive strength of tenascin-C is strongly influenced by additional signals. Whereas, LPA/PDGF renders tenascin-C adhesive and promotes migration, EGF supports the anti-adhesive properties of tenascin-C and fails to promote migration in a fibroblastoid manner. Thus, growth factors may determine whether cells remain in the stem cell niche and in the tumor tissue, respectively. At the mechanistic level, this might be linked to syndesmos, FAK, paxillin and tropomyosins downstream of syndecan-4.

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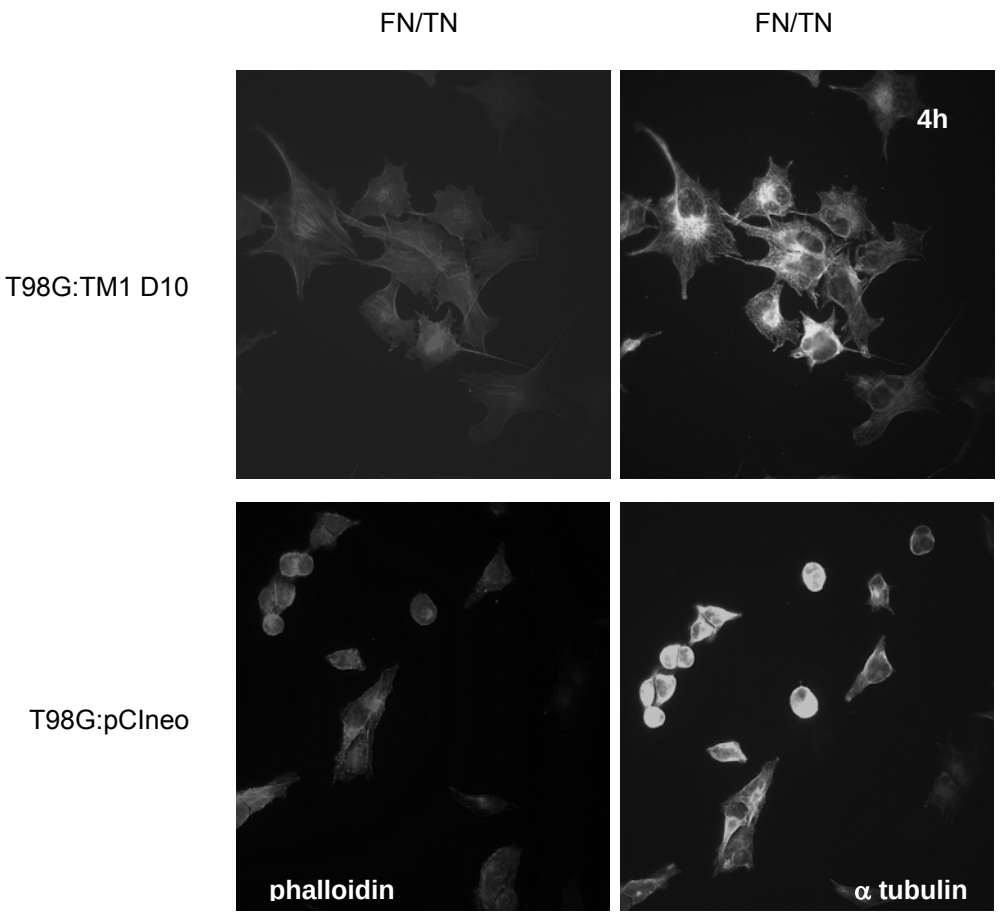
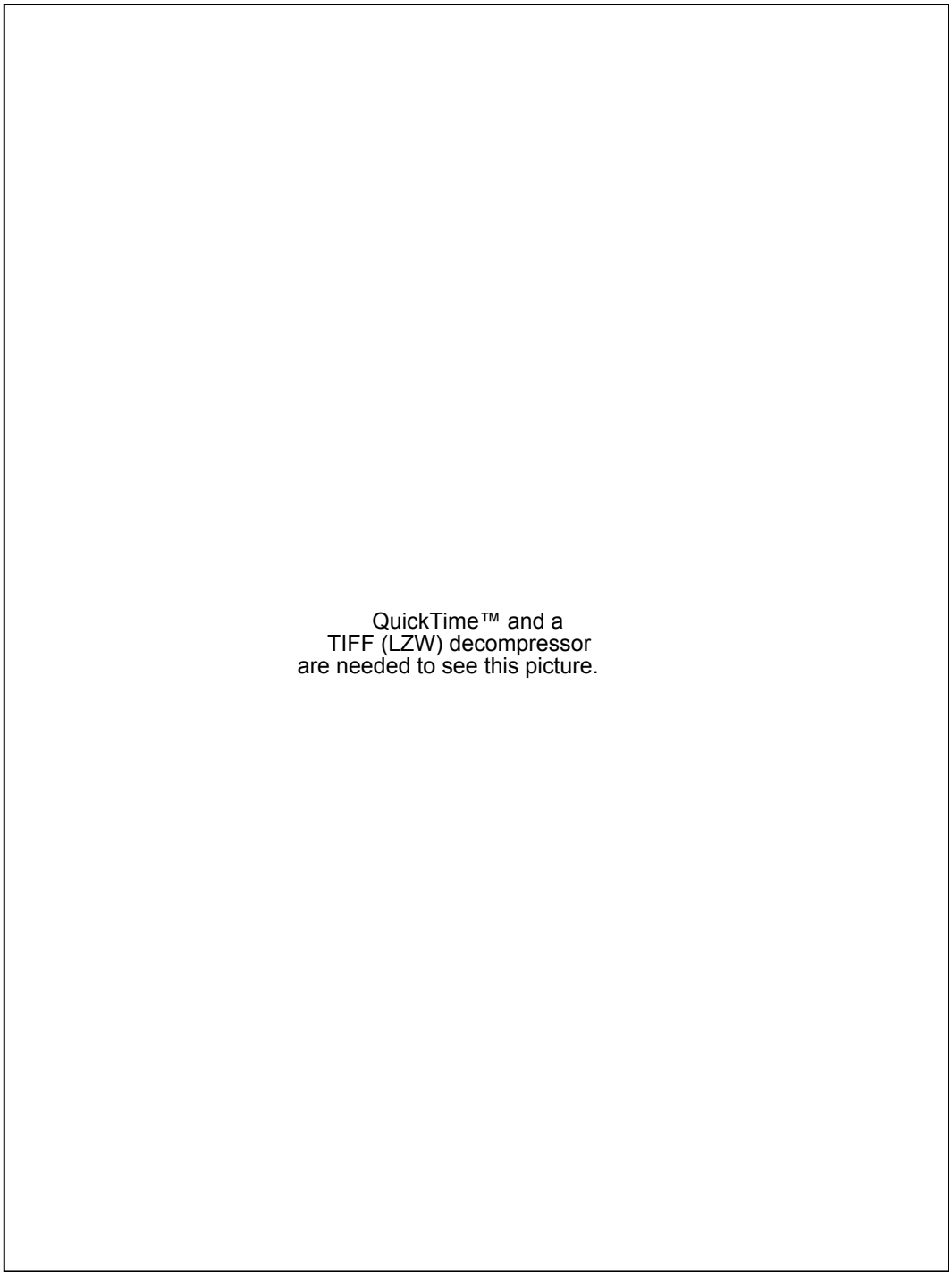
Supplemental Data

