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THE RAC GTPase IN CANCER: FROM OLD CONCEPTS TO NEW PARADIGMS

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Abstract

Rho family GTPases are critical regulators of cellular functions that play important roles in cancer progression. Aberrant activity of Rho small G-proteins, particularly Rac1 and their regulators, is a hallmark of cancer, and contributes to the tumorigenic and metastatic phenotypes of cancer cells. This review examines the multiple mechanisms leading to Rac1 hyperactivation, particularly focusing on emerging paradigms that involve gain-of-function mutations in Rac and guanine nucleotide exchange factors (GEFs), defects in Rac1 degradation, and mislocalization of Rac signaling components. The unexpected pro-oncogenic functions of Rac GTPase activating proteins (GAPs) also challenged the dogma that these negative Rac regulators solely act as tumor suppressors. The potential contribution of Rac hyperactivation to resistance to anti-cancer agents, including targeted therapies, as well as to the suppression of anti-tumor immune response, highlights the critical need to develop therapeutic strategies to target the Rac pathway in a clinical setting.

Keywords

Rac; Rac-GEF; Rac-GAP; cancer signaling; cancer therapeutics

INTRODUCTION

Exactly 25 years ago, two seminal papers by Alan Hall and coworkers illuminated us with one of the most influential discoveries in cancer signaling: the association of Ras-related small GTPases of the Rho family with actin cytoskeleton reorganization (1,2). Those findings set the mechanistic basis for the control of cell motility, invasiveness, and metastasis in response to extracellular receptors. The Rho small (~21 kDa) G-protein family comprises 20 members categorized into Rac (Rac1, Rac2, Rac3, and RhoG), Rho (RhoA, RhoB, and RhoC), and Cdc42 (Cdc42, TC10, Chip, TCL, and Wrch-1) subfamilies, and

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other less studied GTPases that include RhoD, RhoE, and RhoH (3). Rho GTPases control a variety of cellular functions through regulation of actin contractility and peripheral actin structures, including cell morphology, locomotion, and polarity. Accordingly, they are key players in physiological processes such as embryonic development, neuronal plasticity, phagocytosis and stem cell formation. Deregulation of Rho GTPase function in cancer is associated with fundamental hallmarks of progression, including changes in gene expression, cell survival, oncogenic transformation, tumor metabolism, and invasiveness (3–5). Deciphering the key effectors and regulators of Rho family members became a crucial undertaking in cancer cell biology, and significant research efforts have been directed towards targeting Rho-regulated pathways for battling cancer.

Nearly all Rho GTPases act as molecular switches that cycle between GDP-bound (inactive) and GTP-bound (active) forms. Activation is promoted by guanine nucleotide exchange factors (GEFs) responsible for GDP dissociation, a process that normally occurs very slowly, thereby facilitating the exchange for GTP that is present at much higher cytosolic concentrations. On the other hand, GTPase activating proteins (GAPs) inactivate Rho proteins by accelerating their intrinsic rate of GTP hydrolysis. Once in the inactive conformation, Rho GTPases associate with guanine nucleotide dissociation inhibitors (GDIs), a step that contributes to their stabilization and precludes them from getting activated (3,4). In cancer, changes in abundance of Rho GTPases and their regulators, or excessive input signals leading to their activation have been associated with disease progression (3–5). More recently, point mutations and deregulated stability or localization of these proteins have been identified as mechanisms that contribute to tumorigenesis and metastasis, which will be discussed in this review particularly for the GTPase Rac (summarized in Fig. 1 and Table 1).

