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Pleiotropic Functions of Rho GTPase Signaling: a Trojan Horse or Achilles' Heel for Breast Cancer Treatment?

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Abstract

Rho GTPase signaling is altered in human breast tumors, and elevated expression and activation of Rho GTPases correlates with tumor progression, metastasis, and poor prognosis. Here we review the evidence that Rho signaling functions as a key regulator of cell cycle, mitosis, apoptosis, and invasion during breast cancer growth and progression and discuss whether these pleiotropic actions enhance or limit the targetability of this network. We propose that depending on the stage and subtype of breast cancer, targeting Rho signaling may have chemopreventative, anti-tumor, and anti-metastatic efficacy. An understanding of how Rho signaling is perturbed in specific stages and subtypes of breast cancer and how it functions in the context of the complex *in vivo* environment during the stochastic process of tumor formation and progression are necessary in order to effectively target this signaling network for breast cancer treatment.

Keywords

breast cancer; metastasis; Rho; GTPase; GEF; GAP; GDI; inhibitor; statins

BREAST CANCER: A NEED FOR BETTER TREATMENTS

Breast cancer is a highly prevalent disease affecting women in the United States, and in 2009 approximately 62,000 cases of *in situ* breast cancer and 192,000 cases of invasive breast cancer were diagnosed [1]. Despite recent declines in breast cancer incidence and mortality, over 40,000 women died from this disease last year. Breast cancer is not a single disease; rather, it is a collection of heterogeneous diseases. This heterogeneity is reflected by diverse histopathologies, molecular profiles, and responses to therapy. Current therapies fall short of treating this complex disease, and failure to prevent recurrence, and ultimately metastatic disease, underpin the high mortality rate of breast cancer.

THE BREAST IS HIGHLY SUSCEPTIBLE TO TUMORIGENESIS

The breast consists of a branched ductal epithelial tree with each duct terminating in a lobular unit. The ductal epithelium is composed of a bilayer of luminal epithelial cells and basal (myoepithelial) cells situated on an underlying basement membrane (Figure 1). The ductal and lobular epithelium is embedded within connective tissue stroma that is rich in extracellular matrix proteins, fibroblasts, immune cells, and endothelial cells of blood and lymphatic vessels. Complex interactions between the epithelium and surrounding microenvironment are critical for normal breast development and homeostasis, and disruption of these interactions facilitate breast cancer growth and progression [2, 3].

The epithelium of the breast undergoes marked morphological changes driven by changes in gene expression during pubertal development and throughout a woman's reproductive lifespan. Breast development is initiated at puberty when elevated systemic steroid (estrogen and progesterone) and peptide hormones (GH) as well as locally produced growth factors (IGF and EGF) stimulate outgrowth of the ductal tree (For review see [4]). During each menstrual cycle, the breast epithelium is cyclically stimulated by ovarian hormones to proliferate, and in the absence of pregnancy, the expanded epithelium regresses. During pregnancy, progesterone stimulates massive proliferation of the lobular epithelium, which differentiates into alveoli that secrete milk components upon lactation. When lactation is terminated, the alveoli undergo apoptosis and the breast is remodeled to resemble its pre-pregnant morphology. The cyclic stimulation and regression of the breast epithelium under the control of ovarian hormones over decades of a woman's life, as well as the massive cellular expansion, death, and remodeling that occurs during pregnancy, lactation, and post-lactational involution, provides an ideal setting for the accumulation and propagation of tumor promoting mutations [5].

Breast cancer develops along a continuum characterized by histopathological changes whereby the normal morphology of the breast epithelium and associated microenvironment is progressively disrupted (Figure 1). Invasive ductal carcinoma is the most common type of breast cancer and accounts for approximately 80% of breast cancer cases diagnosed annually [6]. Its earliest stage begins as overgrowth or hyperplasia of the epithelial cells, resulting in a pre-cancerous lesion, atypical ductal hyperplasia. Accumulation of epigenetic and genetic insults promotes the formation of ductal carcinoma *in situ* (DCIS), which is a benign lesion because it remains encapsulated by the basement membrane. Invasion of the tumor cells through the basement membrane marks the transition of benign DCIS to malignant invasive ductal carcinoma (IDC). IDC is associated with a significantly elevated risk of development of metastatic disease. Metastatic disease results when tumor cells enter into the blood or lymph vessels and travel to regional lymph nodes and ultimately distant sites within the body. At these new sites, the cells exit the vasculature and colonize the organ to form metastatic tumors. Metastasis is responsible for the majority of breast cancer-related deaths [1], and thus, therapies designed to prevent and treat metastatic disease are required to effectively combat this disease.

Breast cancer is not a homogeneous disease. Molecular profiling has demonstrated at least six subtypes of invasive ductal carcinoma including luminal A, luminal B, ErbB2⁺, basal, normal breast-like, and claudin-low [7, 8]. Each of these subtypes displays unique genetic profiles, histopathologies, and responses to therapies [9]. Approximately 10–15% of breast cancers are lobular carcinomas and an additional ten rare types of breast cancer such as inflammatory breast cancer have been described [10].

Variations in the cell of origin, or tumor initiating cells, from which breast cancer arises may impact the heterogeneity of breast cancer. Accumulating evidence suggests that tumor initiating cells or cancer stem cells drive tumor growth and progression (reviewed in [11]). Furthermore, recent studies have demonstrated that cancer stem cells are resistant to chemotherapy [12, 13], and are thus likely to be responsible for breast cancer recurrence and metastatic progression. This is a particularly active area of research in the breast cancer community (for review see [14] and Paul et al., in this issue of *Current Drug Targets*).

CURRENT BREAST CANCER THERAPIES

Clinically, breast tumors are classified based on histopathology and expression of various biomarkers such as Brca1, Brca2, estrogen receptor (ER), progesterone receptor (PR), and ErbB2/Her2. These markers are used to guide treatment and provide prognostic information.

In addition, molecular profiling tests that allow for analysis of genomic signatures such as Oncotype Dx is in clinical use to help determine risk of recurrence in ER-positive, node-negative patients [15, 16]. It is likely that more molecular profiling tests will become available in the near future.

Standard therapeutic strategies take advantage of the uncontrolled proliferation of breast cancer cells. The majority of these are cytotoxic agents including DNA damaging agents (e.g. anthracyclines, platinum based chemotherapies) and microtubule inhibitors (e.g. taxanes). These cytotoxic drugs kill dividing cells thereby reducing the mass of the primary tumor and inhibiting the growth of disseminated tumor cells at metastatic sites. These agents promote disease free survival to varying degrees depending on the stage and grade of the tumor [17]. Neutropenia, neurologic, cardiac, and gastrointestinal toxicities are a significant problem associated with these chemotherapeutic agents. Furthermore, it is clear that these therapies fail to effectively prevent recurrence and disease progression in all stages and subtypes of breast cancer.

Other therapeutic strategies take advantage of the differential expression of targetable molecules, such as trastuzumab, which is a monoclonal antibody that inhibits Her2/ErbB2 activation. Trastuzumab has had a significant impact on the outcome of Her2-positive breast cancers [18]. Like the cytotoxic agents described above, it also has cardiotoxicity [18]. Since breast tumor growth may also be promoted by estrogen signaling, endocrine therapies such as anti-estrogens (e.g. tamoxifen) and aromatase inhibitors (e.g. letrozole) are administered to women with ER positive tumors. Because of the heterogeneity of breast cancer, the current targeted chemotherapeutic approaches fall short of curing many breast malignancies. For example, triple negative breast cancers express none of the targetable oncogenes ER, PR, and Her2/ErbB2, and are therefore resistant to several lines of therapy. In addition, acquired resistance to targeted therapeutics such as trastuzumab and tamoxifen has an important impact on their utility.

