Minireview: Mouse Models of Rho GTPase Function in Mammary Gland Development, Tumorigenesis, and Metastasis

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Ras homolog (Rho) family small GTPases are critical regulators of actin cytoskeletal organization, cell motility, proliferation, and survival. Surprisingly, the large majority of the studies underlying our knowledge of Rho protein function have been carried out in cultured cells, and it is only recently that researchers have begun to assess Rho GTPase regulation and function in vivo. The purpose of this review is to evaluate our current knowledge of Rho GTPase function in mouse mammary gland development, tumorigenesis and metastasis. Although our knowledge is still incomplete, these studies are already uncovering important themes as to the physiological roles of Rho GTPase signaling in normal mammary gland development and function. Essential contributions of Rho proteins to breast cancer initiation, tumor progression, and metastatic dissemination have also been identified. **(*Molecular Endocrinology* 30: 278–289, 2016)**

Ras homolog (Rho) GTPases have been intensively studied since their initial discovery over thirty years ago. Although best known for controlling actin cytoskeletal organization, Rho proteins impact many signaling pathways to regulate diverse processes including transcription, cell cycle progression, and cell survival (1–4). Most studies describing these functions have been carried out in vitro and in cultured cells. However, in recent years many groups have begun to assess Rho GTPase function and regulation in vivo. The purpose of this review is to summarize the evidence for Rho GTPase function in mouse models of mammary gland development, tumorigenesis and metastasis.

Overview of Rho GTPase regulation

There are 20 Rho proteins in the human genome, with Ras-related C3 botulinum substrate 1 (Rac1), Cell division cycle 42 (Cdc42), and RhoA being the best studied (2). Rho GTPases function as molecular switches, cycling between their active, GTP-bound and inactive, GDP-bound states. However, Rho proteins are extraordinarily slow at exchanging nucleotides and require large families of regulatory proteins to function in the cell. For example, there are nearly 80 Dbl homology and CDM-zizimin homology family Rho guanine nucleotide exchange factors (RhoGEFs) in humans which catalyze GDP release, thereby stimulating GTP binding (5, 6). There are also nearly 70 Rho guanine nucleotide activating proteins (RhoGAPs) in humans (Figure 1) (7). Both families of regulatory proteins exhibit a wide diversity in Rho GTPase affinities, regulatory mechanisms and tissue distributions. Three guanine nucleotide dissociation inhibitors also exist that sequester inactive Rho proteins in the cytosol and in some cases protect them from degradation (8). Each cell type expresses diferent complements of Rho proteins and their regulators. With this dizzying array of possible interactions, an obvious challenge is to identify the critical signaling events that are required for a particular outcome.

ISSN Print 0888-8809 ISSN Online 1944-9917
Printed in USA

Received November 12, 2015. Accepted December 10, 2015.
First Published Online December 17, 2015

Abbreviations: 3-dimensional, 3D; Akt1, v-akt murine thyoma virus oncogene homolog 1; Cdc42, Cell division cycle 42; DLC-1, deleted in liver cancer 1; Dock1, dedicator of cytokinesis 1; E, embryonic day; ER, estrogen receptor; HER2, human epidermal growth factor receptor 2; IRS-1, Insulin receptor substrate 1; MEKK1, mitogen activated protein kinase kinase kinase 1; MLC2, myosin regulatory light chain 2; MLK3, mixed lineage kinase 3; MMTV, mouse mammary tumor virus; MMTV-PyMT, MMTV-polyoma middle t antigen; MRTFa/b, myocardin-related transcription factors a and b; MYPT1, myosin phosphatase, target subunit 1; Net1, neuroepithelial transforming gene 1; Pak1, p21-activated kinase 1; PKN, protein kinase N; PR, progesterone receptor; Rac1, Ras-related C3 botulinum substrate 1; Rho, Ras homolog; RhoGAP, Rho guanine nucleotide activating protein; RhoGEF, Rho guanine nucleotide exchange factor; ROCK, Rho kinase; Stat3, Signal transducer and activator of transcription 3; TEB, terminal end bud; Vav1, Vav guanine nucleotide exchange factor 1.

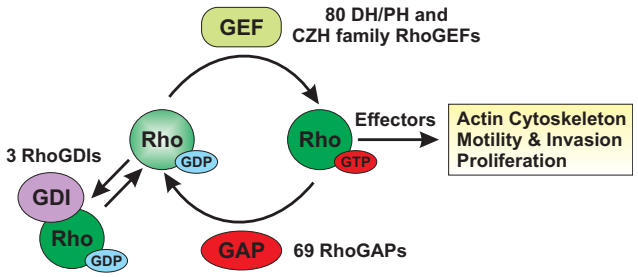

Figure 1. Regulation of Rho GTPase signaling by GEFs, GAPs, and GDIs.