DISTINCTIVE ROLES FOR RHO GTPases IN CANCER INITIATION AND PROGRESSION

The initial evidence that Rho GTPases are positively involved in cancer cell growth arised from studies showing transforming activity by active forms and inhibition by dominant-negative forms (see for example Ref. (6)). Elevated expression of Rac1, the active splice variant Rac1b, and other related GTPases has been frequently observed in human cancer, which in some cases correlated with aggressiveness and poor prognosis (3). Consistently, studies in mouse models supported the requirement of Rac for tumor growth. For example, genetic deletion of the *Rac1* gene in mice impairs the development of mutant KRas-driven cancer in skin, lung, and pancreas (7–9). Loss of Rac2, but not Rac1, delays the initiation of acute myeloid leukemia, although the survival of fully transformed leukemia cells is dependent on Rac1 and Rac2 (10). Similarly, deletion of Rac3 resulted in increased survival of a transgenic mouse model of acute lymphoblastic leukemia (11). The involvement of Rac1 in cancer progression has also been strengthened by means of genetic deletion or silencing of Rac-specific GEFs such as P-Rex1 and Tiam1 in mouse models, resulting in impaired tumorigenic and metastatic phenotypes (12–14). It became increasingly evident, however, that unlike Rac, Rho could exert either pro- or anti-oncogenic actions *in vivo* in different contexts. For example, early studies revealed that ablation of the *RhoB* gene in

mice (a gene deleted in many human cancers) enhances carcinogen-induced skin cancer (15). *Rhob*^{-/-} mice are less prone to form squamous cell carcinomas following chronic exposure to UVB, arguing that RhoB favors early stages of oncogenesis, however RhoB could also limit the progression to highly aggressive tumors (16). Deletion of the *RhoC* gene in the MMTV-PyVT mouse model of breast cancer significantly reduces lung metastasis of mammary tumors (17). However, deletion of *RhoC* or *RhoA* genes failed to suppress KRas-mediated lung tumorigenesis in mice, and RhoA loss rather accelerates the formation of lung adenomas (18), suggesting complex biological scenarios and potential compensatory mechanisms between members of the family. Therefore, Rac1 and Rac1 GEFs likely stand as the most promising cancer therapeutic targets within the Rho GTPase family.

EMERGING PARADIGMS IN RHO GTPase HYPERACTIVATION IN CANCER

Cancer associated mutations in Rho GTPases

Unlike Ras mutations, Rho GTPases were only recently recognized to be mutated in human cancer, as a result of large genomic sequencing studies. Nonetheless, the causal association between these mutations and disease progression remains to be fully determined in most cases. One of the most prominent driver mutations in Rac has been found in melanoma, specifically a hot spot in the *RAC1* gene leading to missense mutations in P29, occurring with frequency of ~5% and up to 9% in chronically sun-exposed melanomas (4,19). Rac1^{P29S}, the most common mutation, leads to gain-of-function and consequently enhances downstream Rac signaling. This mutation confers resistance to Raf and MEK inhibitors, thus having significant clinical therapeutic significance (20). Interestingly, expression of Rac1^{P29S} in melanoma patients correlates with PD-L1 up-regulation, thus potentially contributing to suppression of an anti-tumor immune response (21). These findings could also be extended to other cancers since the Rac1^{P29S} hotspot mutation has been identified in head and neck, and endometrial cancers (22). A 5% incidence in Rac1 mutations (Rac1^{G12V}, Rac1^{G12R}, Rac1^{P34R}, Rac1^{Q61R}, and Rac1^{Q61K}) has been found in germ cell testicular tumors, which makes these tumors, along with melanoma, the cancer types with the highest incidence of Rac1 mutations reported to date (23). Gain-of-function mutants Rac1^{A159V} (paralogous to A146 mutants in KRas) and Rac1^{Q61R} (paralogous to Q61 mutants in KRas) have been also identified in head and neck, and prostate cancer, respectively (22). It should not be assumed that the same scenario is true for other Rho GTPases, since RhoA mutations found in gastric adenocarcinomas, head and neck cancer, and lymphomas could be either gain-of function (for example RhoA^{Y42C}) or inactivating (for example RhoA^{G17V}), further emphasizing the complex behavior of this GTPase in cancer, as described above (3,4). Discerning the functional significance of these mutations in the context of additional oncogenic and tumor suppressing alterations is vital to establish their utility as biomarkers, as well as their role in therapy responsiveness.

Anomalous Rac degradation and localization

Rac degradation is partially modulated by post-translational modifications. Ubiquitination by FBXL19 and HACE1 E3 ligases influences Rac expression levels (24,25). Consistent with the role of Rac in the control of NADPH oxidase complexes, deficiency in the tumor suppressor HACE1 increases reactive oxygen species (ROS) production, and this in turn

leads to an addiction to Gln, a major nutrient source for tumor cells (26). Moreover, HACE1 down-regulation cooperates with ErbB2/HER2 overexpression in mammary cells to induce Rac1 hyperactivation, migration, malignant transformation, and tumorigenesis *in vivo*, effects that are sensitive to pharmacological Rac inhibition (27). A very recent report that identified cancer-associated missense mutations in HACE1 leading to defective Rac1 ubiquitination highlights the relevance of the control of Rac1 expression in cancer cell proliferation (28).