Thus, despite the availability of effective cytotoxic drugs, endocrine therapies, and an increasing number of targeted therapies, there is a clear need for novel therapeutics designed to prevent recurrence and metastasis and to treat late-stage disease. A great deal of effort is aimed at identifying new targetable molecules and developing agents to selectively modify the action of these molecules in breast cancer. One potential signaling network that provides a number of potential new targets is the Rho GTPase signaling network. In this review we will discuss specific Rho GTPases, regulators, and downstream effectors that have been implicated in human breast cancer. We will describe the *in vitro* and the relatively limited *in vivo* studies that have provided information about the specific molecular and cellular mechanisms by which these molecules may contribute to the growth and progression of breast cancer. We conclude that there is a need for more *in vivo* studies and a better understanding of how this signaling network is altered in specific stages and subtypes of breast cancer.

RHO PROTEINS ARE PROMISING YET ELUSIVE TARGETS FOR THERAPY

The Ras-homologous (Rho) family of small GTPases regulates a multitude of vital cell processes such as endocytosis, cell morphology, proliferation, survival, motility, and differentiation [19–21]. Extracellular signals received by cell-matrix adhesion molecules (e.g. integrins), receptor tyrosine kinases (e.g. growth factor receptors), and cell-cell adhesion molecules (e.g. cadherins) are transmitted to Rho GTPases, which in turn activate a variety of effector proteins [22].

Disruption in the normal activity of Rho GTPases has been closely associated with tumorigenesis [23–25] and metastasis [26–29]. Indeed, many tumorigenic processes such as

cell transformation, angiogenesis, tumor cell invasion, and metastasis have been correlated with Rho GTPase activity. Importantly, Rho proteins are frequently overexpressed and hyperactivated in human breast cancer [23, 27, 30]. All of this underscores the importance of Rho proteins in breast cancer development and progression and makes these proteins attractive candidates for targeted drug development. However, because of their numerous roles in essential cellular processes, there are some potential challenges to targeting this signaling pathway for cancer therapy. We will discuss these challenges throughout the review.

Rho proteins cycle between two forms: the inactive guanine diphosphate (GDP)-bound form and the active guanine triphosphate (GTP)-bound form (Figure 2). Guanine nucleotide exchange factors (GEFs) activate Rho GTPases by removing the GDP and replacing it with GTP. As their name suggests, Rho GTPases possess an intrinsic hydrolytic activity that converts GTP to GDP. However, this chemical reaction is generally slow unless catalyzed by GTPase activating proteins (GAPs). GAPs have historically been classified as negative regulators of Rho proteins, but recent evidence suggests a more complex role in Rho protein regulation for GAPs that will be discussed later. A third class of regulatory proteins, GDP-dissociation inhibitors (GDIs), may inhibit Rho protein activation by preventing exchange of GDP for GTP or by sequestering Rho proteins in the cytoplasm.

The activity of specific Rho GTPases is tightly regulated both temporally and spatially within the cell depending on the functional state of the cell [19]. The active form of Rho GTPases binds to the cell membrane where it acts upon effector proteins. This anchorage to the membrane is made possible through lipid modifications that occur post-translationally in the Golgi. Most Rho GTPases undergo geranylgeranylation, but RhoB and a number of other Rho proteins are farnesylated [31]. Extracellular cues can selectively regulate the spatiotemporal localization of Rho protein activity. For instance, using a FRET probe specific for activated RhoA, Pertz et al. found that active RhoA was localized to cell protrusions in randomly migrating fibroblasts, but this localization was absent when protrusions formed in response to a directional cue, platelet-derived growth factor (PDGF) [32].

And if the spatial, temporal, and functional complexity of regulation of Rho GTPase activity is not enough, this complexity is further amplified by the multitude of regulatory proteins. In humans, there are 20 Rho GTPases, and these are regulated by three RhoGDIs, 85 RhoGEFs, and more than 70 RhoGAPs [33]. Several Rho family members (Rnd1, Rnd2, Rnd3, RhoH, RhoBTB1, and RhoBTB2) lack GTPase activity, and are therefore constitutively active [34]. These proteins likely depend upon transcription, phosphorylation, or protein-protein interactions for regulation [35]. Other Rho proteins, Wrch1 and Rac1b, have such high GTP exchange rates that they are essentially constitutively GTP-bound [36, 37]. In addition to direct Rho regulators, Rho activation may be dependent on complex formation around scaffold proteins. Some of these scaffold proteins are Rho protein effectors themselves, including mDia, WAVE, WASP, rhotekin, and IQGAP [38], and these scaffolds may determine the specificity of Rho activation and signaling [39]. Interestingly, certain Rho GTPases are known to antagonize one another. For example, a study in NIH3T3 fibroblasts showed that Rac suppresses Rho activity by a mechanism dependent on p120 catenin and p190A RhoGAP [40, 41]. Additionally, many of the Rho regulators act on more than one Rho protein, and many of the effectors are activated by more than one Rho protein. To add to this complexity, there is also crosstalk between regulators of Rho and non-Rho GTPase pathways [41, 42]. Certain Rho proteins can also be modified by phosphorylation, such as RhoA by protein kinase A (PKA) and RhoE/Rnd3 by Rho kinase I (ROCKI) [43, 44].

MODELS TO INVESTIGATE THE ROLE OF RHO PROTEINS IN TUMORIGENESIS

Much of the evidence that Rho GTPases are involved in cancer development and progression stems from associations between tumor formation or tumor grade and Rho protein expression and activity. Although activating mutations have not been found in RhoA, RhoB, RhoC, or Rac1 [24, 45], elevated transcript levels of RhoC, Rho6, and RhoG and overexpression of RhoA, RhoB, RhoC, Rac1, and Cdc42 proteins have been observed in human breast cancer [23, 24, 26]. RhoC is also overexpressed in human inflammatory breast cancer [46]. In addition, progression of breast tumor grade was correlated with increased expression of RhoA, RhoB, and RhoC protein levels [24], and elevated transcripts of RhoC, RhoG, and Rho6 correlated with lymph node positive tumors and metastases [26]. In another study, elevated protein levels of RhoC correlated not only with breast tumor progression but also with resistance to doxorubicin [47]. The probable role of these proteins in breast cancer is discussed in the following section.

One challenge to studying Rho proteins *in vivo* is that germline knockout of several of the Rho GTPases results in embryonic lethality in mice (supplementary table in [48]). For this reason, the majority of studies investigating Rho signaling in breast cancer have been carried out using overexpression of constitutively active and dominant negative forms of the proteins in breast cancer cell lines cultured on plastic. These models are limited because they do not mimic the complex *in vivo* environment in terms of signaling molecules, extracellular matrix (ECM) components, cellular interactions, or tensional dynamics. Furthermore, the use of dominant negative or active forms of Rho GTPases in breast cancer cell lines on plastic do not recapitulate the complex stromal-epithelial interactions and accumulating genetic alterations that drive the stochastic and multistage process of tumor formation and progression. For a discussion on the strengths and limitations of different models used to investigate breast cancer see [49].

