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PUBLISHER'S NOTE

Expression of Concern: Association of tetraspanin CD9 with transmembrane TGFα confers alterations in cell-surface presentation of TGFα and cytoskeletal organization

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There is an error in *J. Cell Sci.* (2008) **121**, 2265-2274 (doi:10.1242/jcs.021717).

The journal was alerted anonymously to duplicated cell images in Fig. 6D. The authors no longer have any original data and therefore cannot supply the correct panel but do state that the conclusions of the paper are not affected by this error.

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Association of tetraspanin CD9 with transmembrane TGFα confers alterations in cell-surface presentation of TGFα and cytoskeletal organization

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Summary

Ligand presentation is a major determinant of receptor activation. The epidermal growth factor receptor (EGFR), a tyrosine kinase receptor, is activated by growth factors of the transforming growth factor α (TGFα) family. The tetraspanin CD9 interacts with transmembrane TGFα and decreases its ectodomain shedding to release soluble TGFα. Here we report that CD9 has a role in the maturation of transmembrane TGFα and its stabilization at the cell surface, and in the cell-surface distribution in polarized epithelial cells. Furthermore, coexpression of CD9 and TGFα confers changes in cytoskeletal organization with a decrease in actin stress fibers and focal adhesions, and changes in RhoA and Rac1 GTPase activity. These alterations are reversed by blocking EGFR signaling. Finally, we demonstrate changes in cell adhesion and migration resulting from coexpression of TGFα with CD9. These results provide insight into the role of CD9 in the presentation of TGFα in epithelial and carcinoma cells, whose physiology is driven by ligand-induced EGFR activation.

Key words: Epithelial cells, Tetraspanin, Transforming growth factor α

Introduction

Autocrine and paracrine signaling mechanisms mediate the interactions of growth and differentiation factors with their receptors, which drive normal development and a variety of developmental diseases such as cancers (Ariztia et al., 2006). Whereas the nature of the ligand defines the interaction with its cognate receptor and the receptor-initiated signaling mechanisms, the mode of ligand presentation is also a major determinant of receptor activation and has a major role in the biological responses. Thus, proteins that interact with the ligands have been shown to define the affinities of the ligand-receptor interactions, as well as receptor internalization and routing. Although the roles of ligand presentation are apparent for a variety of receptor classes, they are perhaps best illustrated for transmembrane tyrosine-kinase receptors and tyrosine-kinase-associated receptors. Some striking examples are provided by the roles of heparan sulfates and syndecans in the presentation of fibroblast growth factors (FGFs) to their cognate receptors (Guerrini et al., 2007; Horowitz et al., 2002), and the roles of the proteoglycan β-glycan (Esparza-Lopez et al., 2001) and the glycoprotein endoglin (Cheifetz et al., 1992) in the presentation of transforming growth factor β (TGFβ) to its cell-surface receptors.

The transforming growth factor α (TGFα) family of growth factors represents a small family of proteins that are marked by a single 50-amino-acid-long epidermal growth factor (EGF) core domain that is the determinant of binding to EGF family receptors. TGFα-family proteins are expressed as type I transmembrane proteins that can undergo proteolytic cleavage to release a soluble ligand that contains the EGF core – a process called ectodomain shedding (Arribas and Borroto, 2002; Fan and Derynck, 1999). Alternative processing and heterogeneous glycosylation often occur, thus giving rise to multiple forms of the soluble protein (Bringman et al., 1987). TGFα is often considered as a prototype of the TGFα family. Its transmembrane form gives rise to three forms of soluble TGFα, each of which contains the EGF core sequence that is required for EGF receptor (EGFR) activation (Bringman et al., 1987). The differential interactions of these soluble isoforms with the receptor have not been studied. Whereas soluble TGFα allows activation of the EGFR at a distance, transmembrane TGFα can only activate the EGFR in a juxtacrine manner through cell-cell contact. In addition, soluble TGFα induces ligand-receptor internalization, whereas such a scenario is hard to imagine for the interaction of transmembrane TGFα with the EGFR (Brachmann et al., 1989; Ebner and Derynck, 1991; Wong et al., 1989). Thus, the modes of action and biological effects of soluble and transmembrane TGFα are distinct. Despite its importance, little is known about the mechanisms that regulate the presentation of TGFα proteins.

Although TGFα is expressed in most, if not all, cells of neuro-ectodermal origin, its role in carcinoma progression is also well-appreciated. Indeed, most carcinomas express TGFα, often at increased levels in comparison with the normal cells from which they are derived. The increased TGFα-EGFR signaling associated with increased expression of TGFα and/or its receptor is thought to drive cancer progression (Holbro et al., 2003; Kenny and Bissell, 2007; Lee et al., 1995). Accordingly, clinical therapies for carcinomas are being developed based on interference with activation of the EGFR by its ligands (Dancey and Freidlin, 2003).

Transmembrane proteins in the tetraspanin family have been shown to interact with various cell-surface transmembrane proteins. Whether they have an important role in ligand presentation is not

clear. Tetraspanins are characterized by four transmembrane segments in a defined topology, with a large extracellular loop that often interacts with the ectodomain of the associated transmembrane proteins. The tetraspanins form homodimers and heterodimers, and are therefore likely to dimerize their interacting proteins. They are also thought to form – through secondary interactions – a much wider tetraspanin web that may recruit other interacting proteins, thus defining the presentation and localization of these associated proteins (Hemler, 2005; Levy and Shoham, 2005).

Among the tetraspanins, CD9 has been shown to interact with members of the TGFα family of growth factors, specifically TGFα, heparin-binding (HB)-EGF and amphiregulin (Inui et al., 1997; Shi et al., 2000). Originally identified as a surface antigen with a molecular mass of 24-27 kDa on lympho-hemopoietic cells (Kersey et al., 1981), CD9 is also widely expressed on non-hematopoietic tissues and has been implicated in a variety of biological functions including motility (Garcia-Lopez et al., 2005; Hemler, 2005; Longhurst et al., 2002). CD9 expression is often drastically reduced in various metastatic cancers, e.g. lung, colon, breast, bladder pancreatic cancer and squamous cell carcinoma, as well as malignant melanoma, and its expression levels have been inversely correlated with invasion and metastasis (Boucheix et al., 2001).

We have reported that CD9 interacts with transmembrane TGFα and that the interaction results in decreased TGFα ectodomain shedding and an increase in cell-surface transmembrane TGFα (Shi et al., 2000). As a consequence, TGFα induces EGFR activation at levels that are beyond what can be achieved by adding soluble TGFα to EGFR-expressing cells (Shi et al., 2000). We extended these studies and report here that CD9 is involved in the biosynthesis of transmembrane TGFα, its localization and stabilization at the cell surface, and the distribution of TGFα in polarized epithelial cells. Furthermore, coexpression of CD9 and transmembrane TGFα leads to distinct changes in cytoskeletal organization, such as a decrease in actin stress fibers and focal adhesions, accompanied with a change in activation of RhoA and Rac1 GTPases. These results might provide insight into the role of CD9 in the functions of TGFα family proteins in epithelial and carcinoma cells, whose physiology and behavior are driven by ligand-induced EGFR signaling.

Results

CD9 enhances TGFα expression at the cell surface

To examine whether CD9 expression affects the expression and localization of TGFα, we generated Madin-Darby canine kidney epithelial (MDCK) cells that were infected with retroviral vectors to ectopically express CD9, TGFα or both. MDCK cells grow in contact-inhibited monolayer cultures and appear non-transformed. Following two rounds of retroviral infection, using vectors with different selection markers and the corresponding selections, we obtained four types of cell population. Cells either expressed CD9 and TGFα, TGFα and a control vector for the CD9 plasmid, CD9 and a TGFα control vector, or both control vectors. The expression levels for CD9 and TGFα were verified by immunoblotting and immunofluorescence.