Overview of Mammary Gland Development

Mouse mammary gland development occurs during embryogenesis, puberty, and pregnancy (Figure 2) (9–11). During embryogenesis mammary gland development begins at Embryonic day (E) 10.5 and is completed by E18.5, at which time a rudimentary structure is formed consisting of a nipple and a primary duct with 10–15 side branches. The mammary gland then stays dormant until puberty, when estrogen signaling stimulates invasion of the ducts into the mammary fat pad. At the tip of an invading duct is the terminal end bud (TEB), which consists of an outer layer of cap cells surrounding a mass of body cells. Cap cells become the myoepithelial cell layer in a mature duct, whereas body cells eventually form the luminal epithelial cells. Cell proliferation and movement drive extension of a TEB into the fat pad. Complexity of the ductal tree is enhanced by extensive side branching, such that by the end of puberty the ductal tree has filled the fat pad. Further remodeling occurs during pregnancy, when the ends of the ducts differentiate into milk-producing alveoli. After weaning, the mammary gland undergoes involution, during which the alveolar epithelial cells die off to restore the gland to its prepregnancy state. Because all stages of mammary gland development and involution require changes in cell motility, proliferation, and apoptosis, one would predict that Rho GTPases should play prominent roles in these events.

Rho GTPases in Mammary Gland Development

Because many Rho GTPases share overlapping functions with subfamily members, there is a large potential for compensation in gene deletion studies. Thus, results of such studies must be interpreted with caution. Whole body deletion of RhoB or RhoC did not elicit large changes in mammary gland development, as these animals were able to nurse their young (12–14). Mammary gland-specific deletion of Rac1 also did not affect gland development during puberty or pregnancy (15). This was unexpected, as treatment of organotypic cultures of mammary tissue with the Rac1 inhibitor NSC23766 prevented ductal branching (16). This discrepancy may reflect compensation by Rac3, or may indicate that organotypic culture does not fully recapitulate mammary gland development in vivo. Surprisingly, deletion of *Rac1* delayed involution. This was attributed to a delay in signal transducer and activator of transcription 3 (Stat3) activation, which is necessary for lysosomal-mediated death and initiation of mammary gland involution (17, 18).

A limited number of Rho GTPase gain of function studies in mammary gland development have also been conducted. Expression of constitutively active Rac3 (V^{12}Rac3) under the control of the mouse mammary tumor virus (MMTV) promoter did not affect mammary gland development during puberty or pregnancy (19). Because Rac3 and Rac1 interact with the same downstream effectors, this supports the notion that Rac signaling is not required for mammary gland development. However, MMTV-V^{12}Rac3 expressing mothers had difficulty nursing their offspring, which appeared to result from a milk ejection defect. This fit with the observations of others, who found that expression of constitutively active Rac1

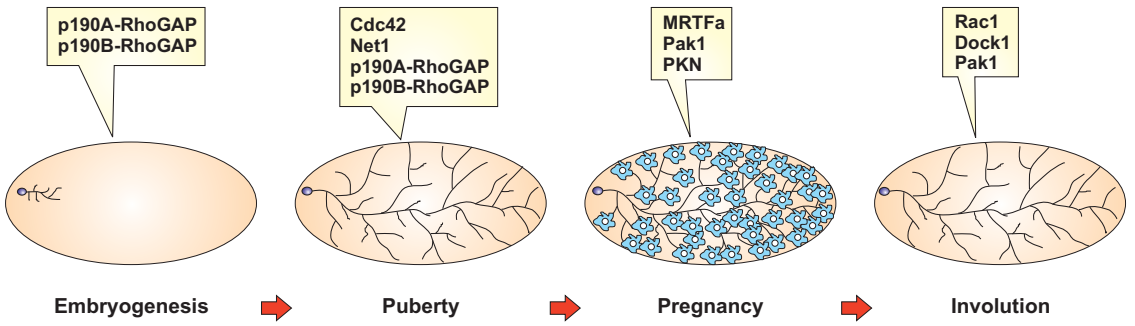

Figure 2. Mouse mammary gland development. The nipple and rudimentary ducts form during embryogenesis. During puberty, the ductal tree expands to fill the mammary fat pad. Milk producing alveoli are formed during pregnancy and disassembled during involution. Rho GTPases, their regulators and effectors that have been shown to control each stage of mammary gland development are shown.

or p21-activated kinase 1 (Pak1) in alveoli in vitro caused myoepithelial cell relaxation (20).

The effect of Cdc42 overexpression on mammary gland development has been explored using a tetracycline-inducible approach (21). Overexpression of wild-type Cdc42 in mammary epithelial cells during mid-to-late puberty caused hyperbranching of TEBs and increased side branching in mature ducts. Branches coincided with gaps in the myoepithelial layer, consistent with a role for myoepithelial cells in suppressing branching (16). Cdc42 overexpression also enhanced mammary epithelial cell motility in vitro and promoted branching in 3-dimensional (3D) culture.