Supplemental Fig. 1 Cell spreading and associated signaling in MEF wt and MEFS4-/- on FN/TN upon activation of syndecan-4 with FNIII13.
Wildtype MEFs (MEF wt) and MEFs lacking syndecan-4 (MEF S4-/-) were plated on the indicated substrata for 4 h in serum free medium. (**A**) documentation of cell shape, (**B**) number of spread cells, (**C**) immunoblotting for the indicated molecules.

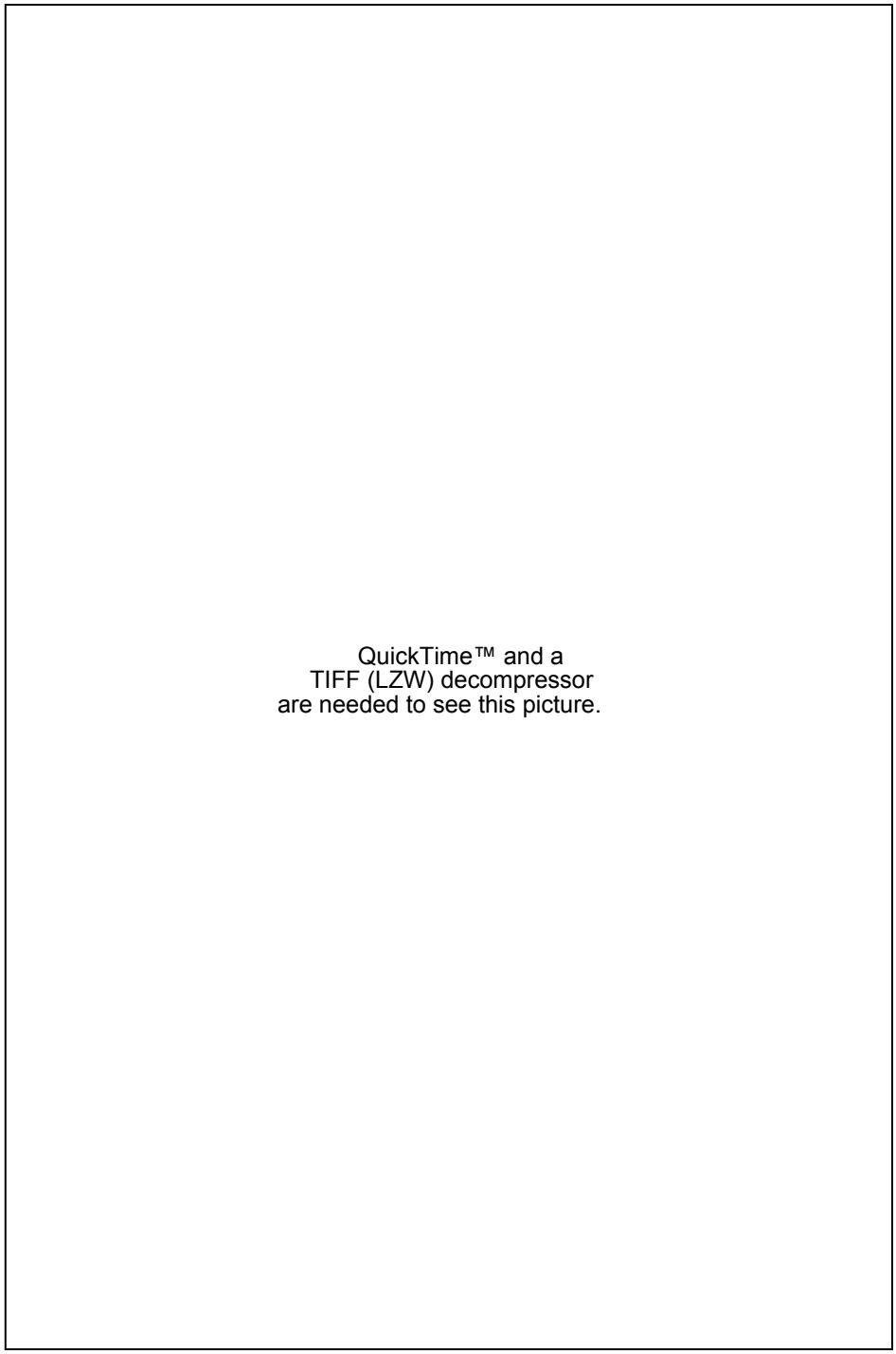
Supplemental Fig. 2



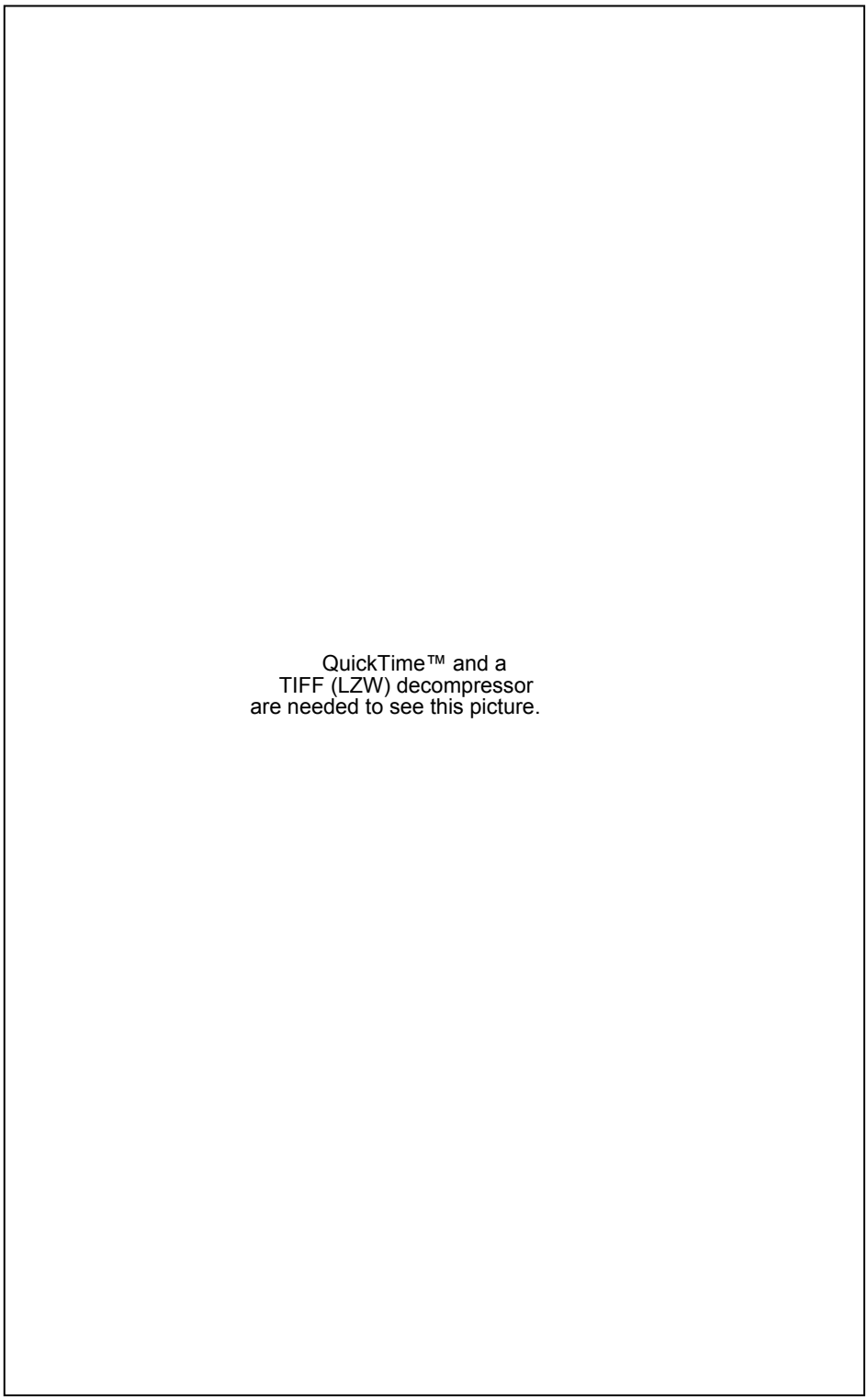
Supplemental Fig. 2 Effect of TM1 expression levels on cell adhesion to FN/TN
Cells ectopically expressing βTM (T98G:TM1D10) or pCIneo (T98G:pCIneo) were plated on FN/TN in the presence of 1% FCS for 4 h before immunostaining with phalloidin or an anti-tubulin antibody.



Supplemental Fig. 3 Effect of signaling inhibitors on LPA/PDGF-induced cell spreading on FN/TN
T98G cells were plated on fibronectin (data not shown) or FN/TN together with LPA/PDGF and inhibitors for PLC (U73122) and JNK (SP600125) (**A**) before lysis and immunoblotting (**B, C**). U73122 was added together with LPA/PDGF (lanes 3 and 4) or 15 minutes before addition of LPA/PDGF (lanes 5 and 6) (**C**). Note, that both conditions of JNK inhibition did not affect cell spreading by LPA/PDGF.



Supplemental Fig. 4 RhoA activation and ROCK inhibition on FN/TN
Cells plated for the indicated time points on fibronectin in the absence or presence of tenascin-C together with LPA/PDGF or no growth factors were lysed and GTP-bound RhoA was detected upon binding to plastic-adsorbed rhotekin by ELISA for RhoA (**A**). Cells plated together with the ROCK inhibitor Y27632 (**B**) were lysed and immunoblotted for the indicated molecules (**C**).