Spatial localization of Rho GTPases is key to fine-tune signaling outputs, and is tightly regulated by post-translational modifications and protein-interactions. In the case of Rac, prenylation of the C-terminal CAAX motif contributes to membrane redistribution from a cytosolic compartment (3). Abnormal localization of Rac, particularly in the nucleus, has been shown to occur in cancer cells. Increased nuclear Rac1 in tumor cells may be the result of an imbalance in the nucleocytoplasmic shuttling of this protein, causing a reduction in active cytoplasmic Rac1 and a concomitant elevation of active RhoA that favors actomyosin contractility required for cell invasion. In addition, nuclear Rac1 regulates actin polymerization at this cellular location, a function important for tumor cells as it confers the nuclear plasticity necessary for invasion (29). Adding more complexity to the role of nuclear Rac in cancer, a recent study demonstrated that deregulated Rac-GEF activity in tumor cells accumulates Rac in the nucleolus, which supports protein biosynthesis required for cancer cell growth (see below) (30).

Deregulation in Rac-GEF function

One of the best characterized mechanisms of Rho protein signaling deregulation involves the hyperactivation of GEFs, either by excessive upstream oncogenic activation or deregulated GEF expression/activity. The multiplicity of cellular outcomes regulated by the more than 70 identified Rho GEFs is dictated by their differential pattern of expression and selectivity for Rho proteins, as well as by the intricate mechanisms governing their activation. Various studies have linked enhanced Rac exchange activity, resulting from GEF overexpression, to an invasive and metastatic phenotype (5).

Among the many Rho family GEFs regulating Rac activity and implicated in cancer progression, Ect2, Tiam1, P-Rex and Vav family members stand as the most prominent ones associated with tumorigenesis and metastasis. Their anomalous expression and/or activation may impact patient outcome prediction and therapeutic management. For example, Vav3 overexpression in ovarian cancer associates with poor prognosis and confers resistance to established therapeutic regimes (31). Genetic alterations in the *VAV1* gene, including activating mutations and fusions, have been found in peripheral T-cell lymphomas, establishing an oncogenic role for this GEF in the development of this disease (32).

The PI3K/Gβγ-regulated Rac-specific GEFs P-Rex1 and P-Rex2 have received significant attention in recent years as they are either overexpressed or mutated in cancer. P-Rex1 up-regulation occurs through enhanced gene expression, epigenetic deregulation, or increased protein stability (12,33–35). P-Rex1 overexpression in luminal breast cancer plays a fundamental role in integrating upstream signals emanating from tyrosine-kinase and G-protein-coupled receptors, and has been associated with metastasis in breast cancer patients.

Indeed, lymph node metastasis and metastatic breast tumors display elevated P-Rex1-positive immunostaining (12). A particular role for ErbB3/HER3 signaling in CXCR4-mediated P-Rex1/Rac1 activation via hypoxia-inducible factor 1 α (HIF-1 α) has been recently described in luminal breast cancer models (36). Deletion of the *PRex1* gene in a melanoma mouse model prevents metastatic dissemination (13). A potential role for P-Rex1 beyond solid tumors has been recently highlighted in acute myeloid leukemia (AML), particularly in AML cells harboring mutant Ras proteins (37). Whereas P-Rex1 mutations are infrequent, a high incidence of P-Rex2 point mutations has been described in melanoma (14%, third in prevalence after BRAf and NRas) (38). Although the biological consequences of the multiple P-Rex2 mutations, observed in melanoma and pancreatic cancer, have yet to be fully determined, a recent study has demonstrated enhanced Rac1-GEF activity and a pro-tumorigenic function of a truncated P-Rex2 mutant (39).