RhoGEFs in Mammary Gland Development

The role of RhoGEFs in mammary gland development has only recently been investigated. Zuo et al examined the role of the RhoA subfamily GEF Neuroepithelial transforming gene 1 (Net1) in mammary gland development during puberty (22). Net1 had previously been shown to be overexpressed in human breast cancer and was necessary for human breast cancer cell motility and invasion in vitro (23–25). Mice with a whole body deletion of Net1 were healthy and able to nurse their young. However, there was a significant delay in mammary gland development during puberty. Mammary epithelial cells in Net1 knockout mice were slower to invade the mammary fat pad and there was less ductal branching. Reduced branching was observed even after the fat pad was filled and was accompanied by a decrease in phosphorylation of the Rho kinase (ROCK) substrates Myosin phosphatase, target subunit 1 and Myosin regulatory light chain 2. As Net1 is not a GEF for Rac1 or Cdc42, these data suggest that RhoA subfamily signaling is important for ductal branching in vivo. There was significantly less proliferation in the TEBs, which was accompanied by reduced expression of estrogen receptor (ER)α and the ERα-regulated genes cyclin D1 and progesterone receptor (PR) A and B. This may have been due to a reduction in RhoA and RhoB activation, as these GTPases have been shown to control ERα expression in MCF7 cells in vitro (26, 27). Interestingly, *Net1* knockout caused focal disorganization of luminal and myoepithelial cells after puberty, reminiscent of the effect of ROCK inhibition on mammary gland development in organotypic cultures (16). In addition, there was a significant elevation of collagen deposition surrounding the ducts. These defects were cell autonomous, as transplantation of mammary epithelial cells from *Net1* knockout mice into immunodeficient SCID/Beige mice recapitulated all of the defects observed in *Net1* knockout mice except for reduced speed of the fat pad invasion.

In support of the idea that Rac1 function is not essential for mammary gland development, deletion of the Rac1 GEFs *Tiam1* or *Dock1* also did not significantly affect mammary gland development (15, 28). However, *Dock1* deletion delayed involution. This delay was greater than after *Rac1* deletion, perhaps reflecting the ability of Dedicator of cytokinesis 1 (Dock1) to activate Rac3 as well as Rac1. Similar to *Rac1* deletion, loss of Dock1 delayed Stat3 activation and prevented epithelial cell apoptosis within the alveoli (15). Because Tiam1 and Dock1 function as GEFs for Rac1 subfamily GTPases, these observations indicate that Rac signaling is mainly important during involution.

RhoGAPs in Mammary Gland Development

p190A-RhoGAP, p190B-RhoGAP, and deleted in liver cancer 1 (DLC-1) have been studied in mammary gland development. DLC-1 is a GAP for RhoA, RhoB, and RhoC (29, 30). p190-RhoGAPs function as GAPs for Rac1, Cdc42, and RhoA in vitro (31, 32), but in cultured cells these proteins have been primarily associated with inhibition of RhoA activity. For example, p190A-RhoGAP suppresses RhoA activation initially during substrate adherence in fibroblasts (33). Nevertheless, the specificity of p190-RhoGAP proteins for particular Rho GTPases in vivo has not been fully resolved.

A role for p190-RhoGAPs in mammary gland development was first suggested when it was shown to be overexpressed in the TEBs of rat mammary glands (34). It was later shown that mice heterozygous for *p190B-RhoGAP* exhibited a delay in mammary gland development that was overcome by adulthood (35). Cap cells within the TEBs proliferated less and expressed less Insulin receptor substrate 1 (IRS-1), IRS-2, and IGF1 receptor. IGF-1 signaling is important for cap cell proliferation (36), and ROCK phosphorylates IRS-1, thereby inhibiting its ability to signal (37). Thus, these phenotypes were consistent with elevated RhoA signaling in *p190B-RhoGAP* heterozygotes. Because homozygous deletion of *p190B-RhoGAP* is embryonic-lethal, E16 mammary anlagens were transplanted into immunocompromised mice. Strikingly, none of the *p190B-RhoGAP*−/− anlagen transplants survived, whereas 75% of the wild-type transplants were successful. Moreover, mammary gland development in the *p190B-RhoGAP* heterozygotes was significantly reduced. The inability of anlagens from *p190B-RhoGAP* knockouts to implant may reflect the

actions of this gene in mammary bud development, because *p190B-RhoGAP* deletion reduces mammary bud size and proliferation, and causes disorganization of the surrounding mesenchyme (38).

The role of p190B-RhoGAP was further explored using a doxycycline-inducible system to stimulate transient overexpression during mammary gland development (39). Short term overexpression of p190B-RhoGAP caused abnormal TEB morphology characterized by increased budding and thickened stroma, whereas longer term overexpression increased ductal branching and resulted in hyperplastic growths after involution. The stroma in p190B-RhoGAP-overexpressing mammary glands exhibited increased collagen deposition and lysyl oxidase expression. In addition, fibroblasts isolated from p190B-RhoGAP overexpressing glands showed increased TGFβ production and signaling which was stimulated by the mammary epithelial cells, indicating a paracrine effect (40).

p190A-RhoGAP also contributes to embryonic and pubertal mammary gland development (41). p190A-RhoGAP is highly expressed in mammary buds during embryogenesis, and in TEBs and ducts during puberty. *p190A-RhoGAP* heterozygotes exhibited a short delay in fat pad filling during puberty and reduced adhesion between cap and body cells in the TEBs. TEB defects were consistent with altered netrin signaling (42), and expression of the netrin receptor neogenin was lower in *p190A-RhoGAP* heterozygotes. To assess the effect of complete *p190A-RhoGAP* deletion, mammary bud epithelial fragments from E18.5 animals were transplanted into immunocompromised mice. Loss of *p190A-RhoGAP* reduced the transplant take rate and inhibited fat pad filling during puberty. Contrary to the effects of *Net1* deletion, these mice exhibited increased expression of ERα and PR, supporting the idea that RhoA signaling may regulate ERα expression in the developing mammary gland.