In order to test the tumorigenic properties of cells with specific genetic alterations or under particular therapeutic interventions xenograft models have often been employed. Such studies have been used to investigate Rho GTPase signaling. In this system, cancer cells are injected subcutaneously or into the epithelial-free mammary fat pads of immunocompromised mice. A closely related technique measures the ability of intravascular cancer cells to colonize distal sites to form metastases. In this model cells are injected into the lateral tail vein of the mice. Another type of xenograft model uses human cancer cells co-injected with human stromal components (the human in mouse (HIM) model), to recapitulate a human tumor within a mouse. Genetic alterations in HIM cells can replicate the same histopathological characteristics found in subtypes of human breast tumors and progress through the same disease stages [50].

Mouse models that overexpress wildtype, hyperactive, or null mutant Rho GTPases have been developed for RhoB, RhoC, Rac1, Rac2, Rac3, Cdc42, RhoH, and RhoG [35]. Knockout of Rac3, RhoG, RhoB, and RhoC are viable and/or normal, while mice with Rac2 or RhoH gene knockout show immune cell defects (supplementary table in [48]). However, knockout of other Rho family genes is embryonic lethal, necessitating conditional knockout models. For example, germline deletion of Cdc42 results in early embryonic lethality [51], and conditional floxed models are now available that have been utilized to investigate the effects of Cdc42 deletion in the skin, liver, brain, and hematopoietic system [52–58].

CELLULAR AND PATHOLOGICAL FUNCTIONS OF RHO PROTEINS

Much work has been published on the roles of different Rho GTPases on cell motility *in vitro*. Rac1 and Cdc42 are important for actin rearrangements at the leading edge of the cell, while RhoA regulates acto-myosin contraction through the downstream effectors Rho kinase (ROCK) and myosin light chain kinase (MLCK) at the rear of the cell to produce forward motion on tissue culture plastic. More recently, Rac1 and RhoA were shown to regulate mesenchymal and amoeboid invasion in three-dimensional (3D) matrices in culture [59]. Cdc42 and Rac1 are also important for establishing the polarity of epithelial cells through the tight junction-associated PAR complex [60]. Another means by which Rho proteins affect cell motility is through regulation of the extracellular matrix (ECM) by modifying matrix metalloproteinase (MMP) activity [61–63]. In addition, both Rac1 and Cdc42 are required for mammary epithelial cell differentiation in response to external cues. One study found that Rac1 activated its effector PAK in mammary epithelial cells plated on basement membrane proteins, in the absence of lactogenic hormones. Conversely, Cdc42 activated PAK independent of matrix interactions, but required hormone (hydrocortisone, insulin, prolactin) stimulation [64].

In addition to these biochemical roles, functions important in tumorigenesis and metastasis have been elucidated for Rho proteins [65, 66]. Rho proteins probably cooperate to promote tumor metastasis. In a study by Bouzahzah et al. [67] highly metastatic MTLn3 cell lines were stably transfected with dominant negative forms of RhoA, Rac1, or Cdc42. For example, each of these cell lines displayed differing effects on cell dynamics in culture. Focal adhesion formation was disrupted by expression of dominant negative (DN) RhoA and Cdc42, but not Rac1. Cell migration was reduced by expression of all three DN Rho proteins, but especially by RhoA. Cell proliferation was significantly reduced by DN Rac1 and Cdc42, but less so by RhoA. Anchorage-independent colony formation was reduced by DN Rac1 and Cdc42. DN Rac1 inhibited cell spreading on collagen whereas, DN Cdc42 and RhoA had little effect. However, despite their disparate effects on cells in culture, all three DN proteins inhibited MTLn3 intravasation and metastasis in xenograft experiments. These studies indicate that all three Rho proteins are important for metastasis.

There are a number of excellent reviews available describing the Rho proteins and their roles in tumorigenesis and cancer progression [21, 65, 66]. We will focus on Rho proteins and Rho pathway regulators and effectors that impinge most directly on breast cancer with a special emphasis on targetable proteins for drug development and on strategies already implemented to inhibit Rho protein signaling pathways.

RhoA

Perhaps the best studied of the Rho GTPases, RhoA has been shown to be involved with a multitude of cellular processes. However, the role of RhoA in tumorigenesis and tumor progression is not entirely clear. RhoA protein is overexpressed in human breast cancer and does not correlate with tumor suppressor p53 or ERBB2/HER2 oncogene status [23, 24]. Curiously, high RhoA expression was found in circulating lymphocytes of breast cancer patients whose tumors overexpress RhoA [68]. In addition to effects on the cytoskeleton, RhoA was shown to activate the mitogen ERK by a pathway involving MMPs and the epidermal growth factor (EGF) receptor [63]. Importantly, primary human mammary epithelial cells (hMECs) experimentally induced to overexpress wildtype or constitutively active RhoA underwent immortalization in cell culture. Interestingly, this ability of RhoA was independent of such well-known RhoA effectors as ROCK, mDia, and PKN [69].

One mechanism by which RhoA may promote tumorigenesis indirectly is through its effects on the estrogen receptor (ER α). RhoA activation causes polymerization of actin, resulting in

release of megakaryoblastic leukemia (MKL)-1, which acts as a co-regulator with ER α and alters its transcriptional functions. In addition, endogenous MKL-1 was found on estrogen response element (ERE)-containing genes in MCF-7 breast cancer cells [70]. As further evidence that RhoA is an oncogene, a recent study revealed that microRNA-31 (miR-31) expression, which functions to suppress RhoA transcription, is inversely correlated with metastasis both in breast cancer cell lines and in human tumors. The same study also found higher levels of RhoA expression in metastases of human breast cancer than in the primary tumors [71].

On the other hand, studies have also shown that RhoA suppresses certain carcinogenic processes. For example, overexpression of RhoA-GTP promotes cortical actin filament formation and dephosphorylation of focal adhesion kinases (FAK) resulting in reduced cell spreading on tissue culture plastic and rounded morphology [63]. Consistent with these activities, a siRNA study that knocked down RhoA expression showed that RhoA suppresses the invasive properties of SUM-159 breast cancer cells [72]. One possible explanation for these results is that altered Rho activity may have differential effects on tumorigenic processes depending on the stage and subtype of breast tumor from which the cells were derived. Furthermore, these studies highlight the need for a more complete understanding of Rho activity and signaling in different stages and subtypes of breast cancer.

RhoB

RhoB is an unusual member of the Rho family. The carboxyl-terminal end of this protein can be modified by either farnesylation or geranylgeranylation, though generally it is farnesylated. Unlike other Rho family members that have been primarily implicated as oncogenes, RhoB is unusual because it appears to have tumor suppressor function. A recent study investigated low RhoB-expressing ovarian cancer cell lines injected into the peritoneum of mice. Ectopic expression of RhoB inhibited tumor growth [73]. Interestingly, another study utilized Ras-transformed NIH3T3 fibroblasts expressing different mutants of RhoB and transplanted these cells subcutaneously into nude mice. The geranylgeranylated (GG) form of active RhoB suppressed tumor growth, whereas the farnesylated (F) form promoted tumor growth [74]. Treatment of invasive breast cancer cells with a farnesyl transferase inhibitor (FTI) was shown to increase the levels of RhoB-GG [75], suggesting that differential prenylation of RhoB may be one mechanism of FTI anti-tumor activity. These results suggest that the specific lipid modification is another regulatory mechanism that can markedly alter Rho GTPase function, which has important implications for the design and use of FTIs for treatment of cancer.

