Tumor progression: small GTPases and loss of cell–cell adhesion

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Summary

Tumor progression involves the transition from normal to malignant cells, through a series of cumulative alterations. During this process, invasive and migratory properties are acquired, enabling cells to metastasize (reach and grow in tissues far from their origin). Numerous cellular changes take place during epithelial malignancy, and disruption of E-cadherin based cell-cell adhesion is a major event. The small Rho GTPases (Rho, Rac and Cdc42) have been implicated in multiple steps during cellular transformation, including alterations on the adhesion status of the tumor cells. This review focuses on recent in vivo evidence that implicates RhoGTPases in epithelial tumor progression. In addition, we discuss different hypotheses to explain disruption of cadherin-mediated cell–cell adhesion, directly or indirectly, through activation of Rho GTPases. Understanding the molecular mechanism of how cadherin adhesion and RhoGTPases interplay in normal cells and how this balance is altered during cellular transformation will provide clues as to how to interfere with tumor progression. ***BioEssays*** **25:452–463, 2003.**

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Funding agencies: We thank the Medical Research Council and Cancer Research UK for generous support. E.L. is supported by a postdoctoral fellowship from the Ministerio de Ciencia y Tecnología, Spain. VMMB is a MRC Senior Research Fellow.
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DOI 10.1002/bies.10262
Published online in Wiley InterScience (www.interscience.wiley.com).

Abbreviations: GAP, GTPase-activating protein; GEF, guanine nucleotide exchange factor; GRAF gene, GTPase regulator associated with the focal adhesion kinase pp125^{FAK}; DLC-1, deleted in liver cancer; MMPs, matrix metalloproteinases; TIMP, tissue-specific inhibitor of matrix metalloproteinases; HGF/SF, hepatocyte growth factor or scatter factor; TGF-β, transforming growth factor β; COX-2, cyclooxygenase 2; EGF, epidermal growth factor; ROS, reactive oxygen species.

Introduction

Tumor progression is a multistage process with each stage being highly complex (Fig. 1). Tumor cells are genetically unstable, and accumulation of mutations (both germ line and somatic) is one of the first steps during transformation (Fig. 1a). Among others, these mutations include genes encoding cytoplasmic signaling molecules that modulate cell growth, cell adhesion and gene expression. By interfering with the function of these proteins together with environmental stimuli, a cascade of major changes in regulatory signaling pathways occurs. Thus, tumor cells are characterized by their ability to subvert the controls on normal cellular processes such as proliferation, senescence, apoptosis, differentiation and migration.

Epithelial tumors account for the vast majority of human cancers. Fig. 1c lists the progression of epithelial tumors. Differentiated epithelial cells are polarized: they show tight cell-cell adhesion, cuboidal morphology and distinct membrane domains (apical and basolateral), with different protein and lipid compositions. During tumor progression, loss of epithelial characteristics results in dedifferentiation and correlates with poor prognosis. The dedifferentiation process involves not only morphological alterations (cell–cell adhesion and cytoskeletal networks), but also changes in attachment to substratum, motility rate and gene expression profile (i.e. epithelia-specific genes are switched off). Thus, dedifferentiation is an important step in epithelial tumorigenesis, as it marks the transition between benign and malignant tumors (Fig. 1).

The reader is referred to interesting recent reviews on the various stages of tumor progression.[1,2] This review will focus on the steps of de-differentiation, invasion and metastasis in epithelial tumors, in particular on the contribution of disruption of cell-cell contacts and inappropriate activation of the Rho family of small GTPases to these processes. In vivo and in vitro evidence will be discussed, together with possible mechanisms for altering cell–cell adhesion and Rho protein activity.

Cadherins and catenins

Epithelial morphological and functional differentiation is maintained by specialized adhesive structures, such as tight junctions, desmosomes and adherens junctions. Tight junctions are membrane complexes that act as a primary barrier to the

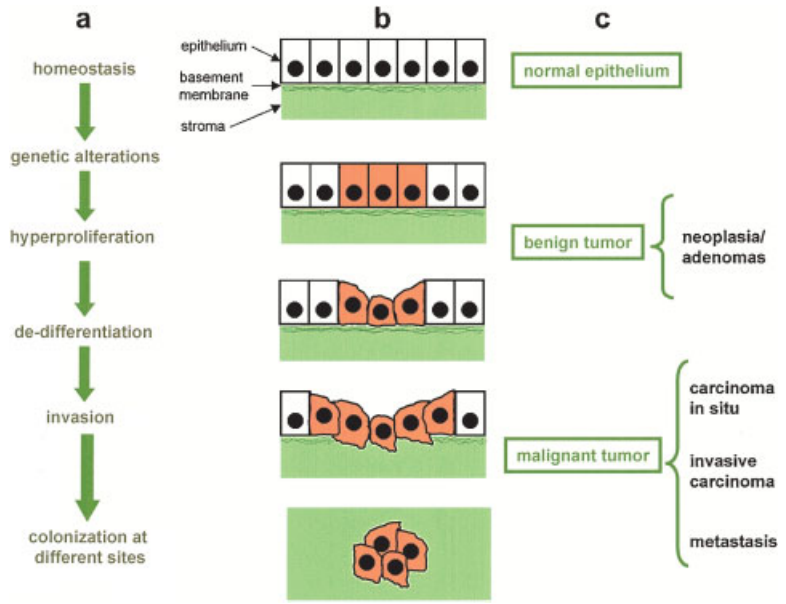

Figure 1. Progression from normal epithelia to benign and malignant tumors. Different cellular processes are perturbed during tumor progression. Benign tumors are characterized by hyperproliferation, but cells have a differentiated morphology and are surrounded by the basement membrane or a fibrous connective tissue capsule. De-differentiation of benign tumors occurs when cells lose the morphological characteristics of epithelia (cuboidal cell shape, tight cell-cell adhesion, distinct apical and basolateral membrane domains—polarity). With the disruption of junctions between neighboring cells, increased motility is observed, and this is facilitated by the degradation of matrix proteins. In malignant tumors, loss of differentiation occurs (epithelial–mesenchymal transition) accompanied by reduced cell–cell adhesion and inhibition of the expression of differentiation markers. Cells are able to infiltrate the neighboring tissues, gain access to blood vessels and colonize distant sites (metastasis). Thus, the de-differentiation process marks the transition between a benign and malignant tumor.