Additional evidence for a role for RhoA in pubertal mammary gland development was derived from a study where one allele of *Dlc-1* was deleted (43). In these mice, *Dlc-1* deletion resulted in an increase in ductal branching and aberrant TEB morphology. This was coincident with an increase in RhoA activity. However, unlike *p190B-RhoGAP* deletion, loss of *Dlc-1* did not affect epithelial cell proliferation, nor did it affect luminal and myoepithelial cell organization within mature ducts. These data support the idea that RhoA activation is important for ductal branching. Moreover, the divergent effects of deletion of different Rho GAPs or GEFs suggest that these proteins form distinct signaling complexes that direct localized RhoA activation.

Rho GTPase Effectors in Mammary Gland Development

Rho GTPases signal through multiple effectors to control cell function, and an important effector for Rac1, Rac3, and Cdc42 is the serine/threonine kinase Pak1 (44, 45). Pak1 expression increases in mammary epithelial cells during pregnancy, reaching a peak during lactation (46). Expression of constitutively active Pak1 T423E from the β-lactoglobulin promoter, which drives expression during pregnancy and lactation, resulted in alveolar hyperplasia during lactation (47), whereas dominant negative Pak1 K299R expression inhibited alveolar differentiation by stimulating apoptosis (46). Because Rac1 does not contribute to alveolar differentiation, it is likely that these effects occurred downstream of endogenous Cdc42. Alveolar hyperplasia was accompanied by increased proliferation and ERα/PR target gene expression. ERα activation was mediated by Pak1-dependent phosphorylation of ERα on S305, which increased ERα transcriptional activity. Surprisingly, inhibition alveolar differentiation by dominant negative Pak1 correlated with reduced Stat5a activation rather than altered ERα signaling. Pak1 phosphorylated Stat5a on the activating site serine 779, and dominant negative Pak1 blocked Stat5a activation during lactation. These data indicated that may Pak1 contribute to alveolar differentiation during pregnancy and lactation by stimulating ERα and Stat5a transcriptional activity, and may also promote alveolar epithelial cell survival by an unknown mechanism.

Rho GTPase signaling stimulates the activity of the myocardin-related transcription factors a and b (MRTFa/b) by promoting their nuclear localization (48, 49). In concurrent studies using distinct gene deletion strategies, 2 groups showed that MRTFa is required for myoepithelial cell function during lactation. *MRTFa* knockout dams were unable to nurse their young due to an inability to eject milk from the mammary gland. This was due to reduced expression of contractile genes, including smooth muscle actin, smooth muscle myosin heavy chain, calponin and caldesmon.

Protein kinase N (PKN) is a RhoA and Rac1 effector that is important for cell migration, survival, vesicle transport and cell division (3). Interestingly, mammary gland-specific expression of dominant negative PKN did not affect development but caused premature involution during lactation. This was due to an inability to expel milk from the mammary glands, which led to an increase in apoptosis. Importantly, tight junction assembly in mammary gland ducts was compromised (50). These data suggest that PKN plays an important role in mammary gland function during lactation, most likely by controlling milk

ejection efficiency. However, it is important to note that *Pkn1* knockout mice are fertile and able to nurse their young, suggesting that dominant negative PKN1 may be interfering with additional signaling proteins in mammary epithelial cells, such as PKN2 or PKN3 (51).

Rho GTPase Function in Mouse Mammary Gland Tumorigenesis and Metastasis

Rho GTPases are overexpressed in human breast cancer and stimulate breast cancer cell motility and invasive activity in vitro (52–55). A role for Rho proteins in breast cancer in vivo was first shown when knockdown of RhoA or RhoC in MDA-MB-231 cells blocked the growth of subcutaneous tumor xenografts. Knockdown of RhoA and to a lesser extent RhoC also inhibited angiogenesis, suggesting a mechanism for reduced tumor growth (56). It was later demonstrated that RhoA transcription was regulated by c-Myc, and that RhoA expression was essential for MDA-MB-231 cell lung colonization after tail vein injection (57).

A role for RhoC in breast cancer metastasis was demonstrated using *RhoC* knockout mice (14). *RhoC* deletion significantly inhibited metastasis in MMTV-polyoma middle t antigen (MMTV-PyMT) mice, which normally develop mammary gland tumors with 100% penetrance and exhibit extensive lung metastasis (58, 59). Interestingly, *RhoC* deletion did not affect any aspect of primary tumorigenesis. Lung micrometastases were detectable but apoptotic. Primary tumor cells grown in vitro also exhibited reduced motility and invasive capacity. These data suggest that RhoC contributes to metastasis by promoting cancer cell motility as well as survival in distant metastatic niches.

