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Dysregulated GPCR Signaling and Therapeutic Options in Uveal Melanoma

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Abstract

Uveal melanoma (UM) is the most common primary intraocular malignant tumor in adults and arises from the transformation of melanocytes in the uveal tract. Even after treatment of the primary tumor, up to 50% of patients succumb to metastatic disease. The liver is the predominant organ of metastasis. There is an important need to provide effective treatment options for advanced stage UM. In order to provide the preclinical basis for new treatments, it is important to understand the molecular underpinnings of the disease. Recent genomic studies have shown that mutations within components of G protein-coupled receptor (GPCR) signaling are early events associated with ~98% of UMs.

Implications—This review discusses the alterations in GPCR signaling components (GNAQ and GNA11), dysregulated GPCR signaling cascades, and viable targeted therapies with the intent to provide insight into new therapeutic strategies in UM.

Introduction

UM is a rare cancer with ~2,500 new patient diagnoses being reported per year in the U.S. Primary UM tumors are treated effectively by radiation plaque therapy or enucleation; however, approximately 50% of patients develop metastatic disease, frequently in the liver (1, 2). In some cases, metastases are found decades after successful treatment of the primary tumors and one likely explanation is dissemination of tumor cells from the primary site followed by cellular dormancy (3). Metastatic UM responds poorly to clinically available therapies and patients often succumb within 1 year of diagnosis of the metastases; hence, there is an urgent unmet need for effective therapeutic strategies for advanced UM (4). There is a low mutational burden in UM tumors, unlike cutaneous melanoma, but identification of

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mutations in components of GPCR signaling in UM tumors may uncover new therapeutic targets in UM. These components include *GNAQ*, *GNA11*, *PLCB4* and *CYSLTR2*, mutations of which are found in ~98% of UM. Here, we review the molecular alterations in GPCR pathway components and discuss the therapeutic possibilities directed at targeting GPCR signaling.

GNAQ and GNA11 mutations

GNAQ and *GNA11* encode the alpha subunits of guanine nucleotide-binding proteins (G proteins), $G\alpha_q$ and $G\alpha_{11}$, respectively. They form a heterotrimeric complex with β and γ subunits and are important intermediates between membrane-bound GPCRs and intracellular signaling cascades. G proteins are normally inactive when bound by guanosine diphosphate (GDP) but agonist activation of GPCRs triggers recruitment of the G proteins to the receptors where a switch from GDP to guanosine triphosphate (GTP) occurs, rendering the G proteins active to bind/stimulate proteins associated with downstream pathways. Normally, the GTPase activity of G proteins hydrolyzes GTP to GDP (5), an inactivation process that is catalyzed by the regulator of G protein signaling (RGS) proteins. *GNAQ* and *GNA11* mutations occur in a mutually exclusive manner in ~93% of UM tumors (The Cancer Genome Atlas (TCGA): *GNAQ* and *GNA11* mutations detected in ~50% and ~43% of UM tumors, respectively) (6). They are almost exclusively found in exon 5 codon 209 (Q209) although mutations in exon 4 codon 183 (R183) have been determined in a minority of cases (7–9). Common substitutions in *GNAQ* are glutamine-to-leucine (Q209L) and glutamine-to-proline (Q209P) whereas in *GNA11*, the most frequent substitution is Q209L. Q209 is crucial for the GTPase activity of G proteins; thus, hydrolysis of GTP is abolished in *GNAQ* and *GNA11* mutants, leading to constitutive activation of the $G\alpha_q$ and $G\alpha_{11}$ proteins in UM. Interestingly, the levels of $G\alpha_q$ Q209L mutant proteins may be regulated by Ric-8A, a molecular chaperone that contributes to folding of the G protein (10). Deletion of *Ric-8A* in *GNAQ* Q209L mutant melanocytes grafted into NSG mice led to a marked reduction in levels of membrane-associated mutant $G\alpha_q$ proteins and inhibition of *GNAQ* Q209L driven tumor progression.