RhoC

RhoC was implicated in a screen of genes whose expression was altered in inflammatory breast cancer, a disease marked by aggressive invasion and rapid progression to metastasis [46]. In immortalized human mammary epithelial cells (hMECs) and SUM-159 breast cancer cells RhoC promotes motility and invasion, and in mice RhoC promotes metastasis [72, 76, 77]. Orthotopic injection of hMECs overexpressing RhoC into the mammary fat pad of athymic mice resulted in tumor formation suggesting that RhoC is capable of promoting malignant transformation [76]. RhoC is overexpressed in human breast cancers [26, 46], and similar to RhoA, the level of RhoC expression positively correlates with disease progression [26, 47]. In addition, a *rhoC* knockout mouse has been developed and crossed with the MMTV-Polyoma Middle T (PyMT) mouse mammary tumor model. In this system RhoC deficiency did not impact normal mammary gland development or tumorigenesis, but significantly inhibited metastasis [77]. These data implicate RhoC as an important mediator of metastasis, especially in a specific subtype of breast cancer, inflammatory breast cancer.

Rac1

The Rho protein Rac1 regulates cell cycle progression, cell polarity, motility, and invasion. For example, Rac1 regulates cell proliferation through effects on cyclin D1. Fournier *et al.* [78] demonstrated that Rac1 is an important mediator of proliferation downstream of E-cadherin signaling in MCF-10A cells. Furthermore, Rac1 mediates lamellopodia formation via actin rearrangement in cell culture [79, 80]. Rac1 is known to drive important functions related to epithelial-to-mesenchymal transition (EMT), which is thought to play a causative role in tumor progression. Studies in breast cancer cells have shown that Rac1 overexpression promotes loss of epithelial cell polarity, lamellipodia formation, cell migration and invasion, and increased metastatic potential [81–83]. These studies suggest that Rac1 contributes to tumorigenesis via pleiotropic mechanisms.

In vivo data corroborates the notion that Rac1 promotes tumorigenesis. A detailed study of Rac1 expression in human breast cancer tissues found that mRNA and protein levels were elevated in tumor tissue compared to normal tissue [37]. In addition, immunohistochemical (IHC) staining demonstrated increased Rac1 staining in benign fibrocystic breast disease and hyperplasias compared to quiescent terminal ductal lobular units, suggesting a role for Rac1 in the earliest stages of human breast tumor formation. Ductal carcinoma *in situ* (DCIS) samples showed strong Rac1 staining, but there was no difference in staining intensity between lymph node metastases and their corresponding primary tumor, suggesting that further upregulation of Rac1 is unnecessary to promote metastasis. Disease-free survival was increased for patients whose tumor tissue showed elevated, but diffuse cytoplasmic staining for Rac1 versus strong, membrane-associated staining. These data suggest that hyperactivation of Rac1 may play an equally important role in disease progression as elevated protein levels.

Although no activating mutations have been discovered in the Rac1 gene, a transcriptional variant, Rac1b, has been described [84]. This isoform has a 19-amino acid insertion including two threonine residues that may be phosphorylated by casein kinase II and protein kinase C (PKC). Rac1b is overexpressed relative to Rac1 in colon cancer [84]. Rac1b is also expressed in breast cancer tissues [37, 84]. In assays using radiolabeled GTP, purified Rac1b showed an increased rate of GTP exchange, suggesting that it has an increased activity level relative to Rac1 [37]. In more recent studies, activation of Rac1b downstream of MMP-3 induced reactive oxygen species (ROS) signaling leading to genomic instability, Snail transcription factor activity, and consequent EMT [62].

Rac3

Closely related to Rac1, Rac3 shows similar cellular functions including promotion of migration, invasion, and metastatic potential when expressed in a constitutively active form [81]. siRNA knockdown of Rac3 in SNB19 glioblastoma cells and BT549 breast cancer cells demonstrated that it plays a significant role in cell invasion, but had a smaller impact on migration and proliferation when compared to Rac1 [82]. In addition, Rac3-GTP was found to be elevated in human metastatic breast cancer cell lines and was demonstrated to induce cell proliferation through its effectors p21-activated kinase (PAK) and Jun kinase (JNK) [85].

Cdc42

Similar to RhoA, RhoC, and Rac1, Cdc42 is also upregulated and hyperactivated in invasive human breast tumors compared to normal tissue from the same patient [23, 24]. Cdc42, is a major regulator of polarization and formation of invasive cytoplasmic protrusions called invadopodia. In mammary epithelial and breast cancer cells overexpression of Cdc42 has been shown to induce loss of epithelial cell polarity, promote cell motility, stimulate PI3

kinase activity, and stimulate ERK activation through the EGF receptor [83, 86, 87]. Cdc42 and Rac1 activate many of the same effector proteins [38], and thus it is not completely clear how they mediate differential effects on cellular processes. In T47D breast cancer cells, Cdc42, but not Rac1, activates the non-receptor tyrosine kinase Ack1 (activated Cdc42-associated kinase 1), which phosphorylates FAK at Tyr 397 promoting formation of the Crk/p130^{Cas} complex associated with focal adhesions, which in turn activates Rac1 and cell motility [86].

Cdc42 associates with the polarity-establishing Par6/atypical protein kinase C (aPKC) complex located at tight junctions that connect adjacent epithelial cells near their apical surface. Besides establishing polarity, Cdc42 participates in cell proliferation and mitosis (reviewed in [35]). For example, Hirsch et al. [87] demonstrated that Cdc42 prevents EGF receptor degradation and therefore regulates mitogenic signals. In addition, overexpression of Par6, which is upregulated in breast cancer cell lines as well as human breast cancers, was demonstrated to increase cell proliferation via aPKC and Cdc42 in a manner independent of epidermal growth factor (EGF) signaling [88].

RhoE/Rnd3

One of the so-called “atypical” Rho GTPases, RhoE/Rnd3 lacks GTPase activity and is therefore constitutively active. Thus, unlike the canonical Rho GTPases, it is not regulated by GEFs and GAPs [34]. RhoE antagonizes RhoA activity and promotes apical junction formation to restore cell polarity in mammary tumor cells overexpressing constitutively active RhoA [89]. RhoE also inhibits ROCK1 activity [90], and ROCK1 reciprocally phosphorylates RhoE [44]. RhoE and the related Rnd proteins Rnd1 and Rnd2, were found to bind the middle domain of p190B RhoGAP that is distinct from its GAP domain and GTPase domain [91]. Rnd protein overexpression in fibroblasts leads to the collapse of actin stress-fibers and “rounding-up” of the cells. This rounded morphology was reduced in p190A or p190B RhoGAP null fibroblasts overexpressing Rnd1 or Rnd3, indicating that the morphological effects of Rnd proteins are at least partially dependent on p190-A and p190-B. Taken together, a putative effect of RhoE/Rnd3 overexpression may be suppression of the proto-oncogenic RhoA/ROCK pathway. These activities of RhoE may impact human breast cancer development. For example, a small microarray study demonstrated that RhoE mRNA was overexpressed almost 10-fold in normal mammary epithelium of parous women versus non-parous women [92]. The effects of parity and the resultant gene expression changes that contribute to the reduced breast cancer risk for parous women has been a long-standing interest in the breast cancer research community (For review see [93]). Although further studies are required, these data suggest that RhoE may be one gene that contributes to the decreased breast cancer risk associated with parity (reviewed in [93]).

Other Rho GTPases

Other lesser-known Rho proteins may nevertheless play important roles in tumor development. Transcripts of Rho6 and RhoG were upregulated in breast cancer tissue compared to normal tissue [26], and RhoBTB2 was deleted in some breast tumors and a large number of breast cancer cell lines [94]. These data suggest that similar to RhoB, RhoBTB2 may function as a tumor suppressor in certain stages or subtypes of breast cancer, and further highlight the complexity of Rho GTPases in breast cancer pathology.