Ect2, a GEF that displays exchange activity on Rac, Rho, and Cdc42, plays an essential role in cell division. *ECT2* gene co-amplification with *PRKCI* and *SOX2* genes has been described in human tumors (40,41), although other mechanisms may also account for deregulated Ect2 expression. Targeted shRNA depletion of Ect2 from cancer cells impairs tumorigenic growth in a manner that is independent of its role in cytokinesis (40). Ect2 is required for the growth of stem-like tumor-initiating cells and thereby contributes to KRas-driven lung cancer (30). In normal cells, Ect2 is sequestered within the nucleus. Upon breakdown of the nuclear envelope, Ect2 diffuses throughout the cytoplasm and associates with the mitotic spindle, promoting cytokinesis via RhoA activation. In cancer cells, however, a significant fraction of Ect2 mislocalizes to the cytoplasm, where it promotes Rac activation and transformed growth via the MEK/Erk pathway (5,30,40,42,43). Furthermore, a recent model described the activation of Rac1 by Ect2 in the nucleolus of cancer cells, where it promotes the synthesis of ribosomal RNA (rRNA), a major component of the ribosomal machinery. rRNA and protein biosynthesis are key requirements for abnormal cancer cell proliferation, and rRNA biogenesis inhibitors are currently in clinical trials as anti-cancer agents (30,42). The recent intriguing finding that nuclear Tiam1 is a negative regulator of colorectal cancer cell proliferation and invasion via suppression of a TAZ/YAP genetic program, and that it serves as a good prognostic factor for colorectal cancer patients (44) highlights the complexities of Rac-GEF signaling in different cancer types. Overall, these new findings indicate that different subcellular pools of Rac controlled by specific Rac-GEFs play distinctive roles in cancer progression.

Deregulation of Rac-GAPs: opposite roles in cancer

Based on their roles as catalyzers of Rac mediated GTP hydrolysis, the general assumption has been that Rac-GAPs would act as tumor suppressors. Although cellular-based analyses consistently demonstrated anti-growth and anti-migratory properties for most Rac-GAPs, only a few studies identified tumor suppressor activities for these proteins *in vivo*. Furthermore, this view has been recently challenged by the identification of Rac-GAPs with unexpected oncogenic properties in certain cancers.

The Rac-specific GAP β 2-chimaerin was originally identified as a potential tumor suppressor protein down-regulated in human glioblastoma (45). This GAP negatively

regulates Rac-mediated processes such as cell cycle progression and migration, although the effects vary depending on whether cancer cells are in epithelial or mesenchymal stages (46–48). A recent study demonstrated that genetic ablation of the $\beta 2$ -chimaerin gene (*Chn2*) in the MMTV-Neu/ErbB2 mouse model of breast cancer accelerates tumor onset and increases the number of preneoplastic lesions. However, tumor progression is substantially delayed, and tumors in a $\beta 2$ -chimaerin-null background are less aggressive, unveiling a dual role for $\beta 2$ -chimaerin as a suppressor of tumor initiation and a promoter of tumor progression (48).

Reduced expression of SrGAP2 and SrGAP3, two members of the Slit-Robo GTPase-activating proteins (srGAPs), has been observed in human osteosarcomas and invasive ductal breast carcinomas, suggesting a tumor suppression function for these proteins. Notably, while down-regulation of SrGAP2 contributes to a more aggressive phenotype, most likely by enhancing cell migration, down-regulation of srGAP3 promotes Rac1-dependent, anchorage-independent cell growth (49,50).

The Rac-GAP ArhGAP24 (also known as FilGAP) is down-regulated in renal tumors, and this reduced expression correlates with poor survival. Conversely, ectopic overexpression of ArhGAP24 in renal cancer cells reverts their tumorigenic potential by inhibition of G1/S cell cycle transition, induction of apoptosis, and reduction of invasion (51). ArhGAP24 also has an important role in breast cancer metastasis, although contrasting results reported both pro- and anti-metastatic effects of this protein in triple negative breast cancer (TNBC). Most notably, inhibition of ArhGAP24 by RASAL2 (a Ras-GAP) enhances mesenchymal invasion by increasing Rac activity, a mechanism that is clinically relevant since high RASAL2 expression is predictive of poor outcome in TNBC patients (52).