diffusion of solutes through the intercellular space. They are formed by transmembrane molecules (occludin, claudins and Junctional Adhesion Molecules, JAMs) that associate with cytoplasmic proteins (Zonula Occludens, ZO-1, ZO-2 and ZO-3). Adherens junctions and desmosomes are calcium-dependent cell-cell adhesion complexes both composed of transmembrane proteins of the cadherin superfamily. Desmosomal cadherins (desmoglein and desmocollin) interact heterotypically and link to the intermediate filament network through association with cytosolic proteins (desmoplakin, plakoglobin and plakophilin). Adherens junctions play a fundamental role in embryonic development and in the maintenance of tissue architecture in adults.[3] In adherens junctions, classical cadherins (E-, P- and N-cadherin) are the best-studied family members of the cadherin superfamily.[4] They are anchored to the actin cytoskeleton via proteins called catenins (α-catenin, β-catenin, plakoglobin and p120^{ctn}).

The predominant cadherin type expressed in epithelia is E-cadherin and this molecule plays a causal role in the establishment and maintenance of the differentiated epithelial phenotype. During tumor progression, decreased cadherin function correlates with de-differentiation, metastasis and poor prognosis. After cadherin adhesion is lost, cells undergo a conversion from an epithelial to a mesenchymal phenotype, which involves complex changes in their morphology, adhesive status (to their neighboring cells and to the extracellular matrix), gene expression and migration. It is believed that these effects derive from a combination of disruption of cell–cell adhesion and perturbation of signaling pathways activated by cadherin receptors (reviewed by Refs. 5,6). Thus, cadherin expression can function as a tumor and invasion suppressor, due to its participation in processes such as morphological differentiation and contact inhibition of growth and motility.

Several different mechanisms for perturbation of cadherin function in epithelial tumors have been proposed: (1) transcriptional or genomic regulation of E-cadherin expression, (2) mutations/deletion of cadherin or catenin genes and (3) regulation of adhesive function by intracellular signaling (Table 1). Irrespective of the mechanism used to abolish cadherin function in different tumors, the end result is the same: epithelial cells de-differentiate, and become more invasive and metastatic. According to the first mechanism, the E-cadherin molecule is truncated or non-existent, and it is not possible to envisage a treatment that would delay or prevent the de-differentiation process. According to the latter two mechanisms, (and when catenins are mutated), the E-cadherin receptor is present, but non-functional. Thus, the best strategy to generate therapies to prevent de-differentiation would be to manipulate intracellular signaling pathways that interfere with cadherin function. This approach may lead to a substantial reduction in the ability of tumor cells to colonize distant sites.

Two main features are important for the regulation of cadherin adhesiveness from the cytoplasm: the association of cadherin cytoplasmic tail with catenins, and the interaction with the cortical cytoskeleton. In addition, a growing number of studies point to distinct signaling pathways that can modulate cadherin-dependent cell–cell contacts, in particular pathways activated by Rho small GTPases (reviewed by Ref. 6).

Table 1. Possible mechanisms for the downregulation of cadherin-dependent adhesion during tumor progression

(1) Transcription/genomic regulation of E-cadherin expression:
- Transcription factors (snail, E12/47 and SIP1)
- DNA hypermethylation of E-cadherin promoter
- Switch expression to a different type of cadherin (i.e. N-cadherin)

(2) Mutations/deletions of cadherin or catenin genes:
- Premature stop codons
- In frame deletions
- Germ line mutations

(3) Regulation of adhesive function by signaling pathways:
- Cleavage of cadherin extracellular domain (metalloproteases, presenillins?)
- Post-translation modifications
- Regulation of cytoskeleton attachment
- Increased turnover of cadherin complexes

(1) Transcriptional downregulation. The transcription factors Snail, E12/E47 and SIP1 bind directly to E-boxes sites present in the E-cadherin promoter (Cano et al., Nature Cell Biol 2:76–83, 2000; Batlle et al., Nature Cell Biol 2:84–89, 2000; Perez-Moreno et al., J Biol Chem 276:27424–27431, 2001; Comijn et al., Mol Cell 7:1267–1278, 2001). DNA hypermethylation of the E-cadherin promoter (thereby making the promoter inaccessible for transcription) has been observed in human breast, prostate and renal tumors[89] (Nojima et al., Mol Carcinog 32:19–27, 2001; Graff et al., J Biol Chem 275:2727–2732, 2000; Nass et al., Cancer Res 60:4346–4348, 2000). Finally, N-cadherin is expressed, instead of E-cadherin, in human squamous carcinoma cells (Islam et al., Biochem 78:141–150, 2000) and after TGF-β-induced transdifferentiation.[41] N-cadherin is not able to support the epithelial phenotype, and as polarity is lost, N-cadherin facilitates the invasion and migration of cancer cells into the surrounding stromal tissue (which also expresses N-cadherin; Nieman et al., J Cell Biol 147:631–644, 1999; Hazan et al., J Cell Biol 148:779–790, 2000). (2) Mutations or deletions of cadherin or catenin genes (reviewed by Nollet et al., Mol Cell Biol Res Comm 2:77–85, 1999). (3) Regulation of adhesive function by intracellular signaling. In examples in which cadherin and catenins are expressed and remain intact, altered signaling pathways involving growth factor receptors, oncogenes and small GTPases may change cadherin adhesive properties by different means. These include: cleavage of cadherin extracellular domain, post-translation modification of cadherin complexes (i.e. phosphorylation), increased turnover of cadherin receptors and/or regulation of cytoskeletal attachment (see text for details).