REGULATORS OF RHO GTPASES

Rho proteins are regulated by guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs) and GDP dissociation inhibitors (GDIs). Several of these

regulatory proteins may be important in tumorigenesis and/or tumor progression in breast cancer.

GEFs

RhoGEFs are important positive regulators of Rho protein activity and are frequently found to be upregulated in cancer. For example, MyoGEF activates RhoA and RhoC, proteins associated with metastatic breast cancer. MyoGEF localized to the leading edge of migrating cells and increased invasion of MDA-MB-231 cells in culture [50]. Also, the RhoGEF Dbp, or its hyperactive cleaved form, onco-Dbp, stimulated Cdc42 and Rac1 activities to promote T47D breast cancer cell motility [86]. High expression levels of the RhoGEF Trio was associated with poor prognosis in breast cancer patients, and two other GEFs, Vav1 and Tiam1, showed a similar trend [27]. Additionally, a genetic polymorphism (Lys526Gln), which may be an activating mutation in the RhoGEF AKAP13, was correlated with familial breast cancer. Expression of the RhoGEF Fgd1 correlated with aggressive breast and prostate cancer [95]. Fgd1 was localized to sites where invadopodia would form ahead of fibrillar actin (F-actin) formation in A375MM melanoma cells, suggesting a role in tumor cell invasion. Knockdown of Fgd1 expression led to reduced invadopodia formation, reduced matrix degradation, and decreased Cdc42-GTP, whereas constitutively active Fgd1 increased invadopodia and matrix degradation. Invasion in this system was found to be dependent on Cdc42 but not Rho or Rac. Thus, RhoGEFs are altered in human breast cancers and affect Rho GTPase dependent cell processes that contribute to breast tumorigenesis and invasion.

Recent studies from the Band laboratories demonstrated that overexpression of the RhoGEF, Vav2, in immortalized hMECs disrupts mammary acinar architecture and promotes invasion in 3D culture [96]. These effects were dependent on Rac1 and Cdc42 because their knockdown abrogated the phenotype. Interestingly, knockdown of RhoA intensified the aberrant acinar morphogenesis and invasive phenotype. These results indicate that Rho GTPases are likely critical for normal MEC morphogenesis and that disruption of their activities is sufficient to induce loss of polarity, abnormal proliferation, and invasion, all of which contribute to tumorigenesis. Furthermore, these studies highlight the complexity of Rho GTPase function during MEC morphogenesis and demonstrate unexpected roles for these signaling molecules during mammary morphogenesis.

Caution should be exercised when generalizing the functions of Rho pathway regulators in tumorigenesis. For example, the Rac-specific GEF Tiam1 promotes tumorigenicity in an oncogene-dependent manner. Knockout of Tiam1 in MMTV-Neu mice delayed the onset of tumors and reduced the number of metastases, but knockout of Tiam1 in MMTV-myc mice had no effect [97]. In fact, others have found that Tiam1 levels decreased with increased breast tumor grade and that Tiam1 expression negatively correlated with tumor size, proliferation, expression of plasminogen activator inhibitor-1 (PAI-1), and tumor suppressor p53 status. Low Tiam1 levels were associated with low estrogen receptor (ER) expression, and Tiam1 was more highly expressed in tumors of lobular origin than ductal or mixed background [28]. Thus, depending on the stage and subtype of breast cancer, altered GEF expression may have differential effects on tumor formation and progression.

GAPs

RhoGAPs have traditionally been classified as negative regulators of Rho proteins because of their GTPase activating function that facilitates conversion of the active GTP bound to inactive GDP bound Rho protein. GAPs themselves are regulated by intramolecular interactions, protein-protein binding, protein-phospholipid binding, phosphorylation,

localization within the cell, and proteolysis. Unlike Rho GTPases, mutations in GAPs have been found that underlie disease (reviewed in [98]).

In general, RhoGAPs, which deactivate Rho proteins, are believed to inhibit the tumorigenic and metastatic processes promoted by Rho GTPase and RhoGEF overexpression. For example, the RhoGAP DLC negatively correlated with tumor progression in breast cancer patients [99], and knockdown of DLC-1 by RNAi in myc-transformed hepatoblasts orthotopically injected into mice caused increased RhoA activity and formation of hepatocellular carcinoma [100]. The authors suggest that loss of DLC-1 may create a targetable dependence on RhoA. As another example, the RacGAP β 2-chimerin inhibits Rac activation, cyclin D1 expression, and retinoblastoma protein (pRb) phosphorylation and induces G₁ cell cycle arrest. In addition, β 2-chimerin expression was reduced in human breast cancer cell lines and tumors [101]. The RhoGAP p190A has been shown to cooperate with Ect2 to regulate RhoA activity at the cleavage furrow in MDA-MB-468 breast cancer cells during cytokinesis, and overexpression of p190A resulted in failed cytokinesis and multinucleated cells [102, 103]. Similarly, overexpression of the related RhoGAP, p190B, in MCF-7 breast cancer cells induces multipolar spindle formation, failed cytokinesis, and multinucleation (T Vargo-Gogola and JM Rosen, unpublished results). Because aneuploidy can be both tumor promoting and tumor suppressive [104, 105], more studies will be required to determine whether the effects of altered p190A and p190B RhoGAP expression during mitosis and cytokinesis enhance or inhibit mammary tumorigenesis.

On the other hand, some GAPs may act as oncogenes and even stimulate Rho protein activity *in vivo*. For example, a study of the RhoGAP ARHGAP8 found that it was transcriptionally overexpressed in the majority of colorectal tumors tested [106], suggesting a correlation between tumorigenesis and ARHGAP8 expression. In a recent study from our laboratory p190B RhoGAP deficiency was shown to suppress mammary tumorigenesis, angiogenesis and lung metastasis in MMTV-Neu mice. These phenotypes were associated with decreased activity of Rac1 [29]. Conversely, p190B overexpression in MMTV-Neu mice resulted in increased tumor formation and metastasis, which was associated with elevated Rac1 activity (Vargo-Gogola & Rosen unpublished results). Together these data suggest that p190B has unpredicted effects on Rho GTPases *in vivo* and that it may act as an oncogene. Computational modeling has suggested that GAP activity regulates Rho GTP cycling rates to promote optimal GTPase activity [107], and increased Rho GTPase cycling rates enhance transformation and tumorigenicity of NIH3T3 fibroblasts [108]. Because activating mutations have not been found in Rho GTPases in human breast cancers [24, 45], it is likely that the balance between GEF and GAP expression plays an important role in driving the oncogenic actions of the Rho GTPases.

GDI

RhoGDIs constitute a smaller, but no less important, class of regulators that can inactivate Rho proteins by binding to the inactive GDP-bound form and preventing activation (reviewed in [109]). RhoGDIs may act as chaperone proteins to ensure correct localization of the isoprenylated Rho protein as it exits the Golgi. A study by Lin *et al.* found that mutating residue 66 of Cdc42 prevented its binding to RhoGDI and caused accumulation of Cdc42 in perinuclear microsomes. Expression of constitutively active Cdc42 expressed in NIH-3T3 fibroblasts caused transformation. However, expression of constitutively active Cdc42 RhoGDI-binding mutant or expression of constitutively active Cdc42 in the presence of siRNA-mediated knockdown of RhoGDI prevented transformation. These results suggest that the interaction between Cdc42 and RhoGDI is essential for Cdc42 exit from the Golgi and later activity [110]. In addition to binding to Rho protein-GDP, RhoGDIs can bind to GTP-bound Rho proteins *in vitro*, but this may not be relevant *in vivo*, since Rho-GDI was found to bind only non-membrane associated (GDP-bound) Cdc42 in cells [111].