The effect of CD9 on TGFα expression was examined by confocal microscopy in cells grown in monolayer culture. When expressed alone, CD9 was distributed all over the cell, but was highly concentrated at sites of cell contact (Fig. 1A, top panel). When TGFα was expressed alone, the protein showed a perinuclear localization, consistent with its accumulation in the Golgi complex, but had little if any localization at sites of cell contacts (Fig. 1A, middle panel). By contrast, when CD9 and TGFα were coexpressed, the TGFα protein showed dispersed staining in the cells, but colocalized with CD9 at the sites of cell contacts, whereas CD9 distribution was not changed (Fig. 1A, bottom panel). Thus our observations show that expression of CD9 together with TGFα, relocalizes TGFα from the Golgi to the cell surface and to cell-contact sites.

To further examine the effect of CD9 on the level of TGFα at the cell surface, cells were cooled to 4°C to slow endocytosis, and cell-surface immunofluorescence staining was performed. As controls, cells were fixed and permeabilized before immunofluorescence staining. The presence of CD9 in cells coexpressing CD9 and TGFα resulted in a pronounced increase in TGFα at the cell surface (Fig. 1B). To quantify this observation, cell-surface proteins of intact cells were labeled with biotin and analyzed by western blot. Cell-surface expression of TGFα was substantially increased in the three CD9-TGFα-expressing cell populations, when compared with cells expressing TGFα alone (Fig. 1C).

We have shown previously that the interaction of transmembrane TGFα with CD9 required the 50-amino-acid-long TGFα core sequence that is located in the ectodomain of transmembrane TGFα. Thus, TGFαΔE, which has this core sequence replaced with an extracellular Myc tag, did not associate with CD9 in coimmunoprecipitation experiments (Shi et al., 2000). To test the role of the TGFα-CD9 association in the cell-surface presentation of transmembrane TGFα, we coexpressed CD9 and TGFαΔE. Without CD9 co-expression, TGFαΔE had a largely intracellular localization (Fig. 1D), similarly to wild-type TGFα (Fig. 1A). In contrast to wild-type TGFα, the localization of TGFαΔE was not affected by co-expressing CD9 (Fig. 1D). Thus, whereas CD9 enhances the cell-surface presentation of wild-type TGFα, thereby resulting in co-staining of TGFα with CD9 at cell contacts (Fig. 1A), no such co-staining of CD9 with TGFαΔE was apparent (Fig. 1D).

These results show that CD9 facilitates the transport of TGFα from its predominantly intracellular location to the cell surface and/or stabilizes TGFα at the cell surface, where the two proteins colocalize; and that this effect of CD9 depends on the association of TGFα with CD9.

Processing of TGFα is affected by the concomitant expression of CD9

The increased levels of TGFα at the cell surface in the presence of CD9 suggested that CD9 might affect the maturation and processing of transmembrane TGFα. To test this hypothesis, we established stable derivatives of CHO cells that express TGFα, CD9 or both. The lack of EGFR in this cell line eliminates the possibility of endocytosis of transmembrane or soluble TGFα upon binding to the EGFR. The biosynthesis of TGFα was monitored by pulse-chase labeling, followed by SDS-PAGE analysis of the ^{35}S-labeled proteins.

TGFα appears as three bands on an SDS-gel: (1) a ~15 kDa band (form I), the precursor form with its unglycosylated prodomain at the N-terminus and primarily located in the endoplasmic reticulum; (2) a ~25 kDa diffuse band (form II) with N-glycosylation of the prodomain, which occurs primarily in the Golgi; and (3) a ~10 kDa band (form III) with the prodomain proteolytically removed. Finally, ectodomain shedding of the TGFα forms that have or have not retained their prodomain, releases soluble TGFα and generates an extracellularly truncated transmembrane protein that is no longer

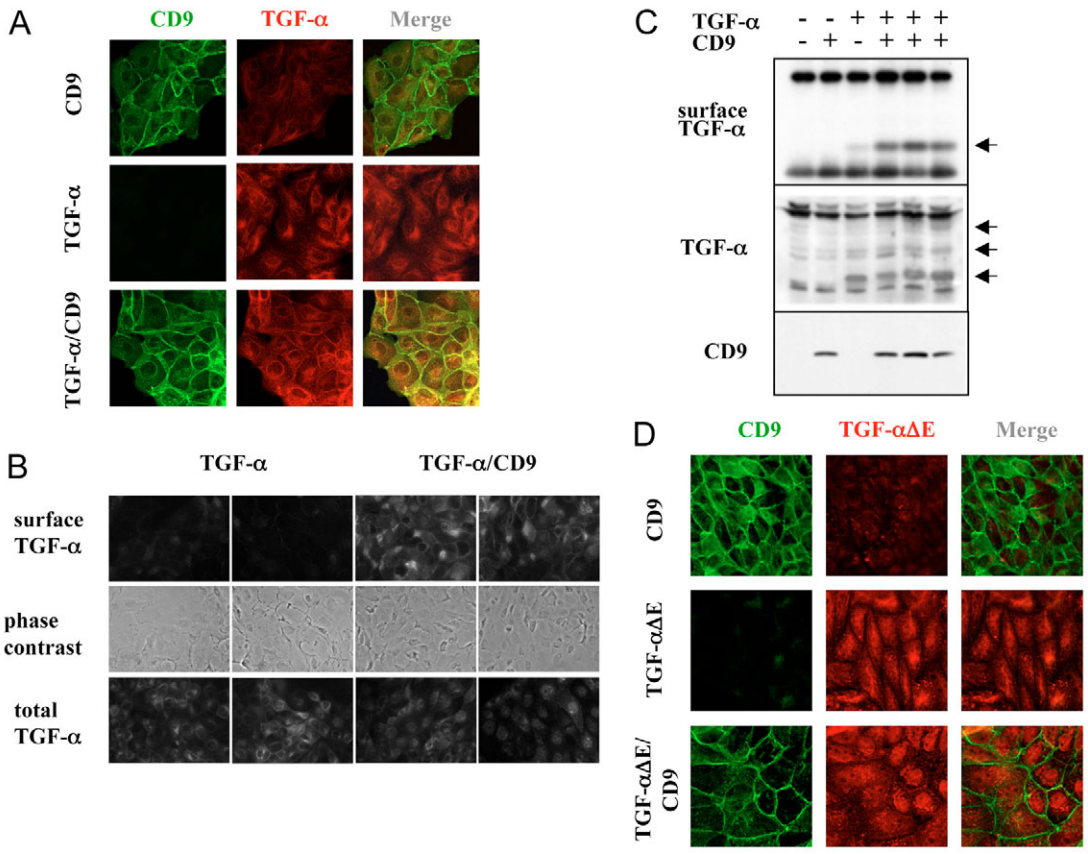

Fig. 1. CD9 alters the localization and presentation of TGFα. MDCK cells stably expressing CD9 or TGFα, or coexpressing CD9 and TGFα were analyzed. (A) Cells were fixed, permeabilized, stained and analyzed by confocal immunofluorescence microscopy (green, CD9; red, TGFα). (B) Cell-surface immunostaining of TGFα of MDCK cells that express TGFα, or CD9 and TGFα together. Top panels show cell-surface staining of non-permeabilized cells on ice, whereas the lower panels shows total immunofluorescence for TGFα of permeabilized and fixed cells. The middle panels show phase-contrast microscopy of the stained cells in the top panels. (C) The cell-surface proteins labeled by biotinylation were subjected to immunoprecipitation for TGFα, thus showing the level of cell-surface TGFα in cells expressing CD9, TGFα, or CD9 and TGFα together (top panel). Middle and lower panels show immunoblotting for the expression levels of total TGFα and CD9, respectively. Arrows in the middle panel mark the three differentially processed TGFα forms (Bringman et al., 1987; Shi et al., 2000) (see also Fig. 2A). The arrow in the upper panel marks biotinylated cell-surface TGFα, corresponding to unglycosylated transmembrane TGFα without its pre-sequence (Bringman et al., 1987); this band corresponds to the lower one in the middle panel. (D) CD9 does not alter the distribution of TGFα that lacks the TGFα core sequence. MDCK cells stably expressing CD9, TGFαΔE or both were fixed, permeabilized, stained and analyzed by confocal immunofluorescence microscopy (green, CD9; red, TGFαΔE).

recognized by anti-TGFα antibodies. As shown in Fig. 2, the pulse-chase profiles of form I were similar in the presence or absence of CD9. By contrast, forms II and III showed increased stability in the presence of CD9. Thus, CD9 does not affect the expression and biosynthesis of transmembrane TGFα per se; rather, CD9 stabilizes the glycosylated and prodomain-removed transmembrane forms of TGFα.