The remarkable complexity of Rac-GAP function is also well illustrated by studies showing a pro-tumorigenic function for RacGAP1 (MgcRacGAP), whose elevated expression has been linked with aggressiveness in several human cancers and has been recently identified as a metastatic driver in uterine carcinosarcoma (53). Similarly, high expression of the Rac/Cdc42 GAP CdGAP correlates with poor prognosis in breast cancer patients, and its expression is particularly elevated in the basal breast cancer subtype. Interestingly, CdGAP is required for TGF β and Neu/ErbB2-induced cell motility and invasion independently of its GAP activity. Through its proline-rich domain, CdGAP forms a functional complex with the transcription factor Zeb2, leading to repression of E-cadherin, and thus favoring epithelial-to-mesenchymal transition (EMT) (54). Another intriguing example is p190B RhoGAP, whose overexpression in the mammary gland results in Rac1 activation and enhanced ErbB2-driven tumorigenesis and metastasis (55). Thus, most probably the oncogenic activity of these GAP proteins involves functions independent of their ability to accelerate GTP hydrolysis from Rac.

TARGETING Rac GTPases: CAN WE OVERCOME THERAPY RESISTANCE?

Given the involvement of discrete Rho proteins in malignant transformation and metastasis, they became attractive targets for cancer therapeutics. Rac1 inhibitors have been developed, which are currently at an experimental/preclinical stage. The first selective Rac1 inhibitor, NSC23766, prevents the interaction of Rac1 with GEFs, particularly Tiam1 and Trio, and

displays anti-tumorigenic and anti-metastatic effects *in vivo*. Other agents acting via inhibition of Rac1-GEF interactions, albeit with potentially different GEF specificity, include EHOp-016, ZINC69391, and 1A-116. Compounds, such as EHT 1864, interfere with nucleotide binding to Rac1, thus preventing the GTPase from entering the GDP/GTP exchange cycle. Therefore, EHT 1864 should have a broader effect than the inhibitors of Rac/Rac-GEF interaction, and could be beneficial for cancers with Rac1 activating mutations such as melanoma (3,56).

In addition to the potential role for Rac1 in the control of PD-L1 expression and suppression of anti-tumor immune responses described above (21), it is becoming increasingly evident that Rac1 is involved in acquired resistance. Ectopic expression of Rac1 GEFs, Rac1 activators such as BCAR3/AND-34, or constitutively active Rac1, promotes resistance to anti-estrogens, a widely used endocrine therapeutic approach for estrogen receptor-positive (ER+) breast cancer patients (57,58). This effect is likely mediated by Rac activation of its main downstream effector, Pak1, which has been involved in tamoxifen resistance by promoting the phosphorylation of ER at Ser305, resulting in increased cyclin D1 expression. Notably, pharmacological inhibition of Rac1 restores the anti-proliferative effects of tamoxifen by reducing ER Ser305 phosphorylation (59). Similarly, Rac1 activity has been associated with resistance of ErbB2/HER2-positive breast cancer patients to the monoclonal antibody trastuzumab. Trastuzumab-resistant breast cancer cells display increased Rac1 activity, which inhibits the ability of trastuzumab to down-regulate ErbB2/HER2 by blocking its internalization (60). A recent study in prostate cancer assigned a crucial role to the Rac-GEF P-Rex1 in promoting resistance to VEGF/VEGFR-targeted therapy (35). Elevated VEGF expression is a hallmark of prostate cancer and a predictor of poor prognosis. The poor response to anti-VEGF (bevacizumab) and anti-VEGFR (sunitinib) therapy in prostate cancer may relate to autocrine VEGF signaling that sustains survival of resistant cancer stem cells (CSCs). These resistant cells exhibit hyperactivation of Rac1, as well as elevated expression of Rac-GEFs P-Rex1 and Tiam1. Notably, silencing P-Rex1, which abrogates Rac1 hyperactivation in the resistant cells, improves the sensitivity to bevacizumab and sunitinib. This study set the basis for the rationale use of Rac inhibitors in combination therapies. Conceivably, VEGF-targeted therapy could be efficient if combined with inhibitors of the P-Rex1/Rac1 pathway. Along the same line, P-Rex1 has been recently postulated as a biomarker for prediction of sensitivity of breast cancer cells to PI3K inhibitors (61). In addition, Rac inhibitors are also very efficient agents against leukemias and lymphomas (62,63). The antagonism of resistance toward fludarabine in chronic lymphocytic leukemia (CLL) by impairing Tiam1/Rac1 interaction with NSC23766 is a great example of the potential use of Rac inhibitors in chemoresistance (63).