Surprisingly, although RhoGDIs have traditionally been thought to inhibit activation of Rho proteins, they also play a positive role in regulating a wide range of GTPase activities (reviewed in [112]). Indeed, in a number of studies RhoGDIs have demonstrated a positive role in tumorigenesis. For example, RhoGDI α was found in a screen for differentially expressed proteins in breast cancer cell lines [113] and was overexpressed in a small sample of breast tumors [23, 24]. RhoGDI α blocked caspase 3-dependent cleavage of Rac1 [114], thus promoting cancer cell survival. Another RhoGDI, D4-GDI (RhoGDI β), increased β_1 -integrin expression and breast cancer cell invasion *in vitro* [115]. In addition, D4-GDI bound selectively to Rac1 and Rac3, and siRNA knockdown of D4-GDI caused activation of Rac1 *in vitro* and inhibited tumor growth and lung metastasis of highly invasive MDA-MB-231 breast cancer cells *in vivo* [116]. These tumor-promoting effects may arise from the ability of RhoGDIs to bind active Rho proteins, thereby constitutively activating them. Alternatively, RhoGDIs may function as chaperones to increase the cytoplasmic pool of Rho proteins. An interesting finding, especially when considering Rho signaling pathways as potential drug targets, is that RhoGDI overexpression induced resistance to cancer drugs (etoposide and doxorubicin) in MDA-MB-231 breast cancer and JLP-119 lymphoma cells [114].

While the studies described above showed RhoGDI overexpression in breast cancers and positive effects on tumorigenic processes, a separate study in patients who underwent mastectomy found that mRNA levels of RhoGDI γ were reduced in breast tumor tissue and that reduced protein levels of RhoGDI α and RhoGDI γ correlated with lymph node positive tumors and metastases [26]. The authors of these studies did not report the subtype and histopathological grade of the tumors under investigation. It is possible that dysregulation of RhoGDIs during different stages of disease or in distinct subtypes of breast cancer may impact whether RhoGDIs have oncogenic or tumor suppressive actions.

RhoGDIs also have pleiotropic functions, and this may be one explanation for their counterintuitive effects in tumorigenesis. For example, a study in MCF-7 cells using coimmunoprecipitation and mobility shift assays demonstrated that RhoGDI α binds directly with the estrogen receptor (ER α) in the nucleus, modifies its interaction with the estrogen receptor element (ERE) in target genes, and alters transcription of ERE reporter plasmids. In addition, siRNA knockdown of RhoGDI α modified the expression of genes downstream of ER α , namely the progesterone receptor (PR) and pS2 [117]. Upregulation of RhoGDI α may, therefore, promote tumor progression by disrupting estrogen signaling. Another possible explanation for its pleiotropic effects is that upregulation of RhoGDI may alter the relative stability of Rho proteins. A recent study showed that RhoB protein levels were suppressed by RhoA levels, and overexpression of RhoGDI α increased RhoB levels by increasing its half-life [118]. Thus, RhoGDI may be a limiting factor in Rho protein stabilization thereby shifting the relative influence of specific Rho proteins in the cell.

EFFECTOR PROTEINS OF RHO GTPASES

Active (GTP-bound) Rho GTPases bind and activate approximately 45 effector proteins including Ser/Thr, Tyr, and lipid kinases, at least one phosphatase, lipases, and numerous scaffold proteins (reviewed in [38]). The p21-activated kinase (PAK) family of effectors is regulated by an autoinhibitory domain that is deactivated by a conformational change induced by members of the Rac and Cdc42 subfamily of Rho proteins [119]. The mechanism of activation of other Rho protein effectors is not well understood.

Rho-associated kinases

One of the best-characterized effectors of Rho proteins are the Rho-associated kinases (ROK or ROCK) I and II. ROCKs are members of the PKA/PKG/PKC family of serine/threonine

kinases, which have become increasingly attractive targets for treatment of a variety of diseases including cancer (for review see [120]). In one study, ROCK I and II were found to be more highly expressed in metastatic breast cancer cell lines than in non-metastatic cell lines, and in human tumors ROCK I overexpression correlated with increased tumor grade [121]. In another study, only elevated levels of ROCK I were found in tumor tissue compared to normal tissue, while increased tumor grade, increased stage, and poor prognosis were inversely correlated with ROCK II mRNA expression [122], suggesting diverse tumor-related activities for these two proteins. ROCK is a target of both RhoA and RhoC and is well known for inducing stress fiber formation and actomyosin contraction in fibroblasts. In studies of endothelial cells using the ROCK inhibitors H-1152 and fasudil, ROCK proteins negatively regulated protein activities important for EMT (MT1-MMP and MMP2) and for cell growth and survival (PI3K/Akt) [123, 124]. However, these inhibitor studies did not distinguish between the two forms of ROCK. Overexpression of the ROCK kinase domain conferred metastatic potential in MCF-7 cells, which are poorly invasive *in vivo*. Conversely, inhibition using Y-27632 or shRNA knockdown of ROCK I suppressed bone metastasis from metastatic SUM1315 cells [121]. Knockdown of ROCK I or ROCK II also inhibited the motility and invasiveness of aggressive MDA-MB-231 breast cancer cells. However, ROCK I/II knockdown did not significantly alter MDA-MB-231 tumor growth *in vivo* [122]. This study did not evaluate the ability of MDA-MB-231 cells to metastasize under altered conditions of ROCK I/II expression.

Another potential mechanism by which ROCK II may contribute to breast cancer growth and development is by affecting mitosis. ROCK II acts downstream of cyclinE/cdk2 and nucleophosmin/B23 to regulate centrosome duplication [125]. Centrosomes were amplified when ROCK II was overexpressed, and this can lead to aberrant mitotic spindle assembly, and ultimately, aneuploidy. These studies further support an important role for the Rho signaling network during mitosis and cytokinesis, and it is attractive to speculate that aberrations in these processes due to altered Rho signaling may promote genomic instability thereby facilitating breast tumor formation and progression.

Another intriguing role for one or both ROCK proteins in mammary tumors is that of realignment of extracellular matrix (ECM) collagen fibers. Collagen density has been associated with breast cancer risk [126], and it was shown that increased collagen density *in vitro* increases matrix stiffness, activates Rho proteins, elevates focal adhesion kinase (FAK) signaling, promotes epithelial cell proliferation, and stimulates invasion by non-transformed mammary epithelial cells (MECs) [127]. Likewise, *in vivo* data from MMTV-Neu mice showed that stiffness of the mammary gland tissue, along with the presence of linearized collagen fibers, correlated with tumor progression [128]. In another study, multiphoton second-harmonic generation (SHG) imaging revealed that tumor cells invade into the ECM at sites where collagen fibers are aligned perpendicular to the border of the cell mass. Studies with tumor cells grown in three-dimensional (3D) collagen gels demonstrated that the Rho/ROCK/myosin pathway is essential for realignment of the collagen fibers, but when the fibers are pre-aligned this pathway is not necessary for tumor cell migration [129]. The authors conclude that Rho/ROCK signaling is important for initial stages of tumor invasion, though it may be dispensable for later stages of tumor cell motility. This study was carried out with the ROCK inhibitor H-1152, and did not distinguish between ROCK I and ROCK II.