Catenins link cadherin molecules to the actin network. In addition, catenins have multiple functions when they are not associated with the cadherin complex. For example, β-catenin promotes transcription of genes relevant to cell cycle progression (myc and cyclin D1).[7] The relationship between the cadherin-bound and the cytosolic pools of β-catenin is somewhat unclear. However, in colorectal tumor cells, E-cadherin suppressed cell growth by sequestering the signaling pool of β-catenin.[8] Thus, at least part of the tumor suppressor function of E-cadherin may be due to inhibition of β-catenin signaling. Plakoglobin, a protein closely related to β-catenin, can also associate with the cadherin cytoplasmic tail. There is some evidence that suggests that this protein may play a role in tumorigenesis also.[7,9]

α-catenin directly links the cadherin complexes to the actin cytoskeleton, and is essential for cadherin adhesive function.[10] Recent studies suggest that α-catenin may also regulate proliferation and participate in tumor progression. For instance, in keratinocytes, ablation of α-catenin leads to enhanced proliferation, focus formation and invasion in vitro.[11] Interestingly, this process appears to be independent of cadherin-mediated adhesion. Finally, another catenin, $p120^{ctn}$, has also been implicated in processes relevant to tumor progression. $p120^{ctn}$ binds directly to the cadherin tail, but its role in cell–cell adhesion is somewhat controversial. Reports suggest that $p120^{ctn}$ can either promote or perturb cadherin-dependent cell-cell contacts (reviewed in Ref. 12). Similar to β-catenin, $p120^{ctn}$ cytoplasmic pools can also participate in the regulation of gene expression and other signaling pathways (discussed below).

Role of Rho GTPases in tumorigenesis: in vivo evidence

The Rho family of GTPases contains four main subfamilies: Rho, Rac, Cdc42 and those that lack GTPase activity.[1] Some members of the Rho (RhoA, RhoB, RhoC), Rac (Rac1, Rac2, Rac3, Rac1b) and Cdc42 (Cdc42) subfamilies are best known for their role in the modulation of the actin cytoskeleton. In fibroblasts and other cell types, RhoA activation induces formation of stress fibers, Rac1 stimulates lamellipodium formation and Cdc42 induces filopodia.[13] However, they can also regulate cell proliferation, cell adhesion and tumorigenesis. When activated (GTP-bound), these proteins interact with a variety of effectors to trigger distinct signaling pathways (Fig. 2a).

In fibroblasts, a number of activated small GTPases are weakly transforming by themselves and can participate in transformation downstream of different oncogenes.[14] However, although the majority of human tumors are epithelial in origin, to date very little work addresses the involvement of Rho GTPases in transformation of epithelial cells. This is significant, since there is evidence that epithelial and fibroblast cells differ in terms of which pathway activated by Ras is sufficient for transformation.[15]

There are at least three mechanisms by which the activity of Rho proteins could be enhanced in tumors: (a) activating mutations in the Rho GTPases themselves, (b) elevated expression of the Rho proteins, and (c) increased GTP loading (activation) (Fig. 2b). Irrespective of the mechanism, the end result is an upregulation of signaling pathways triggered by small GTPases. As regards the first mechanism, so far there is no evidence for activating mutations in Rho GTPases in human tumors.[16,17] This is distinct from the Ras subfamily of small GTPases, in which a high frequency of mutations is observed in tumors of different origins. Two reports show alterations in the RhoH gene in lymphomas.[18,19] In one study, mutations occur in the non-coding sequence and so may have

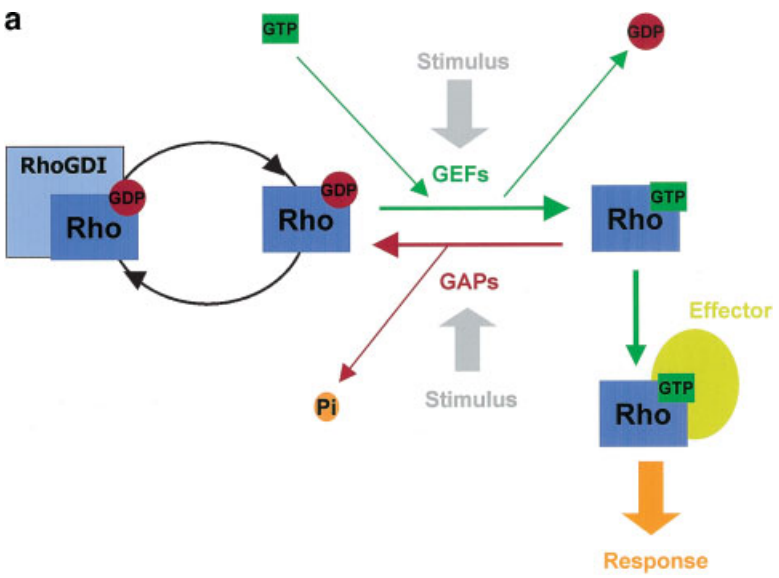

Figure 2. a: Activation cycle of Rho family of small GTPases. Inactive, GDP-bound Rho proteins are found in the cytoplasm associated with RhoGDI. Upon activation, Rho proteins associate with GTP, translocate to membranes and interact with a variety of effector proteins.[1] Inactivation occurs by hydrolysis of GTP, liberating phosphate. This cycle is tightly regulated in the cell by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs). In particular, one Rho effector plays an important role during tumorigenesis: the serine/threonine kinase ROCK (see text for details). Other targets discussed in the text are NADPH oxidase and synaptotagmin (both are Rac effectors). **b:** Possible mechanisms for the upregulation of small GTPase activity in tumors: activating mutations in Rho GTPase genes, increased protein levels or enhanced activation (GTP loading).

an effect on RhoH expression levels.[19] In another study, rearrangements of the RhoH gene and fusions between LAZ3 and RhoH have been identified in non-Hodgkin's lymphoma.[18] The functional consequence of such rearrangements is not clear. Nevertheless, future work may reveal that more of these mutations do exist in Rho GTPases genes.

Elevated expression levels of a number of small GTPases have been demonstrated in human cancer, mostly in the Rho and Rac subfamilies (Table 2). In some cases, there is an increase in the levels of mRNA encoding the Rho GTPases, suggesting enhanced transcription or mRNA stability (Table 2). However, increased mRNA levels do not necessarily imply increased protein levels.[17] In other cases, enhanced Rho protein levels have been demonstrated, which may lead to higher levels of activity. Interestingly, elevated expression levels of Rho (RhoA, RhoB and RhoC) have been correlated with tumor stage or enhanced metastasis in tumors, including breast cancer, melanomas, pancreatic ductal adenocarcinoma and testicular germ cell tumors (Table 2). In addition, increased Rac protein levels also correlate with tumor progression in breast cancer (Table 2).