QuickTime™ and a
TIFF (LZW) decompressor
are needed to see this picture.

Supplemental Fig. 4 RhoA activation and ROCK inhibition on FN/TN
Cells plated for the indicated time points on fibronectin in the absence or presence of tenascin-C together with LPA/PDGF or no growth factors were lysed and GTP-bound RhoA was detected upon binding to plastic-adsorbed rhotekin by ELISA for RhoA (**A**). Cells plated together with the ROCK inhibitor Y27632 (**B**) were lysed and immunoblotted for the indicated molecules (**C**).

Supplemental Fig. 5 Cell migration on FN/TN upon stimulation with LPA/PDGF and modulation of syndesmos and tropomyosin expression
Cell migration of the indicated cell lines on FN/TN is documented in the presence of LPA/PDGF in comparison to serum free medium at the indicated time points. Whereas 1000 pCIneo and shRNA control cells were plated, 2000 cells were seeded for the other cell lines. Note, that very little T98G:syndesmos, T98G:TM1D10 and T98G:αTM3D2 cells migrated despite the presence of twice as many cells as in the controls.

Supplemental Table 3

Supplemental Table 3 Modulators of tenascin-C inhibition of cell spreading

Cells	Treatment on FN/TN	Effector	Spreading	P-FAK	P-Paxillin	RhoA	TM1
T98G, MEF wt	no GF	Syndecan-4	0	0	0	0	0
T98G, MEF wt	no GF, FNIII13 [a]	Syndecan-4	1	1	1	1	1
T98G:sh S4, MEF S4-/-	no GF, FNIII13	Syndecan-4	0	0	0	0	0
T98G, MEF wt	LPA/PDGF	LPAR, PDGFR	1	1	1	1	1
T98G:sh S4, MEF S4-/-	LPA/PDGF	LPAR, PDGFR	1	1	1	1	1
T98G	LPA	LPAR	0	0	0	0	0
T98G	PDGF	PDGFR	0	0	0	0	0
T98G	EGF	EGFR	0	0	0	0	0
T98G	LPA/PDGF, Wm	PI3K, [b]	0	0	0	0	0
T98G	LPA/PDGF, UO126	MEK1/2, [b]	1 [c]	0	0	0	1
T98G	LPA/PDGF, SP600125	PLC, [b]	1	1	1	1	1
T98G	LPA/PDGF, U73122	JNK, [b]	1	1	1	1	1
T98G	LPA/PDGF, Y27632	ROCK, [a]	1	1	1	1	1
T98G:CD2-FAKYF	LPA/PDGF	FAK, [b]	0	0	0	0	0
MEF S4-/-:si paxillin	LPA/PDGF	Paxillin, [b]					
T98G:syndesmos	no GF	syndesmos	1	1	1	1	1
T98G:syndesmos, si paxillin	LPA/PDGF	syndesmos, paxillin, [b]					
T98G:TM1 D10	no GF	TM1	1	1	1	1	1
T98G:sh αTM	LPA/PDGF	TM1-3, [b]	0	0	0	0	0

Summarised are cell spreading and activation of downstream effectors presented in this study. The following cells were used: T98G parental cells or control cells stably expressing pCIneo or shcontrol RNA (T98G), T98G cells stably expressing syndecan-4 shRNA knock-down (T98G:sh S4), dominant negative FAK (CD2-FAKYF), syndesmos (T98G:syndesmos), TM1 (T98G:TM1 D10), αTM shRNA knock-down (T98G:shαTM), wildtype mouse embryo fibroblasts (MEF wt) and MEFs from syndecan-4 knock out mice (MEF S4-/-). Knock down of paxillin was accomplished with transient siRNA expression. The presence (1) or absence (0) of cell spreading (Spreading), on fibronectin/tenascin-C is indicated as deduced from immunofluorescent staining for polymerised actin and for vinculin or by determination of cell shape by phase contrast microscopy. Experiments were done in the absence of growth factors (no GF) or upon addition of EGF (epidermal growth factor), LPA (lysophosphatidic acid) or PDGFBB (PDGF). Data on the effect of the indicated inhibitors Wm (PI3K), UO126 (MEK), SP600125 (PLCγ), U73122 (JNK) and Y27632 (ROCK) are shown on phosphorylation of FAK at Y397 (P-FAK), and expression of RhoA and TM1 in cells grown on fibronectin/tenascin-C. Expression on FN/TN reached levels as on fibronectin (1) and, was very low or absent (0), respectively. a) addition of cells to a FN/TN/FNIII13 substratum. b) receptors for LPA and PDFG-BB. c) elongated motile cell shape. Reduced expression of P-FAK, P-paxillin and RhoA on both substrata.

3 Discussion

Cell adhesion to ECM involves signaling mechanisms which control attachment, spreading and the formation of focal adhesions and stress fibers (Woods et al., 2000). During tumor progression, some of the primary tumor cells as well as the tumor microenvironment undergo characteristic molecular changes, which are essential for the metastatic dissemination of tumor cells. Tumor cell-intrinsic alterations include the loss of cell polarity and alterations in cell-cell and cell-matrix adhesion as well as deregulated receptor kinase signaling, which together support detachment, migration and invasion of tumor cells (Kopfstein and Christofori, 2006). In addition to cell-intrinsic changes, alterations in the tumor microenvironment are causally linked to tumor progression. Tenascin-C alone or in combination with fibronectin is anti-adhesive for most cells and prevents activation of syndecan-4, which causes cell rounding. Previously, it was shown that the function of FAK (Huang et al., 2001; Orend et al., 2003), RhoA (Wenk et al., 2000), and TM1 (Ruiz et al., 2004) is compromised in cells grown on a mixed substratum of FN/TN. This occurred in a syndecan-4-dependent manner.