CD9 redistributes TGFα to the apical compartment in polarized epithelial cells

Since CD9 redistributed TGFα from a predominantly intracellular location to the cell surface in nonpolarized cells, we also evaluated whether CD9 affects TGFα membrane localization in polarized cells. In MDCK cells and other epithelial cells, TGFα and most related TGFα family growth factors as well as the EGF receptor are expressed in the basolateral compartment (Brandli et al., 1991; Dempsey and Coffey, 1994), thus causing autocrine stimulation in the basolateral, but not the apical compartment, of epithelial cells (Hobert et al., 1999). By contrast, CD9 is located on both the basolateral and apical surfaces (Fig. 3) (Yanez-Mo et al., 2001).

To investigate whether CD9 influenced the polarized transport of TGFα, we cultured the four types of MDCK-cell derivatives on Transwell filters, thus allowing the cells to acquire and display their polarized phenotype. We examined the localization of CD9 and TGFα using confocal microscopy (Fig. 3A). MDCK cells expressing TGFα showed cell-surface expression of TGFα predominantly in the basolateral compartment, as reported previously (Dempsey and Coffey, 1994). Cells expressing CD9 showed apical as well as basolateral localization of CD9. The seemingly higher levels of CD9 at the apical surface may reflect the presence of microvilli, which increase the density of the apical membranes. In cells that coexpress TGFα and CD9, TGFα was redistributed from the basolateral compartment to both the apical and basolateral compartments. By contrast, CD9 expression showed a similar distribution in the presence or absence of TGFα. These findings indicate that CD9 can direct TGFα to colocalize with it, and thus to acquire the same cell-surface distribution as CD9.

To examine whether this redistribution depended on the association of TGFα with CD9, we examined the effect of CD9 on the cell-surface distribution of TGFαΔE, which lacks the TGFα

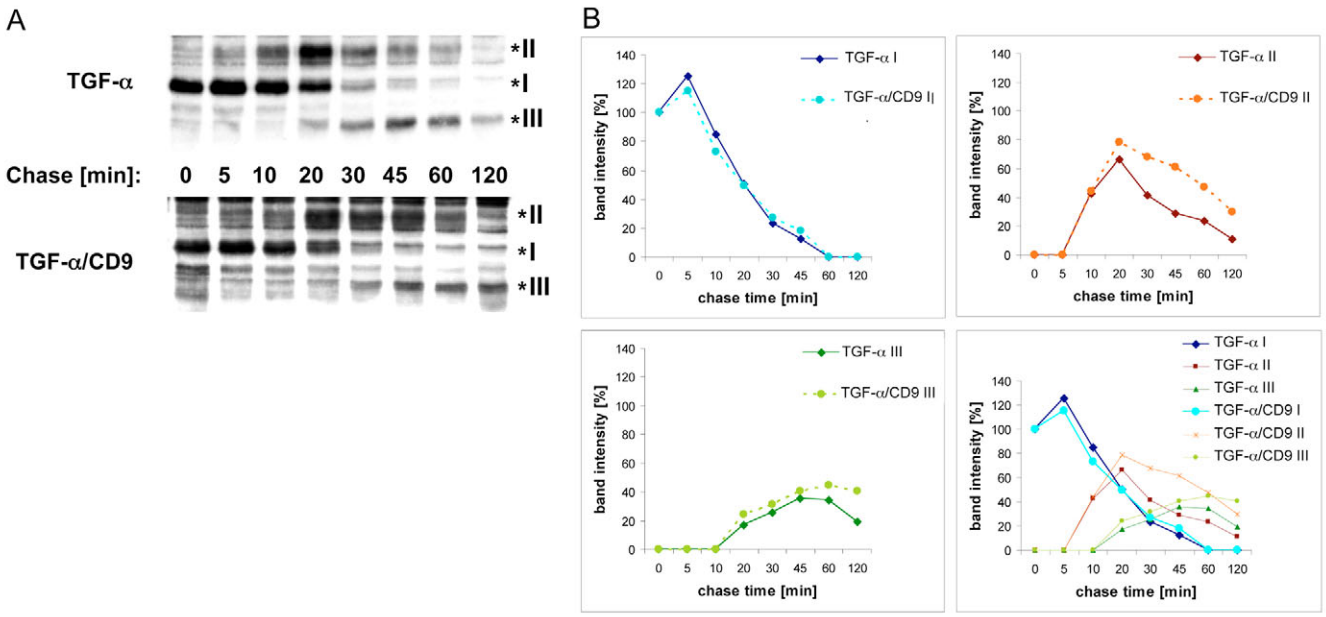

Fig. 2. The tetraspanin protein CD9 alters the stability of the processed forms of transmembrane TGFα. CHO cells expressing TGFα or coexpressing CD9 and TGFα were ^{35}S-Met–^{35}S-Cys pulse-labeled for 12 minutes, chased for times indicated, lysed and subjected to immunoprecipitation with anti-TGFα antibodies. (A) SDS-PAGE analyses of immunoprecipitated ^{35}S-labeled TGFα. (B) Quantification of the transmembrane TGFα bands as a function of the chase time.

core sequence and does not associate with CD9 (Shi et al., 2000). Without the expression of CD9, TGFαΔE showed a similar basolateral distribution in polarized cells as wild-type TGFα. This distribution of TGFαΔE was not affected by concomitant expression of CD9 (Fig. 3B). Thus, whereas CD9 confers a non-polarized redistribution to wild-type TGFα, the inability of CD9 to associate with TGFαΔE prevents such redistribution.

The redistribution of TGFα in the presence of CD9 was confirmed by cell-surface biotinylation of the proteins expressed at the basolateral versus apical cell surfaces (Fig. 3C). Consistent with the immunofluorescence results, TGFα was only detected in the basolateral compartment of MDCK cells expressing TGFα alone. By contrast, concomitant expression of CD9 resulted in the additional presence of TGFα in the apical compartment. These data also demonstrate that TGFα can reach higher levels at the cell surface in the presence of CD9, whereas CD9 levels are reproducibly increased in the presence of TGFα.

The relocalization of TGFα by CD9 might result from a general loss of cell polarity, which could be owing to an increase in TGFα-induced EGFR signaling in the presence of CD9, as we previously reported. In fact, one could imagine that the increased TGFα/EGFR signaling may lead to the loss of polarity, a first step towards the transformed phenotype, even though the cells remained contact inhibited. To test this hypothesis, we examined the distribution of the basolateral markers E-cadherin and p58, and the apical marker gp135 (O'Brien et al., 2001), in the MDCK-derived cells. E-cadherin and p58 retained their predominantly basolateral localization, whereas gp135 was found apically in the TGFα-CD9 expressing MDCK cells (Fig. 3D). We therefore conclude that CD9 specifically redistributes TGFα to a non-polarized cell-surface localization and that CD9 expression at the same time does not affect the polarized phenotype of the cells.