Elevated expression of RhoC has been noted in human inflammatory breast cancer (60), which may be required to maintain a tumor initiating cell population (61). RhoC was shown to be highly expressed in aldehyde dehydrogenase-positive SUM149 cells, which are a human inflammatory breast cancer cell line. RhoC knockdown in these cells reduced orthotopic tumor formation in SCID mice after injection of limiting numbers of tumor cells, suggesting that RhoC was necessary for tumor initiating cell function. RhoC knockdown also prevented lung metastasis. Conversely, expression of constitutively active RhoC in MCF10A cells promoted lung metastasis in SCID mice even when palpable tumors were not detectable.

RhoB is generally perceived as a tumor suppressor gene (62, 63). Consistent with this, *RhoB* deletion enhanced the frequency and reduced the latency of tumor formation in MMTV-PyMT mice (13). However, tumors that formed in *RhoB* knockout mice grew more slowly and had reduced angiogenesis. Moreover, wild-type tumor cell transplants into *RhoB*−/− SCID mice exhibited slower growth. Taken together, these data confirmed a tumor suppressor role for RhoB but also indicated that RhoB function in the stroma may be essential for angiogenesis and tumor progression.

A tumor suppressor role for Rho family GTPase 1 (Rnd1) (RhoE) and RhoD was suggested when it was noted that low expression of these mRNAs correlated with increased risk of metastasis in human breast cancer (64). Stable knockdown of Rnd1 in MCF10A cells caused an epithelial to mesenchymal transition, followed by accumulation of DNA damage and senescence. Mechanistically, Rnd1 knockdown stimulated H- and K-Ras activity due to disinhibition of the Ras and RhoGAP activities of plexin B and p190A-RhoGAP, respectively. Importantly, Rnd1 knockdown in Comma-D cells caused them to become tumorigenic in NOD-SCID mice. Concordantly, Rnd1 overexpression in MMTV-Neu tumor cells inhibited lung colonization after tail vein injection. Rnd1 overexpression in 4T1 mouse breast cancer cells also suppressed tumor formation and lung metastasis after orthotopic injection in Balb/c mice.

The evident role for Rho proteins in driving human cancers has promoted researchers to screen for therapeutics to inhibit their function, and Rac1 inhibitors have been tested in breast cancer models. EHop-016, a derivative of the Rac1 inhibitor NSC23766, blocked MDA-MB-435 tumor growth and metastasis in nude mice xenografts (65). EHop-016 also inhibited interaction between Rac1 and the RhoGEF Vav guanine nucleotide exchange factor 1 (Vav1) and prevented activation of Rac1, Pak1 and v-akt murine thyoma viral oncogene homolog 1 (Akt1) in cells. A distinct Rac1 inhibitor, ZINC68391, blocked productive interaction between Rac1 and Tiam1 (66), and administration of ZINC68391 or its derivative 1A-116 blocked lung colonization of F3II mouse breast cancer cells after tail vein injection. Both of these studies support the importance of Rac1 in metastasis.

RhoGEFs in Mammary Gland Tumorigenesis and Metastasis

The role of RhoGEFs is surprisingly specific for particular breast cancer subtypes. For example, deletion of *Tiam1* delayed tumorigenesis caused by MMTV-Neu and mutant APC, but not by MMTV-Myc (28, 67). MMTV-Neu models human epidermal growth factor receptor 2 (HER2)-driven breast cancers, whereas MMTV-Myc re-

capitulates aspects of both basal and luminal B subtypes of human breast cancer (68, 69). *Tiam1* deletion did not affect the growth of MMTV-Neu tumors once they had formed, but did reduce lung metastasis. Tiam1 may also play a role in stromal fibroblasts, because normal mammary fibroblasts with Tiam1 knockdown no longer inhibited SUM1315 cell metastasis after coinjection in SCID mice (70).

A strong case for the Rac1 GEF P-Rex1 in luminal type breast cancers has also been demonstrated. P-Rex1 transcripts are overexpressed in human luminal but not basal type breast cancers, and correlate with ERα and HER2 expression (71). In addition, P-Rex1 expression was required for Rac1 activation and cell motility in the human luminal breast cancer cell lines T47D, MCF7, and BT-474. Stable knockdown of P-Rex1 in BT-474 cells also suppressed tumor formation after orthotopic injection in nude mice. P-Rex1 was activated by phosphorylation on S695 and S1169 after neuregulin treatment in MCF7 cells, and stable P-Rex1 knockdown in MCF7 cells inhibited tumor formation after orthotopic injection (72).

The RhoGEFs Vav2 and Vav3 appear to cooperate in promoting lung metastasis in orthotopic models. Vav3 is overexpressed in human luminal breast cancers, and knockdown of Vav2 and Vav3 in 4T1 mouse breast cancer cells slowed tumor growth and inhibited lung metastasis (73). Surprisingly, it did not affect metastasis to the lymph nodes or spleen, indicating an organ-specific effect. Inhibition of lung metastasis correlated with reduced Rac1 activation rather than RhoA. Vav2 and Vav3 also stimulated changes in gene expression independent of Rac1, and different genes within this transcriptional program were necessary for distinct aspects of metastasis. For example, Integrin Linked Kinase up-regulation was important for extravasation, but not tumor cell survival. Importantly, the Vav2/3-dependent transcriptome accurately predicted lung metastasis in a retrospective analysis of human breast cancer patients.