The third mechanism, increased GTP loading on Rho proteins, has been supported by a number of recent reports, in particular the discovery of fast cycling (cycle between on and off state at an increased rate) and GTPase-deficient Rho proteins (hydrolyse GTP very inefficiently). A splice variant of Rac1, Rac1b, acts as a fast-cycling mutant, and is upregulated in colorectal tumor samples and some types of breast tumors (see Table 2 and Ref. 17). An example of GTPase-deficient protein is RhoH, in which rearrangements and mutations in tumors have been described above.[20] In further support of increased GTP loading playing a role in tumorigenesis, high levels of active Rac3 have been demonstrated in human breast cancer cell-lines and in breast tumor tissue, though total levels of Rac3 protein were unchanged (Table 2).

Enhanced GTP-loading on Rho proteins may result from reduced activity of GAPs or increased activity of GEFs (Fig. 2a, b). There are a few examples of mutations in GAPs in human

Table 2. Evidence for the upregulation of mRNA and/or protein levels of Rho small GTPases in carcinomas

Tumor	RHO GTPase	mRNA/protein?	Reference
Breast	RhoA	Protein	Fritz et al., 1999 [17]
	RhoB	Protein	
	RhoC	mRNA	van Golen et al., 2000
		Protein	Kleer et al., 2002[17]
	Rac1	Protein	Fritz et al., 1999[17]
		Protein/mRNA	Schnelzer et al., 2000
	Cdc42	Protein	Fritz et al., 1999
	Rac3*	Unchanged	Mira et al., 2000
Colon	RhoA	Protein	Fritz et al., 1999
	Rac1b	mRNA	Jordan et al., 1999
Head & neck SCC	RhoA	Protein	Abraham et al., 2001
	Rac2*	Protein	Abraham et al., 2001
Lung	RhoA	Protein	Fritz et al., 1999
Melanoma	RhoC	mRNA	[84]
Pancreatic ductal adenocarcinoma	RhoC	mRNA	[90]

Variations in the levels of Rho proteins exist among tumors originating from distinct tissues as well as within different samples of the same type of tumor. For example, the Rac1 splice variant, Rac1b, mRNA is upregulated in colon tumors but not in breast tumors. Increased RhoC mRNA levels are an indicator of malignancy in melanomas and pancreatic carcinomas, but not in breast tumors. Due to the high homology among Rac subfamily members, it is not clear whether antibodies against Rac3 and Rac2 show strict specificity (Fritz et al., Anticancer Res 19:1681–1688,1999; Fritz et al., Brit J Cancer 87:635–644, 2002; van Golen et al., Cancer Res 60:5832–5838, 2000; Kleer et al., Am J Path 160:579–584, 2002; Schnelzer et al., Oncogene 19:3013–3020, 2000; Mira et al., PNAS 97:185–189, 2000; Jordan et al., Oncogene 18:6835–6839, 1999; Abraham et al., Laryngoscope 111:1285–1289, 2001; Kamai et al., BJU Int 87:227–231, 2001).

tumors. First, a fusion between the human GRAF gene (GTPase regulator associated with the focal adhesion kinase $pp125^{FAK}$) and Mixed Lineage Leukemia (MLL) gene has been identified in juvenile myelomonocytic leukaemia.[21] The full-length GRAF gene encodes a GAP for Rho GTPases; however, the GAP domain is not retained in the MLL–GRAF fusion. Furthermore, this study reported three other cases in which both GRAF alleles were disrupted, indicating the importance of GRAF for the development of this type of leukemia.[21] Second, the p190-A RhoGAP gene maps to a region of chromosome 19 that is frequently rearranged in a number of human tumors, including pancreatic carcinomas and gliomas. Loss of heterozygosity of several markers flanking the p190-A gene has been identified in some glioblastoma/astrocytoma cases.[22] Finally, loss of heterozygosity of another RhoGAP gene, Deleted in Liver Cancer (DLC-1), has been reported in primary hepatocellular carcinomas tumors and cell lines.[23]

Increased GEF activity may originate from amino-terminal truncations, activating mutations, increased protein levels or post-translation modifications. In vitro, a number of activating amino-terminal truncations of Rho GEFs have been identified by their tumorigenic ability in fibroblasts.[24] However, evidence for such mutations/ truncations of GEF genes in human tumors is not frequently reported. Most examples occur in acute myeloid and acute lymphocytic leukemias, where fusions between a GEF and other genes are found, i.e. LARG (a GEF for RhoA) and Bcr (a GEF for Rho, Rac and Cdc42), respectively.[24] In a small number of renal cell carcinoma samples, a mutation in Tiam1 (a GEF for Rac) was found, which may enhance its in vitro transforming activity.[25] Increased expression of Tiam1 was also observed in breast cancer.[26] However, Tiam1 knockout mice show resistance to the development of skin tumors and those that do develop grow slowly.[27] Interestingly, a greater proportion of the tumors that do appear in $Tiam1^{-/-}$ mice progresses to malignancy. These results suggest that Tiam1 plays an important role in tumor initiation and growth and in the suppression of metastasis.[27]

Even when there are no genetic alterations or increased protein levels, GEF activity could be enhanced by expression of oncogenes and decreased expression of tumor suppressors.[24] For instance, GEF activity could be regulated by phospholipids, phosphorylation and interactions with other proteins.[24] Taken together, the above data suggest that there are multiple points at which the activity of Rho GTPases can be altered during tumor progression. These involve not only genetic alterations in GEF genes, but also regulation of the stability of mRNA/protein levels of small GTPases and GTP loading on Rho proteins. It is likely that overexpression of oncogenes and hyperactivation of other signaling proteins that feed into small GTPase pathways are also important players in the upregulation of Rho function during cancer.