Finally, we also examined the effect of CD9 expression on the expression of TGFα in MDCK cells embedded in growth-factor-reduced basement-membrane extract from Engelbreth-Holm-Swarm tumor (EHS) extracellular matrix with a composition similar to that of Matrigel^{TM}. Under these conditions, single MDCK cells grow in 4-5 days to form hollow cysts that consist of polarized cells resting on a basement membrane surrounding the cyst (O'Brien et al., 2006). Confocal examination of MDCK cells that do not ectopically express TGFα or CD9 showed a very low level of diffuse immunostaining of the endogenous TGFα throughout the cells (Fig. 4). Ectopic expression of CD9 concentrated the endogenous TGFα with predominant visualization at the luminal (apical) side, where the dense microvillous organization confers a high concentration of the plasma membrane (Williams and Clark, 2003). Ectopic expression of TGFα resulted in an increased, but diffuse TGFα immunostaining throughout the cells. By contrast, coexpression of CD9 and TGFα resulted in TGFα staining at the cell surface, both at the basolateral and apical surfaces. These data thus confirm that CD9 enhances the TGFα expression at the cell surface and does so in a non-polarized manner.

Increase in EGFR internalization in cells that coexpress CD9 and TGFα

We previously showed that coexpression of CD9 results in increased EGFR activation by TGFα (Shi et al., 2000), related to and presumably as a consequence of the increased cell-surface expression of transmembrane TGFα. We therefore explored the activation of several parameters known to be regulated by EGFR signaling. One parameter that reflects the level of EGFR activation is the internalization of the EGFR, which can be assessed using confocal microscopy. In polarized cells, the ectopic expression of TGFα resulted in an increased level of granulated intracellular EGFR staining, a reflection of the level of EGFR internalization. The intracellular EGFR staining in TGFα-expressing cells was further increased when CD9 was ectopically expressed at the same time, whereas CD9 by itself had no detectable effect (Fig. 5A).

Consistent with this result, concomitant expression of CD9 enhanced the TGFα-induced EGFR phosphorylation (Fig. 5B) (Shi

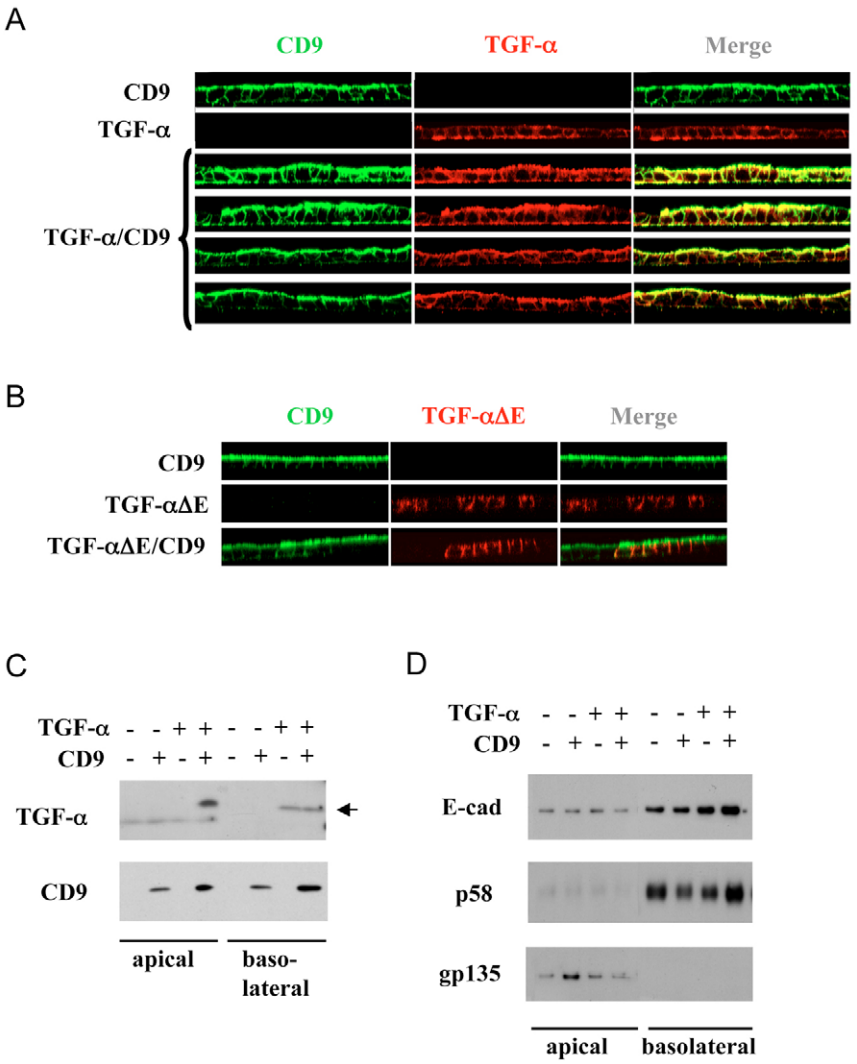

Fig. 3. Expression of CD9 redistributes TGFα to the apical site in polarized epithelial cells. MDCK cells that express CD9, TGFα or both proteins were grown in Transwell chambers to allow them to fully establish their polarized phenotype. (A) Cells were fixed and stained for CD9 or TGFα, and immunofluorescence was visualized by confocal microscopy (*xz* section). (B) Expression of CD9 does not alter the distribution of transmembrane TGFα that lacks the TGFα core sequence in polarized epithelial cells. MDCK cells stably expressing CD9, TGFαΔE or both were grown in Transwell chambers to establish a polarized phenotype. Cells were fixed, stained for CD9 or TGFαΔE, and immunofluorescence was visualized by confocal microscopy (*xz* section). (C) Cells were subjected to biotinylation of cell-surface proteins in the apical or basolateral compartments, and cell-surface TGFα and CD9 were visualized by immunoblotting. Arrow indicates the position of TGFα form III. (D) Same experiment as in C, but visualizing the biotinylated E-cadherin or p58, two proteins that are largely expressed in the basolateral compartment, and the apical marker gp135.

et al., 2000). Ligand-induced activation of the EGFR leads to several downstream events, including activation of MAP kinase signaling pathways. Whereas EGFR activation is known to activate Erk/MAP kinase, p38 MAP kinase and Jnk signaling, concomitant expression of CD9 did not enhance the TGFα-induced activation levels of these three types of MAP kinases compared with cells expressing TGFα alone (data not shown).

Effect of CD9 on the cytoskeletal organization

The MDCK cells containing control vectors or ectopically expressing CD9, TGFα or both all maintained their epithelial characteristics, as apparent from the expression and subcellular localization of the cell-junction proteins ZO-1, desmoglein and E-cadherin (data not shown). However, as there were some slight differences in morphology, we stained the cells for filamentous actin. Control MDCK cells and MDCK cells expressing TGFα or CD9 showed a similar organization and abundance of well-defined F-actin stress fibers (Fig. 6A). By contrast, cells expressing TGFα and CD9 showed a change in F-actin stress fiber pattern. F-actin was primarily localized at the periphery towards the surfaces of cell contacts. Since actin is linked to integrins and the extracellular matrix via focal adhesions, we examined whether this drastic change correlated with the distribution of focal adhesions. The control MDCK cells and those ectopically expressing TGFα or CD9 had overall similar patterns of the focal adhesion proteins paxillin (Fig. 6A) and vinculin (data not shown). By contrast, the cells expressing CD9 and TGFα showed altered distribution of paxillin and vinculin that resulted in a strong reduction in the number of focal adhesions (Fig. 6A; data not shown). Treatment of MDCK cells with soluble TGFα or EGF at saturating levels (5 ng/ml) for short and long periods, did not result in the actin organization and focal adhesion distribution observed in MDCK cells that expressed CD9 and TGFα. However, exposure of the cells to 100 ng/ml TGFα for 24 hours resulted in a reduction of actin and paxillin staining that appeared intermediate between the phenotype of cells that expressed CD9 and TGFα, and those that only expressed TGFα (data not shown).