Here, I investigated the mechanism, by which tenascin-C inhibits the formation of focal adhesions and actin stress fibers, and how tenascin-C affects expression and function of the three downstream targets FAK, RhoA and TM1. First, I examined whether inhibition of syndecan-4 by tenascin-C is potentially linked to reduced expression of RhoA and TM1 and inhibition of FAK. Indeed, MEFs that lacked syndecan-4 did not spread on FN/TN upon activation of syndecan-4 with its ligand FNIII13 4h, and this was linked to the lack of FAK activation. In contrast, syndecan-4 expressing cells spread on FN/TN in the presence of FNIII13, and exhibited a restored expression and function of FAK, RhoA and TM1. Thus, repression of FAK, RhoA and TM1 could explain the lack of actin stress fiber and focal adhesion formation on FN/TN. Syndecan-4, working in cooperation with the $\alpha 5\beta 1$ integrin, can stimulate a Rho-dependent pathway, that elicited focal adhesion and actin stress fiber formation in CBD-adherent cells (Saoncella et al., 1999). Furthermore, syndecan-4 signaling can be bypassed in CBD-adherent fibroblasts by directly stimulating the GTPase Rho with LPA (Saoncella et al., 1999). These data indicate an important role of syndecan-4 in RhoA activation. In addition, syndecan-4 interacts

with important cytoplasmic adaptor proteins such as paxillin. Paxillin binds several other focal adhesion-associated proteins such as vinculin, FAK and syndesmos.

The actin cytoskeleton plays a central role in the regulation of cellular processes linked to metastasis including cell proliferation, apoptosis, anchorage-independent cell growth, cell migration, and invasion (Jaffe and Hall, 2002). TMs play a major role in regulating the actin cytoskeleton, by stabilizing actin filaments during actin polymerization (Cooper, 2002), and inhibition of actin depolymerization from the pointed end. Since tenascin-C downregulated TM1-3, I analyzed the role of tropomyosins in cell adhesion in the presence of tenascin-C in more detail. Published data suggest that TM1 expression and function is regulated by at least two pathways that involve ROCK and MEK. Since the ERK/MAPK signaling pathway is hyper-activated in T98G glioblastoma cells on FN/TN (Ruiz et al., 2004), it is possible that this signaling contributes to repression of TM1 by tenascin-C (Orend, 2005). This possibility was supported by the observation that inhibition of MEK caused high levels of TM1 and resulted in cell spreading on FN/TN (Lange et al., 2007). The RhoA downstream effector ROCK also seems to be required for TM1 expression, since inhibition of ROCK decreased TM1 expression (Janssen et al., 2003; Pawlak and Helfman, 2002a, 2002b). But, inhibition of ROCK did not interfere with high TM1 expression upon stimulation with LPA/PDGF on FN/TN. Signaling by LPA/PDGF potentially compensated for the lack of a signal downstream of ROCK. Epigenetic mechanisms involving de novo methylation and acetylation of the TM1 promotor region have been shown to contribute to the loss of TM1 function in breast cancer cells (Bharadwaj and Prasad, 2002). I was interested to know whether promoter methylation of TM1 in T98G cells could be involved in TM1 downregulation on FN/TN. In collaboration with Mirco Meningatti (research group of Prof. Schär, University of Basel), a methylation-specific PCR showed no changes in promoter methylation up to 6 days of culture (unpublished results). It is possible that the chosen time points were not long enough, therefore a potential epigenetic regulation of TM1 by tenascin-C should be addressed in the future.

Whereas expression of TM1 was not regulated by tenscin-C at the transcriptional level, it turned out that tenascin-C repressed RNA levels of two other HMW tropomyosins, TM2 and TM3, which are far less expressed in T98G cells than TM1. Apparently, lowered levels of TM2 and TM3 affected TM1 protein heterodimer stabilization through proteasomal degradation, that was largely enhanced on FN/TN.

Enhanced protein degradation by tenascin-C was supported by the observation that inhibition of the proteasome by the inhibitor ALLN, restored TM1 expression on the mixed substratum (Lange et al., 2007). These data suggest that tenascin-C does not only repress gene expression of HMW tropomyosins but also enhances their proteasome-mediated protein degradation. Repression of TM1 with tumor suppressor-like activity, by tenascin-C might be relevant in cancer, since low levels of TM1 can protect breast cancer cells from apoptosis (rev in Orend, 2005).

Here, it was shown that concomittant restoration of the expression and function of all three downstream molecules FAK, RhoA and TM1, is necessary to induce cell spreading on a FN/TN substratum, which points at these molecules as a minimal set of important targets of tenascin-C. I observed that activation of EDNRB induced spreading in the presence of tenascin-C, which was dependent on PI3K, PLC and JNK, since chemical inhibitors of these enzymes blocked EDNRB-induced cell spreading on FN/TN. Signaling by EDNRB was linked to activation of FAK and paxillin as well as to restoration of TM1 and RhoA expression, again supporting the notion that inactivation of these molecules is critical for tenascin-C-induced cell rounding (Lange et al., 2007). Whereas phosphorylation of FAK at S910 and of paxillin at Y118 was absent or very low in the presence of tenascin-C in serum-free medium, EDNRB signalling restored phosphorylation in FAK and paxillin. It is possible that phosphorylation of FAK at S910 occurs through ERK2, and that P-S910FAK phosphorylates paxillin, thus restoring cell spreading on FN/TN. Localization of paxillin into focal adhesions is dependent on syndecan-4, which involves binding of syndesmos to syndecan-4. But tenascin-C impairs syndecan-4 activation and prevents localization of paxillin into focal adhesions. Thus, EDNRB activation restores focal adhesion formation on the mixed FN/TN substratum presumably through bypassing the requirement of syndecan-4 for the recruitment of paxillin into focal adhesions.