Overexpression of the Rac1 GEF Dock1 was shown to correlate with reduced survival in human HER2-positive and basal-type breast cancers (74). Accordingly, *Dock1* knockout slightly delayed tumorigenesis and largely inhibited lung metastasis in MMTV-Neu mice. *Dock1*-deleted tumors exhibited less proliferation and increased apoptosis. Dock1 knockdown in Neu-expressing NMuMG cells prevented lung colonization. A gene signature derived from *Dock*1-knockout tumor cells was predictive of disease-free and overall survival in HER2+ breast cancer patients. The related Cdc42 GEF Dock10 is required to lead collective cell invasion of triple negative human breast cancer cells, such as SUM159, in organotypic culture (75). This "trailblazer" population was epigenetically distinct from the bulk population of cells, and its presence was required for lung metastasis after orthotopic injection in NOD/SCID mice.

Less is known about RhoA-specific GEFs in breast cancer. Doxycycline-inducible knockdown of SmgGDS-558 in MDA-MB-231 cells inhibited tumorigenesis after orthotopic injection (76). SmgGDS-558 was also required for proliferation in a panel of human breast cancer cell lines in vitro. RNA interference-mediated inhibition of hPTTG1 expression in MDA-MB-231 cells blocked orthotopic tumorigenesis and lung metastasis in SCID mice. hPTTG1 is a transcription factor that stimulates the expression of multiple genes, including the RhoA-specific GEF GEF-H1. Significantly, ectopic expression of GEF-H1 in hPTTG1 knockdown cells rescued lung colonization after tail vein injection (77).

RhoGAPs and RhoGDIs in Mammary Gland Tumorigenesis and Metastasis

Only p190B-RhoGAP and DLC-1 have been investigated in vivo. Loss of one allele of *p190B-RhoGAP* delayed tumor onset in MMTV-Neu mice, although the number of preneoplastic lesions was increased and tumor growth rate was not affected (78). Tumor angiogenesis was compromised, perhaps due to elevated thrombospondin-1 expression, and mice exhibited fewer lung metastases. Wild-type tumors transplanted into mice heterozygous for *p190B-RhoGAP* grew more slowly and exhibited reduced angiogenesis. Conversely, *p190B-RhoGAP* heterozygous tumors grew as well as wild-type tumors when transplanted into wild-type mice. Thus, p190B-RhoGAP expression in epithelial cells is important for tumor initiation, whereas expression in the stroma is critical for angiogenesis and tumor progression. Accordingly, p190B-RhoGAP overexpression accelerated MMTV-Neu induced tumorigenesis and metastasis (79). However, this was not accompanied by an increase in angiogenesis. Instead, Rac1 signaling may have been altered, as primary mammary epithelial cells overexpressing p190B-RhoGAP exhibited increased Rac1 activity and grew invasively in 3D cultures.

DLC-1 is the human homolog of rat p122-RhoGAP, which is a potent GAP for RhoA but not Rac1 in vitro (29). DLC-1 was originally shown to be deleted in multiple human cancers, including breast cancer, suggesting that DLC-1 was a tumor suppressor (80, 81). DLC-1 mRNA was down-regulated in 12 of 17 human breast cancer cell lines, and DLC-1 reexpression in MDA-MB-361 and MDA-MB-468 cells reduced their proliferation and colony formation in vitro. It also suppressed subcu-

taneous tumor formation in nude mice. DLC-1 expression was also down-regulated in metastatic derivatives of MDA-MB-435 cells, and DLC-1 reexpression inhibited their motility and invasive activity in vitro and suppressed lung metastasis after orthotopic injection in nude mice (82). Bone metastatic variants of MDA-MB-231 cells expressed very little DLC-1, and DLC-1 reexpression suppressed bone metastasis after cardiac injection (30). Moreover, DLC-1 expression inversely correlated with RhoA, RhoB, RhoC, and Cdc42 activation in these cells. In the presence of TGFβ, bone metastatic MDA-MB-231 cells stimulated osteoclastogenesis of bone marrow cells, and reexpression of DLC-1 suppressed this effect. DLC-1 worked by suppressing PTH-like hormone expression and this could be reversed by expression of constitutively active RhoA. Importantly, RhoA activation stimulated ROCK-dependent phosphorylation of Smad3 on serines 204 and 208, which supported transcriptional activation of the PTH-like hormone gene. Thus, DLC-1 suppressed bone metastasis by inhibiting a RhoA/ROCK-dependent gene expression program. In support of these findings, treatment with the ROCK inhibitor fasudil suppressed bone metastasis in mice injected with 4T1 cells (30).