Participation of Rho GTPases in different stages of tumorigenesis

Rho GTPases have been implicated in each of the multiple steps in tumorigenesis: upregulation of proliferation,

de-differentiation, invasion, migration and metastasis. The participation of Rho proteins in hyperproliferation has been reviewed elsewhere.[1,14] As mentioned before, the quickest and most efficient way of inducing morphological de-differentiation during tumor progression is to abolish cadherin-mediated adhesion (see section below). However, recent results point to alternative pathways that can perturb epithelial polarization without disruption of cadherin adhesion. Rho, Rac and Cdc42 play a role in the correct targeting of membrane proteins to the apical or basolateral domain.[28,29] Cdc42 may also interfere with polarization via interaction with the PAR6/PAR3/atypical PKC complex (reviewed in Ref. 6). This complex appears to play a role in modulation of tight junction formation and epithelial polarity in mammalian cells. Overexpression of activated Rac disrupts polarity in breast cancer cells[30] and Rac activity is required for the correct localization of apical proteins in epithelial cysts.[31] Rnd3/RhoE also participates in the de-differentiation and multilayering in Raf1-transformed MDCK cells.[32] As cadherin-dependent adhesion is not significantly affected in the above studies, these results suggest that Rho GTPases may also play a role downstream of cadherin receptors to modulate epithelial polarization.

Rho small GTPases in the regulation of cadherin adhesion

It has been established that the activity of RhoA and Rac1 is necessary for the formation and maintenance of cadherin-dependent cell-cell contacts.[5] The mechanisms via which Rho and Rac can stabilize cadherins at junctions are not clear. It is thought that Rac participates in actin recruitment to cadherin complexes.[33] A recent work suggests that Rac activity is required for lammellipodium formation during initial cell-cell contact, a process characteristic of MDCK cells.[34] In other systems, for example during *Drosophila* dorsal closure, both filopodia and lamellipodia participate in the sealing of dorsal epithelium.[35] In mouse keratinocytes, both filopodia and lamellipodia may be required for initial contacts between cells. However the involvement of Cdc42 or Rac in the formation of these structures during junction formation has not been shown in this cell type (reviewed in Ref. 6).

In apparent contradiction, a number of studies have implicated RhoA and Rac1 in the disassembly of cell–cell contacts.[36–39] Thus, inappropriate activation of Rho proteins or their inactivation may lead to the destabilization of cell–cell contacts. These different results suggest that the activity of Rho and Rac must be tightly regulated (in a local and temporal manner) to ensure stable junctions. The effect of Cdc42 activation on the disassembly of cadherin-dependent cell–cell contacts has not been looked at in detail.

Two possibilities can be envisaged. First, Rho and Rac activation can disrupt cadherin adhesion as part of a transformation programme, in which they cooperate with other signaling pathways triggered by oncogene expression/growth factor receptors. Alternatively, Rho or Rac activation may be sufficient to specifically remove cadherin receptors from junctions. In either case, identifying the pathway that specifically destabilizes junctions is important for the design of new therapies that can prevent the de-differentiation process. The evidence indicating that inappropriate activation of RhoA and Rac1 can perturb cadherin adhesion is discussed below together with possible mechanisms.

RhoA. RhoA and some of its targets have been implicated in the disassembly of cell-cell contacts, migration and metastasis (Fig. 3). Rho signaling pathways can cooperate with distinct pathways in epithelial-mesenchymal transition. Active RhoA is important for HGF-and TGF-β-induced disruption of cadherin contacts in epithelial cells.[40,41] In addition, RhoA activation correlates with cell-cell contact breakdown in Ras-transformed MDCK cells.[42] A recent report suggests that Rho activation per se is sufficient to disrupt cadherin-mediated cell-cell adhesion, and RhoC is more effective than RhoA.[39]

However, junction breakdown by active Rho may not necessarily mean enhanced migration (see below). Thus, the above results need to be reconciled with the fact that Rho activation can either inhibit or promote cell migration in distinct cell types. In addition, it is not clear whether junction disassembly following Rho activation is an indirect consequence of reorganization of the actin cytoskeleton.

It has been proposed that increased contraction generated by RhoA may result in tensile stress at cell-cell junctions, contributing to the destabilization of contacts and inducing cell shape changes.[43] Indeed, after expression of active RhoC, blocking cell contractility using different inhibitors can restore cell–cell contacts (but see below).[39] While it is clear that the transformed cell shape is much more contracted than non-transformed cell shape, it is not known whether Rho-induced contractility is the cause of junction disassembly in epithelial tumors. In normal epithelia, there is already a considerable amount of contraction at junctions to maintain the polarized, cell shape and cohesiveness of epithelial tissues.[5,44]

ROCK (also known as Rho kinase or ROK-α) is a serine/threonine kinase and a key Rho target that induce actin bundling and contraction.[45] Transfection of activated ROCK leads to the disassembly of cadherin adhesion in HCT116 (colon carcinoma cells).[39] In contrast, a dominant negative approach to inhibit ROCK resulted in junction disruption in "non-transformed" MDCK cells,[46] but not in NmuMG mammary cell line.[41] As both activation and inhibition of ROCK can considerably affect actin organization, it is possible that the disruption of the cytoskeleton indirectly destabilizes cadherin receptors. In addition, as ROCK can phosphorylate a variety of intracellular proteins, it is conceivable that