In addition to EDNRB, concomittant activation of the receptors for LPA and PDGF also enabled cells to spread on a FN/TN substratum by a mechanism, which again involved restoration of the expression and function of FAK, RhoA and TM1. By using cells lacking syndecan-4, it was observed that LPA/PDGF also bypassed the requirement of syndecan-4 in cell spreading in the presence of tenascin-C. Knockdown of paxillin prevented LPA/PDGF-induced spreading in these cells on a

mixed substratum, which suggest an essential role of paxillin in LPA/PDGF-induced restoration of cell spreading on FN/TN.

I observed, that TM1 levels were tightly linked to expression of RhoA and activation of FAK, which suggests an interdependent regulation of these molecules. This interdependent regulation could involve molecules that bind syndecan-4 such as Hic-5 and the GTPase dynamin II (Yoo et al., 2005). Upon treatment with LPA, dynamin II and Hic-5 become associated with syndecan-4 at focal adhesion sites. RhoA, which acts upstream of TM1, and TM1 itself are strongly involved in the dynamic regulation of the actin cytoskeleton. Thus, it is possible that expression of RhoA and TM1 is similarly regulated. This possibility is supported by the results that were obtained for several cell lines including REF52, MEF and T98G glioblastoma cells, which show a concomittant expression of both molecules under the chosen conditions. The integrin-linked kinase (ILK) that co-localizes with paxillin in focal adhesions (Nikolopoulos and Turner, 2001) is another candidate that could be involved in cell adhesion on FN/TN. In many cellular processes such as cell migration, ILK, FAK and RhoA work together through paxillin.

Whereas the levels of RhoA expression significantly dropped on FN/TN, an activation of RhoA was not lowered on this substratum in comparison to fibronectin up to 4h. Substratum-independent activation of RhoA may be accomplished in these cells through several mechanisms that potentially involve mDia and MTs (Wheeler and Ridley, 2004; Palazzo et al., 2001; Fukata et al., 2003), Rac (Hall, 1998), which is active on FN/TN (Orend, 2005), as well as a rigid cortex in round cells (Ren et al., 1999; Maddox and Burridge, 2003; Burridge and Wennerberg, 2004). Inhibition of ROCK, a downstream target of RhoA did not interfere with LPA/PDGF-induced spreading but enhanced spreading, which was also reflected by an increased MLC phosphorylation. This result points at a ROCK-independent mechanism of MLC phosphorylation on a FN/TN substratum upon stimulation with LPA/PDGF. Explanations for this result could be the ability of PDGF to induce autocrine signaling through both PDGFRs as well as paracrine signaling, which lead to activation of MAPK and PI3K signaling pathway (Kim et al., 2007; Lokker et al., 2002; Cao et al, 2004). Cell contractility necessary for migration, results in actin-myosin interactions and phosphorylation of MLC. LPA induces the activity of both, the downstream effector ROCK and mDia. Upon inhibition of ROCK, mDia could induce actomyosin-based contractility, which may result in phosphorylation of MLC (Wheeler and Ridley,

2004) independent of ROCK. Furthermore, LPA was identified to induce the formation of stable MTs (Palazzo et al., 2001), which are co-localized with actin filaments during cell migration and result in phosphorylation of MLC.

Activation of the receptors for ET-1 (EDNRB), and LPA/PDGF induced spreading in the presence of tenascin-C by distinct pathways. Whereas EDNRB-induced spreading was dependent on PI3K, PLC and JNK, but not on MEK, LPA/PDGF-induced spreading was dependent on PI3K and MEK, but not on PLC and JNK. Signaling by these factors was linked to activation of FAK and paxillin and, restoration of RhoA and TM1 expression. Together, these results suggest that at least two (and presumably many more) mechanisms exist that modulate the adhesive strength of a FN/TN substratum. Thus, combined signaling from ECM and growth factors can modulate cell adhesion and migration.

Ruiz et al. (2004) described an enhanced expression of EDNRA in the presence of tenascin-C for later time points after seeding. Here, I demonstrated that EDNRA signaling is linked to cell rounding on a FN/TN substratum through inhibition of FAK and paxillin and repression of RhoA and TM1. Because activation of EDNRA and MEK downregulates TM1 expression (Janssen et al., 2003; Pawlak and Helfman, 2002; Pawlak et al., 2004), I examined whether EDNRA-associated cell rounding on FN/TN was dependent on MAPK signaling. Indeed, inhibition of MEK with UO126 blocked EDNRA-induced cell rounding and induced cell spreading, that was linked to a restored phosphorylation of FAK and a stable expression of RhoA and TM1. Thus, the results link TM1 and RhoA protein stability to EDNRA signaling (Lange et al., 2007). Collectively, these data suggest that inhibition of syndecan-4 is responsible for initial cell rounding and that upon induction of EDNRA by tenascin-C, EDNRA prevents cell spreading on a FN/TN substratum at later time points. EDNRA-associated cell rounding on FN/TN seems to be independent on syndecan-4, since it still occured in MEFs lacking syndecan-4 (recent not published data).