RhoGDIs sequester inactive Rho proteins in the cytosol and protect some Rho proteins from proteasome-mediated degradation (8). RhoGDIα has been shown to promote resistance to estrogen antagonists in breast cancer (83). RhoGDIα expression was reduced in tamoxifen-resistant breast cancers and knockdown of RhoGDIα in MCF7 cells made them resistant to tamoxifen. This was due to increased Cdc42, Rac1 and Pak1 activity. Pak1 phosphorylated ERα on S305, which stimulated ERα-dependent transcription. Importantly, MCF7 cells expressing RhoGDIα shRNA formed tamoxifen-resistant subcutaneous tumors in nude mice. A regulatory input controlling RhoGDIα function in breast cancer may be through interaction with 14–3-3τ (84). 14–3-3τ binds to RhoGDIα after phosphorylation on S174 by Pak1. This prevents interaction with Rho GTPases, thereby promoting their activation. Concordantly, overexpression of 14–3-3τ in MCF7 cells increased their ability to form tumors after orthotopic injection in nude mice and enhanced lung metastasis.

Rho Effectors in Mammary Gland Tumorigenesis and Metastasis

The Rac and Cdc42 effectors Pak1 and Pak4 are likely to be important contributors to mammary gland tumorigenesis. Expression of constitutively active Pak1 T423E from a lactoglobulin promoter caused ERα-negative tumors after 1 year in 20% of mice that had undergone at least 1 pregnancy, and in a similar proportion of virgin mice with pituitary-kidney engraftments to stimulate prolactin secretion (85). These tumors had increased p38 MAPK and Mek1/2 activation, which would be expected after expression of active Pak1 (86–88). Expression of the Pak1 autoinhibitory domain in MDA-MB-631 cells, which blocks activation of Paks 1–3, reduced subcutaneous tumor growth and inhibited ERK and Akt activation in SCID mice (89). Moreover, *Pak1* deletion significantly delayed tumorigenesis in MMTV-Neu mice (90). *Pak1*-deleted tumors had reduced ERK1/2 and Akt activation and expressed less β-catenin. Additionally, treatment of subcutaneous BT474 cell xenografts with the Pak1 inhibitor PF-3758309 completely blocked tumor growth, whereas the β-catenin-TCF4 inhibitor iCRT14 was without effect (90). The Cdc42 regulated kinase Pak4, which signals distinctly from Paks 1–3, may also play a role in mammary gland tumorigenesis. Pak4 knockdown in MDA-MB-231 cells restored normal acinar morphology in 3D culture and prevented tumor formation after orthotopic injection in nude mice (91). Moreover, wild-type Pak4 overexpression in immortalized mouse mammary epithelial cells, which stimulates constitutive Pak4-dependent signaling in cells (92), caused tumor formation after orthotopic injection in nude mice (93).

The Cdc42 and Rac1 effectors CIP4, N-Wasp, and IQGAP1 may be important for metastasis. CIP4 is required for epithelial to mesenchymal transition in MCF10.DCIS.com cells after TGFβ treatment in vitro, and for invasiveness after subcutaneous injection into NSD mice (94). Moreover, CIP4 knockdown in MDA-MB-231 or MTLn3 rat mammary adenocarcinoma cells inhibited lung metastasis after orthotopic injection and prevented lung colonization after tail vein injection (95). N-Wasp knockdown in MTLn3 cells inhibited tumor cell motility in rat allografts and reduced tumor cell circulation and micrometastases after orthotopic injection in SCID mice (96). N-Wasp knockdown also altered the morphology of cells leaving the tumor and inhibited collagen degradation, consistent with reduced invadopodia formation. IQGAP1 overexpression in MCF7 cells increased tumor incidence and growth after subcutaneous injection in NOD mice, whereas IQGAP1 shRNA reduced tumor incidence and growth. IQGAP1-overexpressing cells also invaded the underlying skeletal muscle (97).

The MAP3K Mitogen activated protein kinase kinase kinase 1 (MEKK1) contributes mainly to metastasis. MEKK1 binds to Rac1 and RhoA (98, 99), and *MEKK1* deletion in MMTV-PyMT mice did not affect tumorigenesis but reduced lung metastasis (100). This may have

been due to reduced expression of urokinase plasminogen activator, as MEKK1-dependent stimulation of c-Jun N-terminal kinase up-regulates urokinase plasminogen activator expression (101). MEKK1 knockdown in MDA-MB-231 cells also inhibited lung metastasis after orthotopic injection without effects on primary tumor growth (102). Mixed lineage kinase 3 (MLK3) is activated by both Rac1 and Cdc42 (103), and MLK3 knockdown in MDA-MB-231 cells has been reported to delay tumorigenesis and completely suppress lung metastasis (102). However, effects on tumorigenesis are somewhat unclear, as a second group showed that MLK3 knockdown in MDA-MB-231 cells only prevented lung metastasis after orthotopic injection (104). Surprisingly, treatment with the MLK3 inhibitor URMC099, which can penetrate the blood-brain barrier, stimulated brain metastasis after cardiac injection of MDA-MB-231BR cells (105).