Coexpression of CD9 and TGFα results in alterations in the GTPase activity of Rho family members

Actin stress fibers and focal adhesions are regulated by Rho-family GTPases. Among these, RhoA, Rac1 and Cdc42 regulate cell shape through their effects on cytoskeletal organization. RhoA is a key regulator of the formation of actin stress fibers and focal adhesions, whereas Rac1 is associated with the assembly of actin filaments during lamellipodia formation and is generally activated in a reciprocal fashion to RhoA (Burridge and Doughman, 2006). Cdc42 has an important role in the cell polarization (Etienne-Manneville, 2004). We thus explored whether these small GTPases are involved in the changes in actin stress fibers and focal adhesion distribution in the cells expressing CD9 and TGFα. We quantified the levels of activated, GTP-bound forms of RhoA, Rac1 and Cdc42 (Fig. 6B). Control cells and cells expressing CD9 or TGFα all had similar levels of active RhoA, Rac1 and Cdc42. However, in cells that coexpress CD9 and TGFα, the level of GTP-bound RhoA was decreased, whereas the GTP-bound Rac1 level was slightly increased. The active form of Cdc42 showed no significant difference between the cell lines.

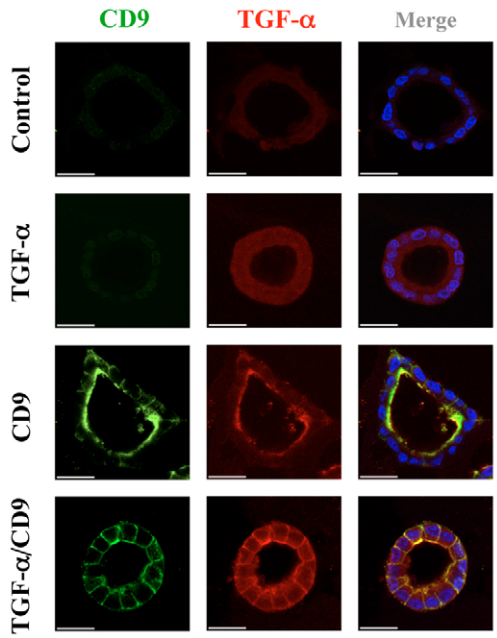

Fig. 4. Effect of CD9 on TGFα cell-surface expression in 3D cell cultures. MDCK cells expressing CD9, TGFα or both proteins were embedded in Engelbreth-Holm-Swarm (EHS) tumor basement membrane extract and grown until cysts had formed. The cells were fixed and stained for CD9 or TGFα, and immunofluorescence images were captured using confocal microscopy. Scale bars: 20 μm.

Changes in cytoskeletal organization and GTPase activity are inhibited by blocking EGFR signaling

Since coexpression of CD9 and TGFα strongly enhances EGFR activation, we evaluated whether the changes in the distribution of actin stress fibers and focal adhesion were related to the EGFR activation. Addition of an antibody that prevents ligand binding to the EGFR ectodomain, reversed the changes in actin stress fiber organization to a phenotype that is similar to that seen in the control MDCK cells (Fig. 6A, right column). Likewise, treatment of the cells that expressed TGFα and CD9 with the anti-EGFR antibody resulted in a change of the distribution of focal adhesion sites back to that observed in control cells or cells expressing CD9 or TGFα only, as visualized by immunofluorescence for paxillin (Fig. 6A, right column) or vinculin (data not shown). In addition, blocking EGFR activation using AG1478 rescued the change in activity of RhoA and Rac1 to the level that was observed in control MDCK cells or cells expressing CD9 or TGFα only (Fig. 6C). Finally, the role of EGFR activation in the phenotypic changes conferred by CD9 and TGFα coexpression was also evaluated in MDCK cells coexpressing CD9 and TGFαΔE, the TGFα derivative that lacks the TGFα core sequence required for EGFR activation. As shown in Fig. 6D, cells coexpressing CD9 and TGFαΔE showed multiple focal adhesions and an actin stress fiber organization that was unlike the changes seen in cells coexpressing CD9 and TGFα, but similar to the pattern obtained when these cells were incubated in the presence of the EGFR antibody (Fig. 6A).

Expression of CD9 in the presence of TGFα results in changes in cell migration and adhesion

Since coexpression of CD9 and TGFα led to a dramatic reduction in actin stress fibers accompanied with a change in the activation state of RhoA and Rac1, we investigated the migration and adhesion properties of the stable cells expressing CD9, TGFα or both. Cells

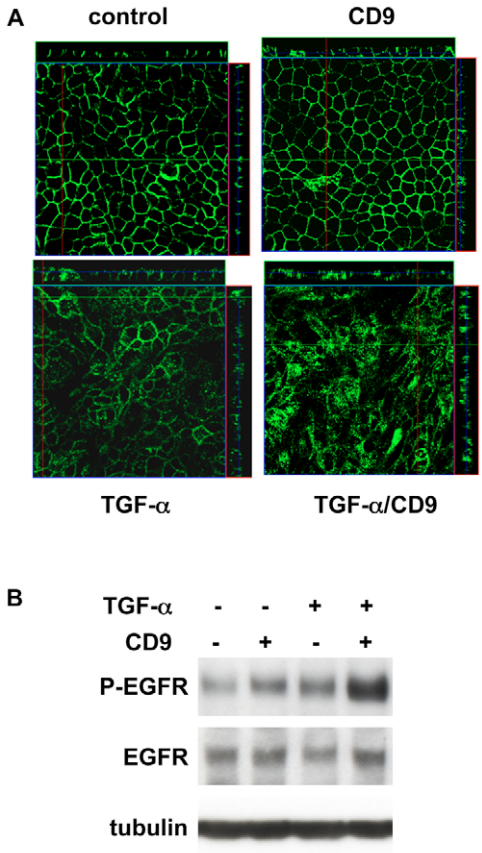

Fig. 5. (A) Increase in EGFR internalization in MDCK cells expressing CD9, TGFα or CD9 and TGFα. Cells were cultured in Transwell chambers, thus allowing full polarization, fixed and immunostained for EGFR. Immunofluorescence was visualized using confocal microscopy (*xy* section). (B) Cells were grown in regular culture medium, lysed, immunoprecipitated with anti-EGFR antibody and immunoblotted with antibodies against EGFR phosphorylated at tyrosine, EGFR or tubulin to detect phosphorylated EGFR (P-EGFR), total EGFR or tubulin.

were examined in a modified Boyden chamber assay in which they migrated through a Transwell filter from 0% to 10% serum. Coexpression of CD9 and TGFα strongly decreased cell migration, whereas cells expressing only CD9 showed a much smaller decrease of migration, and expression of TGFα alone did not affect the migration of the cells (Fig. 7A). Thus, the expression of the tetraspanin CD9 in cells that express TGFα drastically affects the activation of small GTPases, the organization of actin stress fibers, focal adhesions and cell migration.

To investigate cell adhesion, we seeded the cells onto cell culture dishes that had been coated with fibronectin, removed the non-adherent cells after 20 or 40 minutes, and quantified the adherent cells. At 20 minutes after cell seeding, there was only a slight difference in the adhesion between the cell lines. However, 40 minutes after seeding, there was a significant difference in cell adhesion with an increase in adhesion within cells that expressed both CD9 and TGFα (Fig. 7B). This result is consistent with the decrease in cell migration of these cells in the Boyden chamber assays.