In gliomas and other cancers we found a high expression of tenascin-C and EDNRA, that correlated with more advanced stages. This observation supports the possibility that tenascin-C potentially promotes tumor progression through EDNRA (Lange et al., in preparation). Numerous tumors, including carcinomas of the lung (Ahmed et al., 2000), prostate (Nelson et al., 2000), and ovary (Bagnato et al., 1999) produce one or more endothelins and their receptors (Nelson et al., 2003). They play an

autocrine and paracrine role in regulating growth of tumor and tumor associated cells, may inhibit apoptosis (Grimshaw et al., 2002) and promote angiogenesis (Bagnato et al., 2002). Therefore, these findings support notions about a critical role for the ET1/EDNRA axis during tumor progression that might be linked to tenascin-C (Rosano et al., 2006). The ET-1/EDNRA autocrine pathway was shown to be involved in epithelial-to-mesenchymal transition (EMT) in ovarian tumor cells. By using two pharmacologic antagonists of EDNRA: BQ123, a selective antagonist, and ABT-627, a small molecule that potently and selectively inhibits ET1 signaling at the level of interaction with EDNRA (Rosano et al., 2003), both cause inhibition of spontaneous growth rate in ovarian cancer primary cultures and cell lines (Rosano et al., 2005). In contrast, induction of EDNRB pathway promoted tumorigenesis upon activation by ET1 or ET3 in human cutaneous melanomas (Spinella et al., 2007; Bagnato et al., 2004). Blockage of EDNRB by a peptide antagonist BQ788 resulted in growth inhibition and death of melanoma cells in vivo and in vitro (Lahav et al., 1999). Moreover, A-192621, a selective and orally active nonpeptide EDNRB antagonist (Griswold et al., 1999), inhibited cell proliferation and melanoma growth in nude mice (Bagnato et al., 2004). Thus, pharmacological interruption of both EDNRA and EDNRB signaling together with tenascin-C may represent a potent novel therapeutic strategy in the treatment of certain cancers.

Gliomas represent about half of all brain tumors and glioblastoma multiforme is thought to be the most malignant and common intracranial tumor (Vandenberg, 1992). Moreover, tenascin-C is a very prominent molecule of the glioma ECM. Although generally not metastatic, glioblastoma cells exhibit highly migratory and invasive behaviour (Ishiuchi et al., 2002). Therefore, here the mechanism of glioma cell migration on a FN/TN substratum in combination with migration promoting growth factors was examined. Whereas EGF, LPA and PDGF alone did not promote cell spreading and migration on FN/TN, combined signaling by LPA and PDGF triggered spreading and migration. Cell migration is an integrated process that requires coordinated regulation of various signaling molecules, including kinases and phospahatases (Laufenburger and Horwitz, 1996; Webb et al., 2002). Integrin-dependent slow migration with a fibroblastoid cell shape is discriminated from amoeboid cell migration, where cells are round and do not depend on integrin signaling but rather on ECM degrading enzymes such as MMP (Wolf et al., 2003).

EGF activates Akt and ERK1/2, induces morphological changes, and increases cell motility in MCF-7 breast cancer cells (Garcia et al., 2006) and ovarian cancer cells (Oiu et al., 2006). This suggests an involvement of the Raf/MEK/ERK pathway in enhancing EGF-specific migration. But, we observed that EGF did not promote cell migration in a tenascin-C context. This may be explained by an increased dephosphorylation and activation of the actin-severing molecule cofilin at the leading edge by EGF, which lead to inhibition of lamellipodia formation (Song et al., 2006). Furthermore, EGF-induced activation of ERK5 can lead to the loss of actin stress fibers by a mechanism that is distinct from that associated with ERK1/2 (Barros and Marshall, 2005). Hic-5, a member of the paxillin family, showed tyrosine phosphorylation by EGF, which results in a decrease of lamellipodia formation in COS-7 cells (Avraamides et al., 2007). Thus, EGF prevents cell migration on a 2-dimensional substratum via inhibiting lamellipodia formation through disruption of the actin cytoskeleton and focal adhesions. But, in 3-dimensional tissues, a round phenotype may support fast-track amoeboid migration. In this respect, maintained cell rounding on FN/TN by EGF could potentially be relevant in vivo and may promote amoeboid cell migration in tenascin-C enriched tissues. This possibility needs to be addressed in future experiments.

In contrast to EGF-inhibited cell migration, LPA/PDGF triggered cell migration on a FN/TN substratum. LPA induces cell migration both through the $G_{12/13}$-RhoA-ROCK pathway and the G_i-Ras-MEKK1 pathway. Rho is believed to act primarily at the rear of migrating cells where it promotes detachment (Ridley et al., 2003). In contrast to RhoA, Rac acts at the front to stimulate actin-mediated membrane protrusion. Activation of RhoA by LPA directly stimulates actin polymerization through activation of mDia, which induces addition of actin monomers to the barbed, fast-growing end of actin filaments. RhoA, mDia and ROCKs mediate phosphorylation of LIMK and subsequently inhibit cofilin. Polymerization through mDia and inhibition of filament turnover results in an increase of actin filament formation in response to RhoA activation. ROCKs induce actomyosin-based contractility, which is important for cell detachment at the rear. In addition, the concerted action of ROCK and mDia is essential for regulation of cell polarity and organization of microtubules (Wheeler and Ridley, 2004). LPA-induced autophosphorylation of FAK is required for LPA-stimulated cell migration (Bian et al., 2006). FAK is involved in focal adhesion turnover, thus controlling the process of cell attachment and detachment, which is necessary for cell migration (Webb et al., 2004). In contrast to EGF, LPA recruits Hic-

5 to focal adhesions and to pseudopodia, suggesting that recruitment of Hic-5 might directly be linked to endothelial cell migration (Avraamides et al., 2007). Hic-5 may be an important molecule in LPA-stimulated migration through its binding to FAK (Fujita et al., 1998; Nishiya et al., 1999; Nishiya et al., 2002).