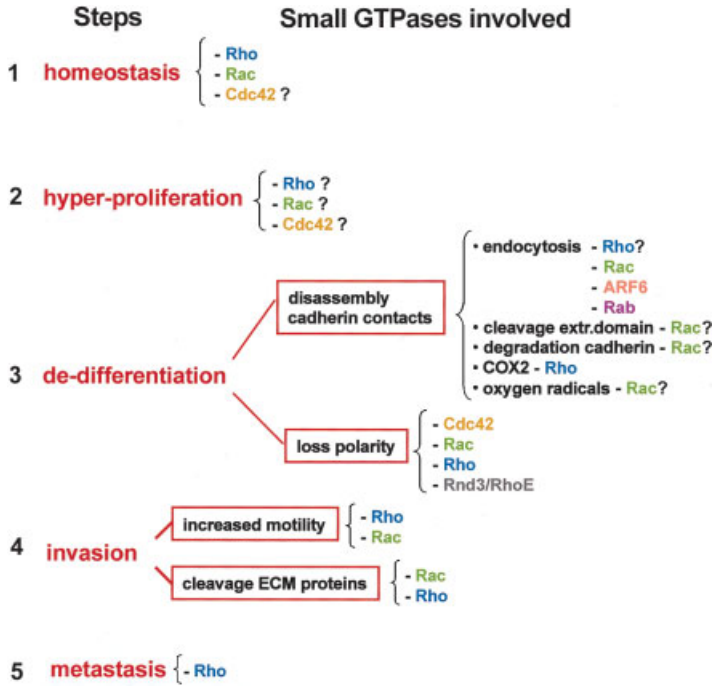

Figure 3. Participation of Rho GTPases in epithelia homeostasis[5] and in different stages of tumorigenesis: hyperproliferation,[1] de-differentiation, invasion and metastasis. Rho proteins participate in each of these steps and the proposed mechanisms are discussed in the text. Question marks indicate that the activity was not formally demonstrated in epithelial cells/tumors or with respect to cadherin function.

phosphorylation of other cytoskeletal proteins may also contribute to junction destabilization.

At least two mechanisms other than cell contraction can be envisaged to explain the effects of active RhoA on junctions: regulation of cadherin endocytosis and modulation by cyclooxygenase 2 (COX-2). The participation of RhoA in cadherin endocytosis has not yet been directly investigated. However, studies on other membrane proteins revealed that active RhoA inhibits endocytic traffic in both polarized and non-polarized cells.[47] Whether cadherin receptors can be regulated in a similar manner is remains to be tested.

The second mechanism involves COX-2. This enzyme catalyzes the production of prostaglandins, lipid signaling molecules that participate in processes such as maintenance of vascular integrity, pain transmission, inflammation and bone remodeling.[48] High levels of COX-2 have been detected in many cancers and in vitro studies show that COX-2 expression can be reciprocally modulated by cell-cell adhesion molecules.[48] For example, inhibition of COX-2 increases expression of a variety of cell adhesion proteins, such as FAT and proto-cadherin 7.[49] It has also been found that inhibition of COX-2 by aspirin reduces in vitro invasion of tumor cell lines by upregulation of E-cadherin, among other genes.[50] In other cell systems, the cadherin-free pool of β-catenin (transcriptionally active) is sufficient to induce COX-2 expression.[51] Rho GTPases seem to be also involved in the regulation of gene expression and in the signaling pathways initiated by COX-2.[52] In that sense, Cytotoxic Necrotizing Factor (CNF, a bacterial toxin) induces RhoA-dependent expression of the COX-2 gene.[53] These results suggest that RhoA GTPase and COX-2 can modulate migration and tumorigenesis by regulating cell-cell adhesion molecules such as cadherins. However, a direct functional connection between COX-2 enzyme, Rho proteins and cadherins during tumor progression remains to be demonstrated in vivo.

Rac1. Rac1 activation per se is sufficient to destabilize cadherin receptors at junctions in keratinocytes.[36–38] This effect occurs in a concentration- and time-dependent manner.[37] In addition, Rac1 activity is necessary for oncogenic Ras-dependent disruption of cell-cell contacts and epithelial morphology.[37,54] Blocking Rac1 signaling in the above examples leads to restoration of cell-cell contacts and inhibition of scattering. Consistent with this, activation of Rac by Tiam1 expression in breast epithelial cells disrupts cell–cell contacts.[26] In contrast, Rac1 activation by Tiam1 in Ras-transformed MDCK cells can reverse the fibroblastoid morphology of these cells and enhance cadherin presence at cell–cell contacts.[55,56] It should be noted that, unexpectedly, in these stable Ras-transformed MDCK cells, Rac1 activity is dramatically downregulated. Thus, by restoring some of the Rac1 activity via Tiam1, junctions are re-formed. It is possible

that further activation of Rac1 would then disassemble junctions.

Therefore, one explanation for the discrepancies present in the literature may be that Rac1 can stabilize or disassemble cadherin complexes, depending on the amount of active Rac1 present (as pointed above), the origin of the cells or the extracellular matrix that the cells are growing on.[5,56,57] Alternatively, these results may reflect the many different ways in which a tumor cell can arise (distinct mutations, oncogenes and altered signaling pathways). In each of these scenarios, the effects of active Rac1 may vary and result from the integration of distinct signaling pathways operating in different cell types.

The molecular mechanisms that mediate the disruptive effects of Rac1 on cadherin-based adhesion are far from clear (Fig. 3). In the context of Ras transformation, Rac activation increases the turnover of cadherin and catenins.[54] It is known that junctions are not destabilized by formation of a lamellipodium, the classical cytoskeletal structure induced by Rac.[37] In fact, formation of lamellipoia and disruption of junctions involve two separate pathways that are independently activated by Rac1.[37] Moreover, the presence of cell-cell contacts appears to suppress lamellipodia in certain cell types.[58] Evidence in the literature suggests regulation of endocytosis is one possible mechanism by which Rac1 could participate in disruption of cadherin-dependent adhesion.

Endocytosis. In polarized MDCK epithelial cells, apical and basolateral endocytosis is inhibited by activated Rac1, in a similar manner to RhoA.[59] In addition, expression of active Rac has been shown to inhibit endocytosis of the transferrin receptor in HeLa cells[47] and EGF receptor in epithelial A431 cell line.[60] In the latter work, Rac inhibits endocytosis by binding to its target synaptojanin 2, a polyphosphoinosite phosphatase implicated in uncoating of endocytic clathrin-coated vesicles.[60] Interestingly, upon Rac1 activation in keratinocytes, E-cadherin complexes are endocytosed through a clathrin-independent mechanism.[38] Although several clathrin-independent mechanisms of internalization have been described in mammalian cells, the effects of Rac1 on these have not been investigated. The diversity in endocytic mechanisms may explain why Rac1 seems to induce or inhibit endocytosis, depending on (a) the type of membrane protein, (b) its localization (apical/basolateral) (c) the cellular context, and (d) the specific endocytic process that takes place (i.e. cadherins could be internalized by pinocytosis, Ref. 44).