Discussion

The TGFα family of growth factors, such as TGFα, HB-EGF and amphiregulin, comprises transmembrane proteins that undergo a regulated cleavage in order to release soluble growth factor. The

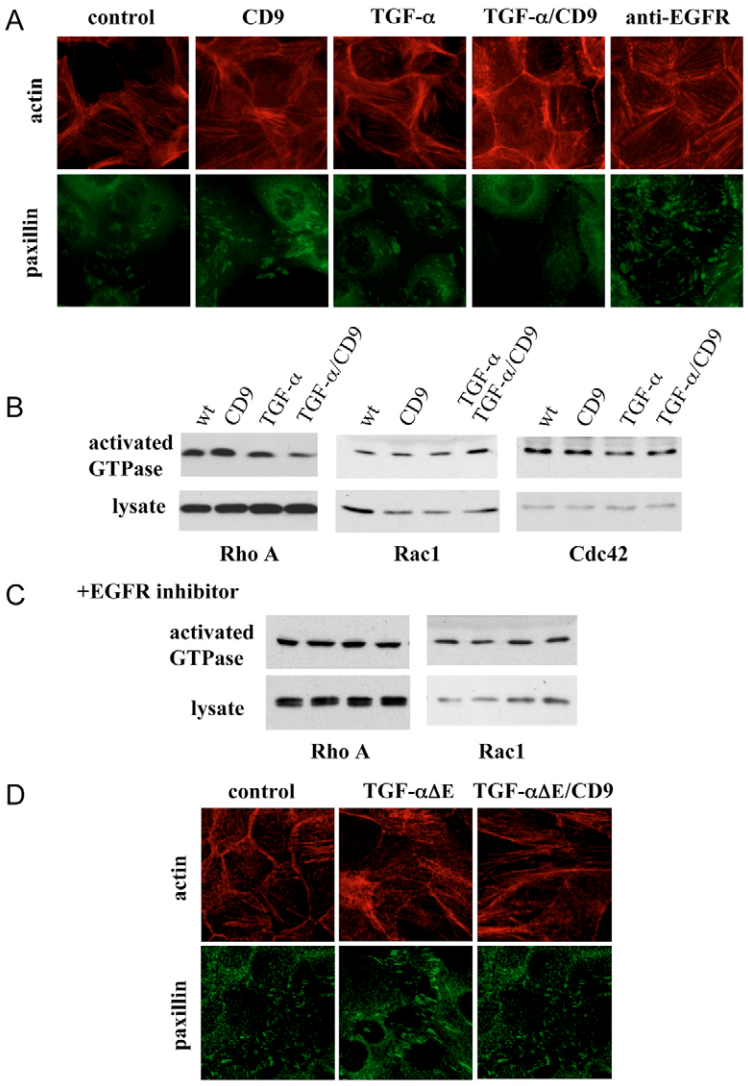

Fig. 6. Coexpression of CD9 and TGFα confers changes in actin stress fibers and activities of Rho family of small GTPases that can be rescued by blocking the EGFR activation. (A) MDCK cells expressing CD9, TGFα or CD9 and TGFα were stained with Alexa-Fluor-594–phalloidin (red) or by immunofluorescence for paxillin (green), and analyzed by confocal microscopy. The last two images (top and bottom) show MDCK cells expressing CD9 and TGFα in the presence of a blocking EGFR antibody. (B) The same cells as shown in A were evaluated for the level of activated, GTP-bound forms of the small GTPases RhoA, Cdc42 or Rac1. (C) RhoA and Rac1 GTPases activities were analyzed in the presence of the EGFR inhibitor AG1478. (D) CD9 expression does not change the distribution of actin stress fibers or focal adhesions when expressed with transmembrane TGFα that lacks the TGFα core domain. MDCK cells that express TGFαΔE, or TGFαΔE and CD9 together were stained using phalloidin (red) or paxillin (green) and analyzed by confocal immunofluorescence microscopy.

transmembrane and soluble growth factors both activate the EGFR, but their modes of signaling differ. The transmembrane forms stimulate EGFRs on adjacent cells or on the growth-factor-producing cells themselves, whereas release of soluble growth factor allows EGFR activation of cells at a distance (Brachmann et al., 1989; Ebner and Derynck, 1991; Wong et al., 1989). Furthermore, EGFR activation by soluble TGFα probably differs from activation by transmembrane TGFα because of differences in internalization of the ligand-bound EGFR. Defects in TGFα ectodomain cleavage have been shown to confer increased EGFR signaling by transmembrane TGFα as well as slower EGFR internalization when compared with soluble ligand (Yang et al., 2000). These results suggest that defective TGFα processing confers increased EGFR signaling, compared with cells showing TGFα ectodomain shedding. In addition, membrane-bound HB-EGF has been shown to be less effective in activating the EGFR but to prolong the EGFR half-life (Miyoshi et al., 1997). Thus, the presentation of a growth factor of the TGFα family as a transmembrane versus soluble polypeptide confers differences in EGFR activation and cell responses.

CD9 enhances TGFα presentation at the cell surface and redistributes TGFα in polarized cells

We provide evidence that CD9, which interacts with transmembrane TGFα (Shi et al., 2000), plays an important role in the presentation of TGFα. CD9 stabilized the transmembrane forms of TGFα in the Golgi and at the cell surface, but not the immature TGFα precursor, which is located in the endoplasmic reticulum. These effects are consistent with the predominant localization of CD9 at the cell surface and may be explained in part by the decreased TGFα ectodomain shedding in the presence of CD9 (Shi et al., 2000). The increased stability of transmembrane TGFα may explain the increase of cell-surface TGFα level in the presence of CD9 (Fig. 1).

Our data also indicate that CD9 defines the distribution of TGFα in polarized cells. In polarized cells, CD9 is expressed in a non-polarized manner at the cell surface, and TGFα is expressed at basolateral surfaces where the EGFR is predominantly localized (Brandli et al., 1991). In the presence of CD9, however, TGFα is distributed at both the apical and basolateral compartments. Since the apical membranes organize themselves as microvilli with a high density of membranes, and tumor necrosis factor α(TNFα)-converting enzyme (TACE) cleaves TGFα at the basolateral membrane (Dempsey and Coffey, 1994), TGFα is predominantly expressed at the apical surface when CD9 is present. This non-polarized redistribution of TGFα by CD9 is not owing to depolarization of the epithelial cells, but reflects an important role of CD9 in the stabilization of TGFα at the cell surface, as well as its cell-surface distribution (Fig. 3).

The effects of CD9 on the cell-surface presentation of TGFα were also apparent in three-dimensional (3D) cultures, in which MDCK cells form spherical structures with an internal lumen that abuts the apical cell surfaces. In such cultures, endogenous TGFα showed a diffuse cellular staining, but concomitant expression of CD9 conferred a striking presence of TGFα at the cell surface, primarily at the apical, luminal membrane with its microvillous organization. The effect of CD9 in stabilizing TGFα and providing a non-polarized distribution of TGFα at the plasma membrane was also apparent in cells that ectopically coexpress both CD9 and TGFα (Fig. 4).

The enhanced cell-surface presentation of TGFα and its redistribution in polarized cells in the presence of CD9 depend on the six-cysteine core sequence of transmembrane TGFα. Since this sequence is required for the interaction of transmembrane TGFα with CD9 (Shi et al., 2000), we conclude that these effects of CD9 on TGFα presentation depend on their physical association. Since CD9

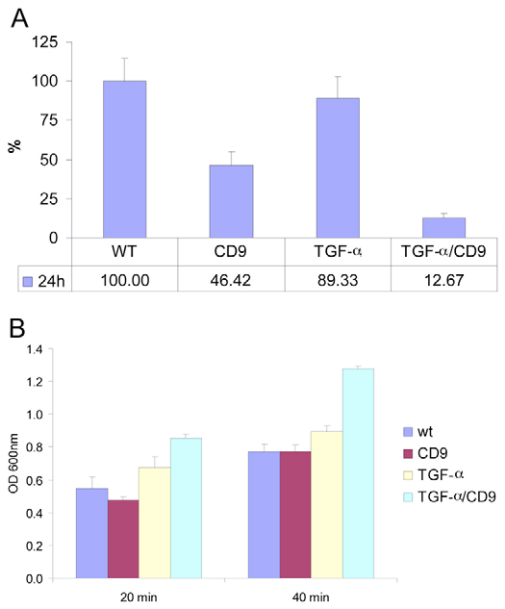

Fig. 7. Coexpression of CD9 and TGFα alters cell migration and adhesion. (A) Migration of MDCK cells expressing CD9, TGFα or both was analyzed in a Boyden chamber assay. Cell migration towards medium with 10% serum was quantified after 24 hours by counting those cells that had migrated through the membrane. Numbers of migrating cells were plotted in percent; 100% migration was defined as the number of migrated wild-type cells. (B) Cell adhesion was examined by Crystal Violet staining of the adherent cells after 20 or 40 minutes after seeding onto fibronectin-coated wells.

also interacts with the TGFα-related HB-EGF and amphiregulin (Inui et al., 1997), we surmise that CD9 also enhances their presentation at the cell surface. Since this core sequence is required for the interaction of CD9 with transmembrane TGFα and because it defines proteins of the TGFα family, CD9 might increase the cell-surface presentation and stability of all TGFα family proteins. The increased availability of TGFα or TGFα-related proteins at the cell surface is expected to greatly enhance juxtacrine activation of the EGFR – as previously shown (Shi et al., 2000).