P21-activated kinases

Another family of well-known Rac1/Cdc42 effector proteins are the p21-activated kinases (PAK1–6), which have been implicated in the growth and progression of various types of cancer including breast cancer (for review see [130, 131]). The gene for PAK1 is located on chromosome 11q13, which is amplified in human breast cancers [132], and PAK1 protein is

overexpressed in human breast cancers [133, 134]. Wang *et al.* [135] found a correlation between PAK1 activity and tumor progression in the polyoma middle T (PyMT) mouse mammary tumor model. Localization of phosphorylated PAK1 changed from nuclear in normal luminal epithelium to mostly cytoplasmic in hyperplasias to nuclear again (and more strongly expressed) in adenomas and adenocarcinomas, suggesting that PAK1 may play different roles at different stages of tumor development. Additional data suggest that PAK1 plays an important role in early tumorigenesis. Expression of constitutively active PAK1 in the mouse mammary gland resulted in hyperplastic lesions associated with increased cyclin D1 protein expression [134]. Constitutively active PAK1 induced a low level of spontaneous mammary tumor formation in hormonally stimulated mice, suggesting that increased PAK activity promotes tumorigenesis [135]. Another study used an inhibitory peptide directed against PAK1 and showed that PAK1 is necessary for angiogenesis *in vitro* and *in vivo* [136]. Intriguingly, Pak1 translocated to the nucleus of MCF-7 breast cancer cells upon stimulation with EGF, where it bound to the promoter regions of several genes and altered their transcription [137], suggesting that PAK proteins may have pleiotropic effects beyond its canonical cytoplasmic functions. Lastly, PAK1 has been implicated in centrosome function and assembly of the mitotic spindle [138], and altered PAK1 expression perturbs spindle formation and mitotic progression [139]. Thus, when altered in breast cancer, PAK1 may contribute to genomic instability to facilitate breast cancer growth and progression.

PAK2 has been implicated in breast cancer cell invasion, proliferation, and survival. For example, overexpression of PAK2 promoted loss of contact inhibition and anchorage independent growth of breast cancer cells. Breast cancer cells that expressed low levels of PAK2 were more susceptible to anticancer drug-induced apoptosis as compared to breast cancer cell lines expressing high levels of PAK2. A cleaved form of PAK2 produced by active caspase-3 in these cells is responsible for the increase in cell death, and elevated expression of PAK2 inhibited caspase-3 expression allowing for resistance to chemotherapeutics and anchorage independent growth [140]. PAK2 as well as PAK1 promotes T47D breast cancer cell invasion, however, the molecular mechanisms by which PAK1 and PAK2 regulate invasion in these cells are distinct [141]. Thus, PAK2 may also contribute to the development and progression of breast cancer, however, whether PAK2 expression is altered in human breast tumors is currently unknown.

IQGAPs

IQGAPs are a family consisting of three proteins, which contrary to the implications of their name, function to stabilize GTP-bound Rho proteins. IQGAP proteins do not possess GAP activity. Rather, they bind to Rac1-GTP and Cdc42-GTP and inhibit their intrinsic GTPase activities, thereby maintaining them in their active states [142, 143]. Paradoxically, IQGAP1 has been implicated as an oncogene, whereas IQGAP2 functions as a tumor suppressor (for review see [144]). IQGAPs act as scaffold proteins at cell-cell junctions to coordinate numerous structural and signaling activities. A recent study showed that IQGAP1 protein levels were elevated in limited set of invasive breast carcinoma tissues as well as aggressive breast cancer cell lines. More importantly, overexpression of IQGAP1 in non-metastatic MCF-7 breast cancer cells produced invasive tumors when implanted into nude mice [145]. IQGAP2 and IQGAP3 are not as well characterized as IQGAP1, and to date no studies have evaluated their expression or role in breast cancer.

PKN1

Protein kinase N1 (PKN1) is another Rho effector protein that may also affect tumor progression. PKN1 was shown to participate in the sealing of tight junctions between cells in the mammary gland. Constitutively active PKN1 in the mouse mammary gland impaired alveolar development during pregnancy, prevented lactation, and induced premature

involution [146]. Radiolabeled sucrose solution injected into the milk ducts on day 1 of lactation was detected at higher levels in the blood of PKN1 transgenic mice compared to control mice, suggesting that the mammary epithelium in the transgenic glands was more permeable. A further experiment in Eph4 mammary epithelial cells showed that constitutively active PKN1 decreases, and dominant negative PKN1 increases, the rate of tight junction sealing [146]. Loss of tight junction integrity may play a role in epithelial-to-mesenchymal transition or in cancer cell intra/extravasation between endothelial cells.

CURRENT INHIBITORS OF RHO GTPASE SIGNALING

A number of strategies have been employed to inhibit the functions of Rho proteins (reviewed in [21]). Small molecule inhibitors of Rho GTPase signaling are summarized in Table 1.

Rho GTPases are dependent on membrane localization for their activity. Therefore inhibition of isoprenylation can block Rho protein signaling. Most of the well-studied Rho GTPases, including RhoA, Rac1, and Cdc42, are tethered to the cell membrane via a geranylgeranyl group enzymatically added to a CAAX motif at their carboxyl terminus. RhoB, on the other hand, is generally farnesylated. Furthermore, a systematic study of post-translational modifications of Rho GTPases demonstrated that Rnd1, Rnd2, Rnd3, TC10, TCL, and RhoD are all selectively farnesylated, while RhoH/TTF can be alternately farnesylated or geranylgeranylated and RhoF/Rif molecules exist with either modification simultaneously in two cellular pools [31].

Because of the important role of geranylgeranyltransferase (GGT) in Rho protein biology, GGT inhibitors (GGTIs) have been developed to inhibit their activation. For example, GGTI-298 was shown to inhibit the membrane localization of RhoA and RhoC and to prevent transendothelial migration in culture [147]. In addition, GGT deficiency in mice suppressed K-Ras induced lung cancer [148]. However, specificity is always a concern when studying prenylation inhibitors. Ras proteins are generally farnesylated but can be geranylgeranylated under certain circumstances. For example, the overexpressed oncogene K-Ras became geranylgeranylated in fibroblasts following treatment with farnesyltransferase inhibitors [149].

Farnesyltransferase inhibitors (FTIs) such as tipifarnib (R115777), which is in phase II clinical trials to treat breast cancer and other malignancies, were first developed to inhibit oncogenic Ras. However, these compounds can also alter the activity of RhoB (and likely several other Rho GTPases), which, similar to Ras, is generally farnesylated. Treatment of invasive breast cancer cells overexpressing RhoC with FTI L-744,832 reversed the invasive phenotype. Interestingly, this effect was due to increased geranylgeranylated RhoB [75], which functions as a tumor suppressor. Furthermore, both FTI-277 and GGTI-298 were found to transcriptionally upregulate RhoB expression in several cell lines, including MCF-7 and MDA-MB-231 breast cancer cells [150], suggesting a second mechanism by which these inhibitors may increase RhoB tumor suppressor activity.

Another strategy takes advantage of the ability of Rho GTPases to modify response to therapy. For example, in a recent study, a trastuzumab resistant clone of SKBR3 cells showed increased Rac1 activity and decreased endocytosis of ErbB2, resulting in accumulation of ErbB2 at the cell membrane and increased downstream ERK signaling. Treatment of the trastuzumab-resistant cells with the Rac1 inhibitor NSC23766 increased endocytosis of ErbB2 and decreased ERK signaling [151]. The authors conclude that combination treatment with trastuzumab and NSC23766 may improve long-term outcome for breast cancer patients with ErbB2 positive tumors.