The possibility cannot be excluded that Rac cooperates with different small GTPases to induce endocytosis. For example, cross-talk has been described between Rho GTPases and members of the ARF and Rab families of small GTPases, regulators of membrane trafficking pathways.[61] ARF6, a member of the ARF family, regulates the recycling of endosomal membrane and other cargo to the cell surface, secretion and actin cytoskeleton remodeling pathways.[61] Sustained expression of active ARF6 in MDCK cells results in the disassembly of adherens junctions and ruffling of the lateral plasma membrane.[62] Similarly to Rac1, these two processes are mediated by different pathways.[37,62] Other studies have also suggested that ARF6 and Rac1 function in the same signaling cascade.[63–66] However, the order in which Rac1 and Arf6 are placed in this cascade varies. The reason for these discrepancies is not known and requires further work to clarify them.

Activated Rab5, a Rab family member, is required for sequestering ligands into clathrin-coated pits and subsequent fusion of vesicles with early endosomes.[67] In MDCK cells, Rab5 participates in the co-endocytosis of E-cadherin with the EGF receptor and c-Met tyrosine kinases, following binding of their respective ligands.[68] Cadherin molecules are then removed from junctions, leading to cell scattering. Surprisingly, endocytosis or recycling of E-cadherin upon removal of calcium ions from the medium does not require Rab5 activation.[68] Thus, Rab5 may be involved in endocytosis of a subpopulation of E-cadherin receptors that are post-transcriptionally modified (i.e. phosphorylated as a result of growth factors activation). These results suggest that there are distinct mechanisms for E-cadherin internalization and are consistent with the effects of small GTPases (Rac, ARF6 and Rab5) on cadherin endocytosis described above. Cross-talk between Rab5 and Rac1 has been described in the case of EGF receptor endocytosis[69] suggesting that a similar co-regulation pathway may act in Rac-induced disassembly of cadherins in epithelial cells. Further studies are necessary to clarify this issue.

Other possibilities. Three additional ways in which Rac may remove cadherin receptors from the cell surface are: cleavage of cadherin receptors, degradation and involvement of Reactive Oxygen Species (ROS). Cadherins can be cleaved by presenilins and metalloproteinase. Presenilins form a complex with E-cadherin in epithelial cells and cleave cadherin molecules at an intracellular site (membrane-cytosol interface).[70] The resulting fragment may behave as a dominant negative and inhibit cadherin function. However, so far, E-cadherin cleavage by presenilins has only been detected during apoptosis and has not yet been investigated during tumor progression.

Rho GTPases play a role in the induction of matrix metalloproteinase expression (Table 3). Matrix metalloproteinases (MMPs) are endopeptidases involved in degradation of extracellular matrix, activation of specific ligands and shedding of extracellular fragments of membrane receptors (reviewed in Ref. 71). MMP cleave and release the extracellular domain of E-cadherin near the plasma membrane (see also below) During tumor progression, cleaved soluble forms

Table 3. Participation of Rho small GTPases in the induction of expression of metalloproteinases (MMP) and tissue-specific inhibitors of MMPs (TIMP)

Small GTPase	MMP or TIMP	Cell type	Effect	Reference
Rho	MMP-1 (collagenase-1)	Human vascular endothelial cells	C3 (Rho inhibitor) inhibits MMP-1 protein expression	Ikeda et al., 2000
	MMP-1	Rabbit synovial fibroblasts	Dominant negative Rho inhibits MMP-1 transcription induced by invasin-coated beads; Activated RhoA triggers MMP-1 transcription	Werner et al., 2001
	MMP-9	THP-1 human monocytic cell line	C3 inhibits LPS-stimulated secretion of MMP-9	Wong et al., 2001
Rac	MMP-1	Rabbit synovial fibroblasts	Activated Rac1 induces MMP-1 transcription and protein expression; Dominant negative Rac activity blocks integrin-induced expression of MMP-1	Kheradmand et al., 1998
	MMP-1	Rabbit synovial fibroblasts	Activated RhoA and Rac induce MMP-1 transcription	Werner et al., 2002
	MMP-2 (gelatinase A)	HT1080 fibrosarcoma cell line	Activated Rac enhances MMP-2 activity	Zhuge et al., 2001
	TIMP-1 TIMP-2	ClearCa-28 human renal carcinoma	Rac activation induces upregulation of TIMP-1 (transcriptional) and TIMP-2 (post-transcriptional)	Engers et al., 2001
??	MMP-2	Bovine smooth muscle cells	Toxin B treatment (inhibits Rho, Rac & Cdc42) induces activation of MMP-2	Koike et al., 2000

Ikeda et al., Hypertension 36:325–329, 2000; Werner et al., J Cell Sci 114:3333–3334, 2001; Werner et al., J Cell Biol 158:357–368, 2002; Wong et al., J Leukocyte Biol 69:959–962, 2001; Kheradmand et al., Science 280:898–902, 1998; Zhuge et al., J BiolChem 276:16248–16256, 2001; Engers et al., Int J Cancer,88:369–376, 2000; Koike et al., Biochem Biophys Res Comm 277:43–46, 2000.

of E-cadherin extracellular domain have been found in cancer patients.[72] These cleaved soluble fragments of E-cadherin can also downregulate cell-cell adhesion in a paracrine manner, by inhibiting adhesion of full length molecules.[73] In mammary cells, enhanced MMP3/stromelysin-1 activity results in epithelial to mesenchymal transition.[71] Conversely, cadherin-dependent adhesion can downregulate MMP-9 expression in pre-malignant oral keratinocytes, suggesting an inverse correlation between the function of these two classes of molecules.[74] Although the small GTPase Rac has been implicated in the expression of MMPs (Table 3), until now, cleavage of E-cadherin as a result of Rac1 activation in epithelial cells has not been demonstrated. This possibility warrants further investigation.