In polarized cells, TGFα and amphiregulin are normally localized basolaterally, where also the EGFR is predominantly localized, resulting in extensive ligand-induced internalization. Low EGFR levels are also found at the apical surface (Hobert et al., 1999). The redistribution of TGFα at the surface of polarized cells in the presence of CD9 might affect where in the cell EGFR populations are activated. CD9 expression in TGFα-expressing cells enhanced EGFR activation and internalization in polarized cells (Fig. 5), implying that the apical ligand is capable of activating the EGFR. It will be interesting to evaluate whether apical versus basolateral activation of the EGFR results in differences in routing of ligand-receptor complexes and functional consequences.

The roles of tetraspanins in protein transport and maturation have only been studied in a few cases. The tetraspanin CD81 facilitates the processing and transport of the glycoprotein CD19, starting with its requirement for exit from the endoplasmic reticulum to the processing and glycosylation of CD19 (Shoham et al., 2006). The tetraspanins uroplakin Ia and uroplakin Ib allow transit of the non-tetraspanin uroplakins UPII and UPIII from the Golgi to the cell surface to form urothelial plaques (Tu et al., 2006). We now provide evidence for a role of CD9 in processing and cell-surface presentation of TGFα, suggesting similar effects on all TGFα-family proteins.

Little is known about how the presentation of TGFα-family proteins is controlled. Cornichon, which transverses the membrane three times, regulates transport of TGFα family proteins in *Drosophila* and mammalian cells (Bokel et al., 2006; Perez-Castro et al., 2007), whereas Rhomboid proteins regulate cleavage of the TGFα-related Spitz and Gurken in *Drosophila* (Urban, 2006). In addition, the cytoplasmic PDZ-domain proteins GRASP55 and syntenin interact with the C-terminal sequence of transmembrane TGFα and control the transport of TGFα to the cell surface (Fernandez-Larrea et al., 1999; Kuo et al., 2000). We now provide evidence that the tetraspanin CD9 regulates both the maturation and stability of TGFα at the cell surface.

Coexpression of CD9 and TGFα alters the epithelial phenotype and the cell behavior

Epithelial cells that coexpress CD9 and TGFα showed distinct phenotypic changes compared with wild-type cells or cells that ectopically expressed CD9 or TGFα. All cells had an epithelial phenotype, independently of whether they expressed TGFα, CD9 or both, as assessed by morphology in monolayer culture and polarized phenotype, and expressed epithelial junction markers such as ZO-1, E-cadherin and desmoglein (data not shown). However, cells that expressed TGFα and CD9 had a striking cortical organization of actin stress fibers, and a much lower number of focal adhesions, as assessed by immunofluorescence for paxillin or vinculin. This phenotype was not apparent in cells expressing only CD9 or TGFα, and was not mimicked by adding soluble TGFα at saturating concentrations (Fig. 6; data not shown). However, when cells that ectopically express TGFα were treated with the metalloprotease inhibitors TAPI-1 or GM6001 (which block TACE-mediated TGFα ectodomain shedding), we observed changes similar to those seen in cells that coexpress CD9 and TGFα (data not shown). These data indicate that coexpression of CD9 and TGFα triggers responses that are not exerted by either protein alone, and that responses to transmembrane TGFα differ from the responses to soluble ligand.

The changes in actin-stress-fiber organization and the decreased number in focal adhesions were accompanied by a decrease in RhoA activation and an increase in Rac1 activation. RhoA regulates the formation of actin stress fibers and focal adhesions, whereas Rac1 activation correlates with actin filament assembly during lamellipodia formation and is generally reciprocal to RhoA activation (Burridge and Doughman, 2006). Interfering with RhoA function perturbs actin stress fibers and focal adhesions, whereas overexpression of RhoA induces the assembly of actin stress fibers (Aspenstrom et al., 2004; Evers et al., 2000). RhoA can also be activated by GPCR ligands, which results in cytoskeletal reorganization (Brown et al., 2006). Considering the functions of RhoA and Rac1, the changes in actin organization and focal adhesion in TGFα-CD9-expressing cells are likely to result from the altered activation of these small GTPases.

The changes in actin organization and in the number of focal adhesions of TGFα-CD9-expressing MDCK cells required EGFR activation. Indeed, these changes were reverted to the organization seen in wild-type cells by a neutralizing anti-EGFR antibody and were not seen in cells coexpressing CD9 and TGFαΔE, which lacks the TGFα core sequence that activates the EGFR. Furthermore, blocking the EGFR activation also inhibited the decreased RhoA and increased Rac1 activity. These results directly link the changes in phenotype and small GTPase activation to EGFR activation, and functionally link the RhoA and Rac1 activation states with the changes in actin organization and focal adhesions. On the basis of

these findings, the observation that inhibition of TGFα shedding in TGFα-expressing MDCK cells confers similar changes in actin and focal adhesion compared with those in CD9-TGFα-expressing cells, and our previous report (Shi et al., 2000), we conclude that coexpression of CD9 and TGFα results in EGFR hyperactivation by transmembrane TGFα to a level that can not be mimicked by soluble ligand, and results in changes in small GTPase activity with consequent phenotypic changes.

Since small GTPases, focal adhesions and actin organization have important roles in cell adhesion and migration, we evaluated the adhesion and migration behavior of TGFα-CD9-expressing cells. These cells showed a decreased cell migration and increased cell adhesion in comparison with MDCK cells that expressed either TGFα or CD9 alone, or with wild-type cells. TGFα signaling through the EGFR is known to have an important role in carcinoma development and progression and, accordingly, the expression of the EGFR or TGFα, or both, is often upregulated in carcinomas (Holbro et al., 2003; Kenny and Bissell, 2007; Lee et al., 1995). However, increased CD9 levels in carcinomas have been correlated with decreased metastatic potential (Boucheix et al., 2001). Our observation of increased adhesion and decreased migration observed in cells that coexpress TGFα and CD9 is consistent with these studies.

In summary, we have shown that CD9 profoundly affects the maturation, cell-surface presentation and cell-surface distribution of transmembrane TGFα. Expression of CD9 results in phenotypic and behavioral changes in epithelial cells that express TGFα, which are mediated by EGFR activation to an extent that can not be mimicked by soluble ligand. Since increased TGFα signaling through the EGFR is a major force in carcinoma progression and because CD9 expression correlates with decreased metastasis, our results may be of relevance for the role of CD9 in carcinoma progression and behavior. Furthermore, because CD9 also interacts with other proteins of the TGFα family, our results might reflect roles of CD9 in the biology of this growth factor family.