GNAQ and *GNA11* are the most frequently mutated genes in UM; however, their mutations do not correlate with UM patient outcome, patient survival or factors that would indicate high risk of metastasis (9, 11). *GNAQ* and *GNA11* mutations occur at similar frequencies in metastasizing and non-metastasizing tumors. Similarly, these mutations are not associated with class 1 (low metastatic potential) or class 2 (high metastatic potential) of UM tumors (11, 12). It has been shown that the Q209 mutation in *GNAQ* and *GNA11* are found in benign nevi such as blue nevi in addition to primary and metastatic UM tumors (7, 8). Particularly, *GNAQ* is frequently mutated in blue nevi (~83%). This suggests that mutations in the G proteins are early events in UM development. Despite this notion, the *GNA11* Q209 mutation is more commonly identified in UM metastases (~57%) and found only in ~7% of benign blue nevi, indicating that in comparison to *GNAQ*, alteration in *GNA11* is associated with higher risk of metastasis of UM (7, 8, 13).

Effector and receptor mutations: *PLCB4* and *CYSLTR2*

In addition to the *GNAQ* and *GNA11* mutations, other, less common, driver mutations in UM have been identified recently by next-generation sequencing. Mutations in phospholipase C $\beta 4$ (*PLCB4*) and cysteinyl leukotriene receptor 2 (*CYSLTR2*) have been identified in 1% and 4 UM tumors, respectively (14, 15) and are mutually exclusive with *GNAQ* and *GNA11* mutations. The alteration in PLC $\beta 4$, D630Y, affects the Y-domain of the highly conserved catalytic core of PLC $\beta 4$ which controls signal transduction (14). Consistent with this finding, PLC $\beta 4$ interacts directly with G α_q (16) (Fig 1). PLC $\beta 4$ mediates signal transduction by catalyzing the conversion of phosphatidylinositol 4,5-bisphosphate (PIP2) in the plasma membrane into inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). Subsequently DAG and IP3 activate downstream signaling components such as protein kinase C (PKC). IP3, particularly, translocates into the cytosol where it induces the release of calcium (Ca²⁺) from the endoplasmic reticulum to activate PKC.

The other alteration in the GPCR pathway in UM is at the level of GPCRs. Out of 136 UM patient specimens analyzed by Moore and colleagues, 4 samples harbored a leucine to glutamine substitution at codon 129 (Leu129Gln) in the *CYSLTR2* gene, and all four samples lacked mutations in *GNAQ*, *GNA11* or *PLCB4* (15). *CYSLTR2* encodes a seven transmembrane GPCR that activates G α_q , a finding that is consistent with the mutual exclusive profile of the mutations (17) (Fig 1). Activation of G α_q by CysLT₂R promotes binding of G α_q /G α_{11} to PLC $\beta 4$. The Leu129Gln mutation is located in the third transmembrane helix of the receptor and promotes ligand independent activation of the GPCR. Expression of Leu129Gln *CYSLTR2* in HEK293 cells increased basal levels of calcium and promoted the growth of melanocyte cell lines *in vitro* and *in vivo* (15). Collectively, these findings raise the possibility of targeting mutant forms of *GNAQ/GNA11*, *PLCB4* and *CYSLTR2* in UMs as well as pathways downstream of mutant *GNAQ/GNA11*.

Dysregulated pathways downstream of mutant *GNAQ/GNA11*

Since the identification of mutations in GPCR signaling components in a high proportion of UM tumors, molecular understanding of pathways downstream of G α_q and G α_{11} has become crucial for development or discovery of effective treatment options for metastatic UM. These pathways include the ERK1/2, Rho/Rac/YAP and PI3K/AKT pathways.

ERK1/2 Pathway—The ERK1/2 pathway usually involves binding of ligands to tyrosine kinase receptors on the cell membrane and activation of downstream intermediates, RAS, RAF and MEK. MEK phosphorylates and activates ERK1/2, which subsequently undergoes dimerization and translocates into the nucleus to regulate cellular processes including proliferation, survival, differentiation and apoptosis (18). The ERK1/2 pathway is frequently activated in UM with 86% of primary UM tumors reported to have elevated ERK1/2 phosphorylation (19). However, unlike cutaneous melanoma, in which *BRAF* is commonly mutated, mutations in *RAS* and *BRAF* are rare in UM tumors (20). Only one patient with choroidal melanoma has been shown to harbor the *BRAF* V600E mutation (21). MEK-ERK1/2 activation in UM is expected to be induced rather by mutant G α_q /G α_{11} proteins. The G proteins activate PLC β and PKC, which then stimulates MEK/ERK1/2 (18) (Fig 1).