Once cadherins are internalized, the molecules may be recycled back to the surface[75] or targeted for degradation via the proteasome.[76] A new cadherin-binding protein, Hakai, plays a role in targeting the E-cadherin complex for degradation by ubiquitination.[76] Similarly to what is observed with Rab5-dependent E-cadherin internalization, Hakai also promotes the endocytosis/degradation of tyrosine phosphorylated E-cadherin receptors.[68,76] Furthermore, Rac-dependent signaling pathways have been linked to the intracellular degradation machinery. So far a direct connection between cadherin degradation and Rac signaling pathways has not been shown in epithelial cells. Further studies may support this interesting possibility.

Finally, a novel interesting way to perturb junctions by Rac activation has been shown in endothelial cells. Active Rac1 induces the removal of VE-cadherin (a cadherin receptor expressed in endothelia) from junctions by promoting tyrosine phosphorylation of the complex. This process requires production of ROS[77] via activation of NADPH oxidase, a known effector of Rac.[45] As different cell types also have the ability to produce ROS, the above results raise the intriguing possibility that E-cadherin stability in epithelial cells may also be regulated by Rac-dependent production of ROS. ROS has additional signaling properties in non-phagocytic cells, such as induction of growth factor expression, proliferation, angiogenesis and extracellular matrix deposition, which may also play a role in tumorigenesis.[78] Thus, it is conceivable that ROS production and signaling may contribute to increased migration and dispersal of tumor cells in multiple ways.

Rho GTPases in invasion and metastasis

During the complex mechanism of tumor progression, several MMPs have been implicated as key proteins in tumor invasion, metastasis and angiogenesis. Invasion in vivo requires the breakdown of the basement membrane that separates epithelial sheets from stromal cells. As shown in Table 3, activation of Rho or Rac leads to increased MMP expression in different cell types; conversely, blocking Rho or Rac function can inhibit transcription of MMPs. However, in other studies active Rac1 promotes upregulation of tissue-specific inhibitors of MMPs, TIMP-1 and TIMP-2 (see Table 3). The reasons for these differences in Rac signaling output are not clear. These differences in Rac signaling may reflect the distinct tumors/cell types studied and the genetic/biochemical modifications that resulted in transformation.[71,79] It should be noted that TIMPs have functions in addition to MMP inhibition. For instance, TIMP1 and 2 can play a role in cell proliferation, apoptosis, angiogenesis and metastasis.[79] Thus, upregulation of TIMPs may also have a positive effect on tumorigenesis.

During tumor progression, the three stages (de-differentiation, invasion and metastasis) are intrinsically related to each other. However, the majority of reports use a single parameter (i.e. increased migration) as a read out for the effect of a signaling pathway in tumor cells. Therefore, it is often difficult to determine the contribution of a given signaling pathway to the other stages. In particular, it would be interesting to know whether there was disruption of intercellular junctions prior to enhanced motility. Thus, it is feasible that a given stimulus could affect primarily cell–cell adhesion, which leads to enhanced migration as a secondary effect.

In vitro, Rho GTPases regulate the migration of a number of cell types including fibroblasts, macrophages and glial cells (Fig. 3, reviewed by Ref. 1). In vitro studies have also demonstrated a direct role for Rac1 activity in epithelial cell invasiveness.[16,30,80] In addition, Rac1 activity is required for migration induced by wounding and the scattering phenotype induced by oncogenic Ras and growth factors such as HGF/SF.[81,82] Blocking Rac1 signaling in the above examples leads to inhibition of scattering or migration.

Early experiments using fibroblasts transformed by RhoA showed the potential in vivo role of Rho during metastasis.[83] In addition, expression profiles for genes implicated in metastasis revealed that RhoC is upregulated in metastatic melanoma tumors (see Table 2). Increased metastasis occurs without alterations in proliferation rate, suggesting that the stronger invasive phenotype may involve Rho-dependent changes in the cytoskeleton.[84] Consistent with these results, in vivo inhibition of ROCK leads to a substantial inhibition of migration and metastasis of prostate carcinoma and hepatoma cells.[85] Blocking Rho function in vitro prevented HGF/SF-induced migration in mouse keratinocytes.[40] However, it should be noted that, in other cell types, activation of Rho can also inhibit migration.[86] Nevertheless, the above results point out that in vivo inhibition of Rho-dependent pathways may be useful in the control of malignancy in certain cell types.

In addition, there exists the phenomenon of cross-talk between cadherins and integrins (cell-matrix receptors), with obvious implications for malignancy.[87] This cross talk coordinates cell–cell and cell–matrix attachment, and ultimately determines a sessile or motile phenotype. For instance, it is known that in the presence of cell-cell adhesion, motility rate is reduced, consistent with contact inhibition of migration. On the other hand, specific subsets of integrin receptors can interfere with the polarization of epithelia in different cell systems. As Rho proteins can regulate both cadherin adhesion and motility, small GTPases are likely candidates to coordinate these processes. Interestingly, recent evidence suggest that the output of Rho signaling can be modulated depending on the attachment to distinct substrate.[31,56] Although the precise mechanisms of how a cross-talk between cadherins and integrins operate are not clear, the data above strongly indicate a central role for Rho small GTPases in this process.

Another interesting possibility is the involvement of $p120^{ctn}$. This catenin has been recently shown to bind directly to Rho1 in *Drosophila*,[88] although similar binding has not been detected in mammalian cells. In different cells, soluble pools of $p120^{ctn}$ (i.e. not associated to cadherin complexes) can alter the activity of Rho GTPases and enhance migration.[12] These results are exciting as they highlight $p120^{ctn}$ as a possible player in the coordination between adhesion and motility.

Conclusions

Epithelial tumorigenesis is a complex process. Issues that contribute to this complexity include the initial transforming mutations, the biochemical and signaling pathways affected, the tissue that the tumor originates from and the multiple ways in which metastasis can be achieved. Because of this multiplicity of factors, the design of therapeutic strategies to prevent tumor progression is a formidable task. Key signaling pathways that will be investigated in the future are the ones triggered by cadherin-mediated adhesion and Rho GTPases, which play a central role in different stages of tumor progression. As outlined in this review, the functions of cadherins and small GTPases intersect in many different cellular processes. Understanding the molecular mechanisms operating at these cross-roads will provide potential therapeutic targets to interfere with metastasis.

Acknowledgments

Due to restrictions on the number of references allowed, we apologize to those whose work has not been cited in this review.

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