Materials and Methods

Antibodies

Mouse monoclonal anti-CD9, anti-E-cadherin, anti-Rac1 and anti-Cdc42 antibodies were from BD Biosciences. Mouse anti-TGFα Ab-1 for western blotting was from Oncogene Research, mouse monoclonal anti-TGFα α1 antibody for surface staining has been described before (Bringman et al., 1987), goat anti-human TGFα antibody for immunoprecipitation was from R&D Systems, and sheep anti-human TGFα antibody for immunofluorescence was a gift from R. J. Coffey (Vanderbilt University, Nashville, TN). Mouse anti-p58 antibody was a gift from Karl Matlin and mouse anti-gp135 antibody was a gift from George Ojakian, rabbit anti-EGFR (1005) and rabbit anti-RhoA antibodies were from Santa Cruz Biotechnology, Inc. Mouse anti-paxillin antibody was from Zymed. Rabbit anti-Myc epitope antibody to detect TGFαΔE was from Sigma.

Construction of expression plasmids

To express CD9 or TGFα, we subcloned cDNAs encoding human CD9 or TGFα into retroviral expression plasmids. The sequence of CD9 8 bp upstream to 58 bp downstream of the coding sequence was amplified by PCR from pRK7-CD9-hygro (Shi et al., 2000) with primers 5′-GTCCAAGAGTTAACCCTCACCATGCCGGTC-AAAGG-3′ and 3′-GTCCATGAGTTAACAAGCTGCAATAAACAAGTTGGGC-5′, thereby introducing *Hpa*I sites, and subcloned into the *Hpa*I site of pLHCX (Clontech). For TGFα, the sequence between –6 bp and +28 bp relative to the coding sequence was amplified by PCR from pRK7-TGFα (Shum et al., 1994) with primers 5′-GTGCTAGAGTTAACGTAAAATGGTCCCCTCGGCTGG-3′ and 3′-CGCTA-TCTGTTAACACCTGGCCAAACTCCTCCTCTG-5′ to introduce *Hpa*I sites, and subcloned into the *Hpa*I site of pLPCX (Choy et al., 2000). The TGFαΔE sequence, which encodes full size TGFα, in which the 50-amino acid TGFα core sequence has been replaced by a Myc epitope tag (Shi et al., 2000), was transferred as an *EcoR*I-*Hind*III fragment from pRK7-TGFαΔE into pLPCX.

Cell culture

MDCK cells were obtained from K. Mostov (University of California, San Francisco, CA) and cultured in minimal essential medium (MEM) containing Earle's balanced salt solution supplemented with 5% fetal bovine serum and antibiotics in an atmosphere of 5% CO_2, 95% air. CHO cells were cultured as described previously (Shi et al., 2000).

Generation of stable cell lines

MDCK cells were infected with the pLPCX-derived vector encoding TGFα, TGFαΔE or control vector, and/or a pLHCX based vector encoding CD9 or control vector. Cells were selected in 0.5 μg/ml hygromycin (pLHCX infections) or 2 μg/ml puromycin (pLPCX infections) for 1-2 weeks. Expression levels of CD9, TGFα and TGFαΔE were verified using western blotting. Cells were maintained in the presence of hygromycin and puromycin.

Metabolic labeling, cell lysis, immunoprecipitation, immunoblot analysis and cel- surface biotinylation

Metabolic labeling and immunoprecipitation were performed as described before (Lee et al., 2003) with the following modifications. Stable CHO cells that expressed TGFα, CD9, or TGFα and CD9 (Shi et al., 2000), were labeled for 12 minutes in Ham's F12 medium without cysteine and methionine, washed and incubated in Ham's F12 medium with 10% fetal bovine serum. Cells were washed five times in PBS and lysed in lysis buffer (137 mM NaCl, 1% Triton X-100, 20 mM Tris, pH 8.0, 2 mM EDTA), supplemented with complete protease inhibitor cocktail (Roche). TGFα immunoprecipitation and western blotting was visualized by Phosphoimager. Immunoprecipitation and immunoblot analyses were carried out as described before (Shi et al., 2000). Cell-surface biotinylation was performed as described (Kuo et al., 2000; Lipschutz et al., 2001). Cell-surface proteins were pulled down by Neutravidin agarose (Pierce), run on SDS-PAGE, and analyzed by western blotting.

Culture of MDCK cells on Transwell filters and biotinylation of cell-surface proteins

MDCK cells were cultured to acquire their polarized phenotype, and apical and basolateral cell-surface protein were biotinylated as described before (Lipschutz et al., 2001) with minor modifications. Briefly, MDCK cells were grown on Transwell filters for 4-7 days, two wells for each cell type and washed three times with ice cold PBS. Sulfo-NHS-biotin was added either apically or basolaterally to the cells, which were then incubated for 30 minutes at 4°C on ice on a rocking table. Cells were washed five times with 10 mM ice-cold TBS. Transwell filter were removed, proteins solubilized with lysis buffer, and apical or basolateral proteins analyzed as described (Lipschutz et al., 2001).

Immunofluorescence and confocal microscopy

Cells were grown on coverslips and, in case of EGFR inhibition, incubated for 4-16 hours with 5 μg/ml anti-EGFR antibody ab-1 (Calbiochem), Immunofluorescence staining was performed as described (Shi et al., 2000) with the following modifications. Cells were fixed with 4% paraformaldehyde (PFA) for 10 minutes, secondary antibodies and phalloidin were from the Alexa Fluor series (Molecular Probes). Cells were mounted with Cytoseal 60 (Richard-Allen Scientific) or Prolong Gold antifade reagent (Molecular Probes). For cell-surface staining, cells grown on coverslips were cooled on ice for 15 minutes, and incubated with anti-TGFα antibody on ice for 30-60 minutes. Cells were washed with ice-cold PBS and fixed for 20 minutes with 4% PFA on ice. After blocking, cells were incubated with secondary antibody. For polarized cell immunofluorescence, cells were incubated and stained as described before (Lipschutz et al., 2001), and immunofluorescence was examined using a Zeiss LSM510 confocal microscope.

3D-cell cultures

3D-cultures were prepared by trypsinizing confluent MDCK cells and then embedding single cells (1.6×10^5 cells/ml) in growth-factor-reduced Engelbreth-Holm-Swarm tumor extract (Cultrex™, Trevigen Inc., Gaithersburg, MD) (Weaver et al., 1997). Cultures were fed every 2 days. Immunostaining was performed as described by (Weaver et al., 1997). Briefly, the cultures were fixed with 2% PFA, embedded in sucrose and frozen in Tissue-Tek O.C.T. (Sakura Finetek, Torrance CA). Immunostaining was performed on sequential 20-μm sections using primary antibodies and Alexa-Fluor-conjugated secondary antibodies. Nuclei were visualized with diaminophenylindole (DAPI, Sigma). Confocal imaging was performed on a Zeiss LSM510 laser scanning confocal microscopy system with LSM 510 software.

Small GTPase activation assay

MDCK cells were grown to 70-80% confluency, starved for 4 hours before adding 20% serum or EGFR inhibitor AG1478 at 1 μM/ml or 5 μg/ml anti-EGFR antibody Ab-1 for 2 hours. Cdc42 and Rac1 GTPase activation were detected as described before (Benard and Bokoch, 2002). RhoA activation was performed following the manufacturer's protocol (Cytoskeleton, Inc.).

Cell adhesion and migration

Cell adhesion assays were performed using 12-well culture dishes that had been coated with 10 μg/ml fibronectin. Cells were starved for 24 hours, trypsinized, washed with PBS and resuspended in serum-free medium. 50,000 cells were added per well, and non-adherent cells were removed after the times indicated. The plates were gently rinsed with PBS and fixed with 4% paraformaldehyde. For quantification cells were

stained with 0.1% Crystal Violet in PBS for 5 minutes, washed with water and dried. 10% acetic acid was added and the absorption of the solution was measured at 595 nm. Each experiment was performed at least three times in triplicates.

Cell migration assays were carried out as described before (Leavesley et al., 1992; Sieg et al., 1998). Serum-starved cells were added to the upper chamber (100,000 cells in 100 μl) in serum-free medium, whereas the lower chamber contained medium with 10% fetal bovine serum. Migration was measured after 24 hours by staining the cell nuclei with DAPI.

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