Here, it was observed that only combined signaling by both growth factors LPA and PDGF triggered migration with a fibroblastoid cell shape. The underlying mechanism may involve an enhanced activation of MEK/ERK- and PI3K/Akt-associated signaling, which is triggered by either of the growth factors already alone. Many pathways are activated by PDGF that potentially promote cell migration on FN/TN. PDGF triggers rapid focal adhesion turnover, and continued adhesion assembly in concert with the spreading protrusion (Melton et al., 2007). LPA/PDGF-induced cell migration was PI3K and ROCK dependent, since inhibition of these kinases blocked LPA/PDGF-induced cell migration on FN/TN. PDGFR may be a target in glioblastoma therapy. Coexpression of PDGF and PDGFR has been shown at all brain tumor stages including low-grade astrocytomas, anaplastic astrocytomas, and glioblastoma multiforme (Maher et al., 2001; Westermark et al., 1995). The selective PDGFR antagonist CT52923 can block autocrine-mediated receptor autophosphorylation and the exaggerated Akt phosphorylation as well as the MAPK pathway, which was also consistently inhibited in some cell lines (Lokker et al., 2002).

By using cells with ectopic expression of syndesmos or reduced expression of TM1-3, we showed that both, a strong as well as loose adhesion blocked LPA/PDGF-induced cell migration on FN/TN. Syndesmos-overexpressing cells exhibited very little motility. Binding of paxillin or Hic-5 to syndesmos may lead to stabilized focal adhesions on FN/TN, which counteracts focal adhesion turnover during migration. Tubulin is another binding partner of paxillin (Denhez et al., 2002) and LPA induces formation of stable MTs (Cook et al., 1994; Nagasaki and Gundersen, 1996). MTs are essential for the polarization, the first step in the migration process as well as in promoting disassembly at the trailing edge. Thus, in cells with ectopic expression of syndesmos not only a stabile actin cytoskeleton but also stabilized mictrotubules might counteract cell migration. Another candidate is the syndecan-4 binding GTPase dynamin II, that colocalizes with syndecan-4 near the nucleus in the cytoplasm (Henley et al., 1998; Jones et al., 1998; Kreitzer et al., 2000). Upon treatment with LPA, dynamin II becomes associated with syndecan-4 at focal

adhesion sites. Thus, LPA/PDGF may promote dynamin II recruitment into focal adhesions and may counteract focal adhesion disassembly during migration.

At the very front of the cell, in the lamellipodium, actin is nucleated and cross-linked into filaments and treadmill rapidly toward the cell body as the filaments elongate from their barbed ends. Filaments in this compartment are short-lived and depolymerize not far from the cell edge. Behind the lamellipodia is a more slowly moving actin network called the lamellum, which contains myosin II and tropomyosins (Gupton et al., 2005; Iwasa and Mullins, 2007). The results presented here, suggest that TM1 downregulation compromise actin treadmilling and through that cell migration on FN/TN. In contrast to the control cell lines, the shTM1 cell lines showed approximately 3-fold decrease in cell migration on the mixed substratum in the presence of LPA/PDGF, which indicates that TM1 is necessary for motility. The first step, actin polarization is supported by RhoA and RhoC (Wheeler and Ridley, 2004), FAK and mDia (Wheeler and Ridley, 2004) and reorientation of the MTOC towards the leading edge (Jaffe and Hall, 2005). LPA induces formation of stable MTs and Rho-GTPase is necessary and sufficient for selective stabilization of MTs (Cook et al., 1994; Nagasaki and Gundersen, 1996). In TM1-overexpressing cells, cell migration is completely inhibited for all time points in the presence of LPA/PDGF. TM1 might protect actin filaments from the severing action of gelsolin (Ishikawa et al., 1989) and from the depolymerising action of ADF/cofilin (Bernstein and Bamburg, 1982; Ono and Ono, 2002; Bryce et al., 2003). Tropomyosins also inhibit the ability of the Arp2/3 complex from nucleating new branch points along the filaments (Blanchoin et al., 2001) and in addition regulate access to the actin filaments of other actin-binding proteins including tropomodulin (Almenar-Queralt et al., 1999) and myosin (Tang and Ostap, 2001). Through TM1-overexpression filament disassembly, necessary to move the cell body forward, may be decreased in T98G cells so that cells are not able to migrate on FN/TN.

If actin filaments can be discriminated and targeted based on their TM composition then this becomes a powerful approach for anticancer therapy. Most importantly, the TMs found in the actin filaments of the contractile apparatus in cardiac and skeletal muscle are quite distinct from those found in the cytoskeleton of non-muscle cells (Stehn et al., 2006). It is therefore likely that targeting of a cytoskeletal TM would not impact on the contraction of heart and skeletal muscle, which identify TMs as an ideal candidate target in anti-cancer therapy. Both disruption and stabilization of TMs

could be desirable because both would interfere with cell migration. High levels of TM1 would also sensitize tumor cells towards anoikis.

In summary, here it was shown that cell adhesion and migration on an anti-adhesive FN/TN substratum can be modulated by additional signaling from growth factors. We identified a minimal set of critical targets of tenascin-C downstream of syndecan-4 that include FAK, paxillin, RhoA and TM1. Induction of EDNRA signaling by tenascin-C provides an additional mechanism that contributes to maintained cell rounding on FN/TN by a mechanism that again affects the same set of tenascin-C targets as those downstream of syndecan-4.

4 References

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Erklärung

I declare that I wrote this thesis “Characterization of tenascin-C-induced signaling in tumorigenesis” with the help indicated and only handed it in to the faculty of science of the University of Basel and to no other faculty and no other university to the dean’s office.

Katrin Lange

Basel, 27.11.2007