The RhoA effector ROCK1 contributes to both tumorigenesis and metastasis. Fasudil treatment inhibited tumor initiation in MDA-MB-231 cells after orthotopic injection, but did not affect tumor growth (106). Conversely, ROCK1 overexpression in MCF7 cells promoted metastasis to the liver, hind limbs and implanted bone cores after orthotopic injection. ROCK1 knockdown in MDA-MB-231 cells, or treatment with the ROCK1/2 inhibitor Y27632, blocked metastasis to implanted bone cores after orthotopic injection. Apart from stimulating actomyosin contraction, ROCK1 may also have promoted invasion by stimulating c-Myc-dependent expression of the promigratory miRNA mIR-17–92 (107). ROCK1 expression was required for tumor cell survival in mouse invasive lobular carcinoma cells, which impacted tumor formation and metastasis (108). Knockdown of the transcription factors MRTFa/b, which control cytoskeletal gene expression and are stimulated by ROCK activity, inhibited MDA-MB-231 cell motility after subcutaneous injection, but did not affect tumor cell proliferation or tumor growth. These cells also did not colonize the lung after tail vein injection (109). Thus, MRTF transcription factors are likely to be important for tumor metastasis, but not primary tumor growth.

Concluding Remarks

Although there is still much to learn about the roles of Rho GTPase signaling in mammary gland development, we can start to draw some general conclusions (Figure 2). Current evidence indicates that Rac-dependent signaling is mainly required for involution, whereas Cdc42 and RhoA signaling are necessary for multiple stages in development. Cdc42 may control ductal branching and alveolar development, whereas RhoA appears to be necessary for ERα-driven epithelial cell proliferation, ductal branching and maintenance of luminal-myoepithelial cell organization.

In breast cancer, Rac1, Cdc42, and RhoA each appear to contribute to tumorigenesis and metastasis (Figure 3). A significant caveat is that much of the data supporting these findings rely on xenografts of a handful of human cancer cell lines, which may not accurately encompass the heterogeneity of human breast cancers and do not account for the role of the immune system. Nevertheless, these data seem to indicate that strategies targeting all three Rho GTPases will have value in treating breast cancers. In this regard, focusing on Rho effectors may be the quickest route to a therapeutic, as many of these proteins are kinases that are tractable drug targets. For example, ROCK1 and ROCK2 are clinically relevant drug targets that appear to contribute to tumorigenesis and metastasis in a variety of human breast cancer cell lines representing distinct breast cancer subtypes. Early data also indicate that particular RhoGEFs may be up-regulated in specific breast cancer subtypes, suggesting that therapies directed at these proteins will have

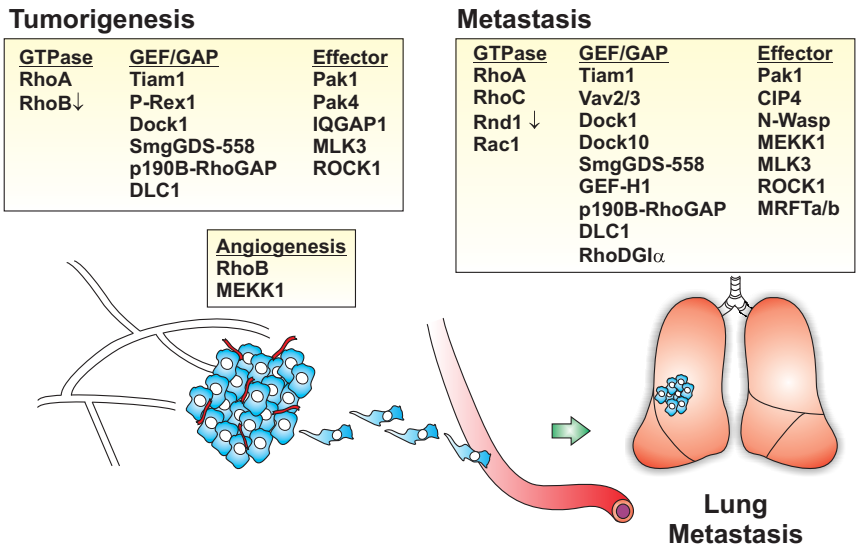

Figure 3. Rho GTPases in mouse models of breast cancer. Breast cancer cells leave a tumor residing in the ductal network, enter the circulation, and metastasize to the lungs. Rho proteins and their regulators that have been implicated in these processes are shown.

value in specific patient subsets. Because of the restricted expression of RhoGEFs, such therapies would also have the potential for fewer side effects.

In the future, it will be important to deepen our knowledge of Rho protein function in mammary gland development and to continue to identify Rho GTPase signaling elements that fulfill specific roles in breast cancer subtypes. It will also be of particular importance to understand the roles of Rho GTPases in the responses of the innate and adaptive immune systems to breast cancer. As critical regulators of cytoskeletal organization, Rho protein signaling is likely to be essential to many aspects of immune cell function (110–113). Thus, therapies targeting particular Rho pathway signaling elements in cancer cells may have unanticipated consequences in the immune system which may have to be taken into account when devising treatment strategies.

Acknowledgments

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This work was supported by the National Cancer Institute Grant CA172129 (to J.A.F.) and a postdoctoral fellowship from the Cancer Prevention Research Institute of Texas, University of Texas Health Innovation in Cancer Prevention Research Training Program, RP140103 (to A.U.).

Disclosure Summary: The authors have nothing to disclose.

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