HMG-CoA-reductase inhibitors (“statin” drugs) inhibit both types of prenylation post-translational modifications in Rho and Ras proteins. Since statins inhibit the potent oncogene Ras as well as Rho proteins, many of which are oncogenic as well, these drugs seem promising as possible cancer treatments. In addition, statins have been prescribed for a number of years to lower serum cholesterol and are well tolerated by patients. In cell culture experiments, fluvastatin blocked membrane localization of RhoA and RhoC [147]. Lovastatin decreased adhesion and migration of aggressive F3II mouse mammary carcinoma cells. Importantly, *in vivo* lovastatin (10 mg/kg/day) intraperitoneal injections reduced the number of metastases in BALB/c mice inoculated intravenously with F3II cells [152]. So far, however, clinical findings of the effects of statins on cancer have been modest or inconclusive [153, 154]. A related strategy inhibits isoprenylcysteine carboxymethyltransferase (ICMT), the enzyme that catalyzes the final methylation step of isoprenylation. The ICMT inhibitor cismethynil blocked the activities of RhoA and Rac1 and increased their binding to RhoGDI. This resulted in reduced cell spreading, adhesion, and migration of aggressive MDA-MB-231 breast cancer cells [155].

Another potential therapeutic opportunity is to inhibit effector proteins activated by Rho GTPases. ROCK has been a popular target for kinase inhibitor development because of its important role in cell motility downstream of RhoA and RhoC. ROCK inhibitors include HA-1077 (fasudil) [156], H-1152 [157], and Y-27632. Y-27632 blocked phosphorylation of cofilin and promoted E-cadherin expression and cell adhesion [158]. Wf-536, a more potent derivative of Y-27632, inhibited cell motility *in vitro* and angiogenesis and metastasis *in vivo* [159, 160]. Fasudil inhibited tumor formation in three types of orthotopic tumor models and is well tolerated by patients in which it is currently used to treat cerebral vasospasm [161]. However, fasudil also inhibits protein kinase N (PKN) [162], another Rho effector protein. Thus, it is possible that fasudil’s anti-tumor activity is due to inhibition of both ROCK and PKN. Another small molecule inhibitor, IPA-3, was recently described that bound specifically to the autoinhibitory domain of PAK. This inhibitor blocked PAK-regulated morphological changes in cells [163]. Another inhibitor, wiskostatin, has also been described for the Cdc42 effector N-WASP, which nucleates actin fibers [164]. FIPI, a small molecule inhibitor of the phosphatidic acid-producing enzyme phospholipase D, which is an effector of Rho proteins, has been developed as well [165]. Neither wiskostatin nor FIPI have been tested for their effects on solid tumors. PI3K interacts with and may also be an effector of Rac and Cdc42. Owing to its important role in a number of cancer types, including breast cancer, a large number of small molecule inhibitors have been developed to inhibit PI3K (see Table 1). Several of these lead compounds have progressed to clinical trials [166–170].

Inhibitors of GEFs, GAPs and GDIs may be of particular interest for inhibitor development because of their ability to regulate Rho protein activity and intracellular distribution. However, few small molecule inhibitors of Rho regulators have been discovered thus far. These regulatory proteins present a challenge to cell biologists because of the complex network of signaling crosstalk and redundancy. One limiting factor in drug development targeted at these regulators is no doubt our limited knowledge of how these proteins interact *in vivo*.

CONCLUSIONS

Rho family GTPases may present a significant opportunity to inhibit tumorigenesis and prevent metastasis. Overexpression or hyperactivation of many Rho proteins and their upstream activators, GEFs, is associated with epithelial cell transformation, hyperplasia, angiogenesis, tumor growth, invasion, and metastasis. However, some Rho proteins may have tumor suppressor functions. Some GAPs have been shown to inhibit tumorigenesis,

while others apparently promote cancer progression. The GEFs studied so far appear to promote tumorigenesis, but it is not clear whether all GEFs can be classified as proto-oncogenes. GDIs have traditionally been classified as inhibitors of Rho proteins; however, their role in Rho GTPase function may be much more nuanced.

Significant challenges remain with respect to understanding the role of the Rho signaling network in breast tumorigenesis. Despite the strong associations of elevated Rho protein activity with tumor progression, we still have scant direct evidence of their role in the stochastic process of tumor development and progression *in vivo*. As more animal models are developed, the role of these proteins in tumorigenesis will become clearer. Another limitation is our lack of understanding the functions of Rho proteins spatiotemporally within the cell and in the complex *in vivo* environment. The use of novel *in vivo* imaging technology is beginning to unravel these mysteries [171–173]. Finally, a complete understanding of the expression patterns of the Rho GTPases, their regulators, and effectors in different stages and subtypes of breast cancer will be required. These challenges to our understanding of Rho signaling biology need to be met before we can effectively design and utilize therapeutics to inhibit the tumorigenic and metastatic processes they regulate.

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Figure 1.
Hypothetical model of Rho family GTPases involved with progressive stages of breast carcinoma.

Figure 2.

The Rho GTPase cycle and current inhibitory strategies.

1: Rho protein bound to GDP and GDP dissociation inhibitor (GDI) remains inactive and sequestered in the cytoplasm. 2: Guanine nucleotide exchange factor (GEF) exchanges GTP for GDP. 3: The active GTP-bound Rho protein binds and activates effector proteins. 4: GTPase activating protein (GAP) hydrolyzes GTP to GDP, returning the Rho protein to its inactive state. 5: Balance between GEF and GAP activity is critical for maintaining rapid cycling and optimal Rho protein activity.

Table 1

Current small molecule compounds with activity against Rho protein signaling pathways.

Small molecule	Target	Molecular action	Biological function	Reference
Fluvastatin [†]	All prenylated proteins [*]	Disrupts membrane localization		[147]
Lovastatin [†]	All prenylated proteins [*]	Disrupts membrane localization	Suppresses metastasis of F3II cells in mice	[152]
Cysmethynil	All prenylated proteins [*]	Blocks final step of prenylation; Disrupts membrane localization		[155]
FTI L-744,832, Tipifarnib (R115777) [†]	Farnesylated proteins ^{**}	Disrupts membrane localization		[75, 174]
GGTI-298	Geranylgeranylated proteins ^{**}	Disrupts membrane localization		[147]
NSC23766	Rac1	Blocks GEF binding		[175, 176]
Fasudil, H-1152, Y-27632	ROCK			[156, 157]
Wf-536	ROCK		Inhibits angiogenesis and metastasis (Lewis lung carcinoma xenografts)	[159, 160]
IPA-3	PAK	Stabilizes autoinhibition		[163]
Wiskostatin	N-WASP	Stabilizes autoinhibition		[164]
SF1126 [†] , XL765 [†] , XL147 [†] , GDC-0941 [†] , BGT226 [†] , NVP- BEZ235 [†] , PI3Ki-A/D	PI3K		Inhibit xenografted tumor growth	[166–169, 177]
FIPI	PLD	Inhibits enzyme activity		[165]

* Prenylated proteins include Ras and other proteins as well as Rho GTPases.

** GG-Rho proteins include RhoA/C, Rac1/2/3, RhoG, Cdc42, RhoBTB1/2. F-Rho proteins include Rnd1/2/3, TC10, TCL, and RhoD. RhoB and RhoH are alternately modified by either For GG, while RhoF simultaneously exists as two pools of F- and GG-modified proteins [31]. F-proteins also include Ras. Atypical Rho proteins Wrch1/2 are palmitoylated [178, 179].

[†]These compounds have entered clinical cancer trials.