Studies have also assigned a role to Rac1 in radioresistance acquisition. Rac1 expression is elevated in breast cancer cells that survive radiation treatment, and survival is mediated by the Erk and NF- κ B pathways. There have been numerous examples of sensitization of cancer cells to ionizing radiation by Rac inhibition, such as the reported effect of NSC23766 and dominant-negative Rac1 in breast cancer cells (64). The fact that all forms of resistance described above can be reverted by inhibition of Rac1 highlight the potential use of the pharmacological inhibition of this GTPase to overcome resistance in the clinic.

FINAL REMARKS

Beyond the pressing demand to fully understand the biology behind Rho GTPase signaling in the context of different cancer types, there is a critical need to translate those basic findings to a clinical setting. With the identification of driver mutations in small GTPases and their activators, such as those described for Rac1 and P-Rex2 in melanoma, new therapeutic avenues open for treatment. The current evidence linking Rac to metastatic dissemination as well as resistance to targeted therapies highlight the need for the development of candidate drugs to support individualized treatment approaches based on Rac inhibition. Unfortunately, the development of drugs designed to inhibit Rac lagged behind relative to other agents targeting crucial cancer signaling pathways. One attractive avenue, based on melanoma studies reporting the correlation between Rac mutant and PD-L1 expression, projects a potential use of Rac inhibitors in combination with anti-PD/PD-L1 antibodies or other agents that facilitate antitumor immune responses.

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Figure 1. Mechanisms of Rac deregulation in cancer

Activating mutations in Rac1 have been recently found in various cancers, including Rac1^{P29S}, identified as a driver mutation in melanomas. Another hyperactive form is the splice variant Rac1b present in a number of cancers. Degradation of Rac1 by the proteasome contributes to the control of Rac protein expression levels, and it could be impaired in tumors as a consequence of missense mutations in the ubiquitin ligase HACE1. Hyperactivation of Rac-GEFs as a consequence of overexpression or mutation is also a prominent cause of Rac1 deregulation in cancer. Rac-GAPs have a complex role in cancer, as their reduced expression contributes to Rac1 hyperactivation in some tumors, however overexpression of specific GAPs has been also linked to aggressiveness, most likely through GAP-independent functions. Abnormal nuclear Rac1 localization is also common in cancer, an effect that has been associated with improper nucleocytoplasmic shuttling. Deregulated

GEF activity in nuclear compartments, such as the nucleolus, may lead to localized Rac1 activation and enhanced ribosomal RNA (rRNA) synthesis.

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Table 1

Common cancer associated alterations in Rac1, Rac-GEFs, and Rac-GAPs.

	Alteration	Examples
Rac1	Up-regulation	Lung and breast cancer
	Point mutations	Rac1 ^{P29S} (melanoma) Rac1 ^{G12V/G12R/P34R/Q61R/Q61K} (germ cell testicular cancer) Rac1 ^{A159V} (head and neck cancer) Rac1 ^{Q61R} (prostate cancer)
	Splice variant	Rac1b (colorectal, breast and lung cancer)
	Mislocalization	Nuclear Rac1 (lung cancer)
Rac-GEFs	Up-regulation	Vav3 (ovarian cancer) P-Rex1 (breast and prostate cancer, melanoma) Ect2 (lung and pancreatic cancer) Tiam1 (breast, prostate and colon cancer)
	Point mutations	P-Rex2 (melanoma, pancreatic cancer) Vav1 (lymphoma)
	Mislocalization	Cytosolic Ect2 (lung cancer) Nucleolar Ect2 (lung cancer) Nuclear Tiam1 (colorectal cancer)
Rac-GAPs	Down-regulation	β 2-chimaerin (glioblastoma) SrGAP2 (osteosarcoma) SrGAP3 (breast cancer) ArhGAP24 (renal cancer)
	Up-regulation	RacGAP1 (uterine cancer) CdGAP (breast cancer) p190B RhoGAP (breast cancer)