Transfection of *GNAQ* Q209L in human melanocytes enhanced phosphorylated ERK1/2 protein levels and was associated with increased anchorage-independent growth (7). Consistently, these results were reversed by siRNA-mediated knockdown of *GNAQ* which decreased phospho-ERK1/2 levels (7). However, it is noteworthy that in some UM cases, ERK1/2 may not be activated by G proteins as a study of 22 UM patient tumors did not observe a correlation between *GNAQ* mutation and ERK1/2 activation although samples with *GNAQ* mutations had a higher average of the total ERK1/2 expression level compared to *GNAQ* wild-type tumors (22).

Rho/Rac/YAP Pathway—A major role of $G\alpha_{q/11}$ is to directly bind and activate PLC β , but additional effectors and signaling pathways may also play an important role. Particularly relevant to UM, $G\alpha_{q/11}$ stimulates Rho and Rac small GTPase-mediated signaling through direct binding and activation of several members of the large Rho guanine-nucleotide exchange factor (RhoGEF) family, including p63RhoGEF and Trio (23, 24) (Fig 1). Trio appears to be a key player in mediating mitogenic signals in UM since knockdown of Trio inhibited tumor growth and DNA synthesis in two UM cell line models (25). Interestingly, Trio is a very large multi-domain protein that has two GEF domains, one that selectively activates RhoA and one that activates Rac1/RhoG. Notably, activation of both RhoA and Rac1 by Trio are required for $G\alpha_{q/11}$ mitogenic signaling (25, 26).

Multiple pathways downstream of Rho/Rac are likely to mediate Trio-dependent cell proliferation in mutant $G\alpha_{q/11}$ UM cells. Depletion of Trio by siRNA did not inhibit ERK1/2 activation, but instead blocked activation of the MAPKs JNK and p38; JNK and p38 can both regulate the transcription factor AP-1 which controls the transcription of a number of growth-promoting genes (25). In addition, two reports strongly implicate two transcriptional co-activators in the Hippo pathway, Yes-associated protein (YAP) and transcriptional co-activator with PDZ-binding motif (TAZ), as being critical mediators of oncogenic $G\alpha_{q/11}$ downstream of Rho/Rac (27, 28). These reports showed that mutationally activated $G\alpha_{q/11}$ induced the cytoplasm to nucleus translocation of YAP/TAZ and transcription of several YAP/TAZ-dependent genes that promote cell proliferation (27, 28). Moreover, siRNA depletion of YAP or inhibition of YAP with the pharmacological inhibitor verteporfin suppressed the growth of UM cells in culture and in a mouse model (27, 28). Robust nuclear localization of YAP was also observed in uveal melanocytes in a recently described zebrafish model for UM (29). The mechanisms underlying Rho and Rac activation of YAP appear to involve both inhibition of upstream kinases, large tumor suppressor homolog 1 and 2 (LATS1/2), as well as increased sequestration to F-actin of the YAP-binding protein angiominin (AMOT), resulting in both cases in increased levels of YAP available to translocate to the nucleus (27, 28, 30). The exciting implication of the above studies is that the Trio/Rho/Rac/YAP pathway of oncogenic $G\alpha_{q/11}$ signaling suggests numerous new therapeutic targets in UM.

ADP-ribosylation factor 6 (ARF6)—Another monomeric small GTPase, ARF6, has been proposed as a key mediator of most pathways activated by oncogenic $G\alpha_{q/11}$ (31). Inhibition or depletion of ARF6 in UM cells inhibited cell proliferation and the downstream signaling targets PLC β , ERK1/2, Rho, Rac and YAP. Activation of ARF6 by $G\alpha_{q/11}$ appears

to be mediated by direct binding of $G\alpha_{q/11}$ to the ARF-GEF, GEP100. A provocative mechanism for the role of ARF6 in controlling $G\alpha_{q/11}$ signaling was proposed, in which ARF6 promoted localization of mutationally activated $G\alpha_{q/11}$ to cytoplasmic vesicles, rather than the plasma membrane, and these cytoplasmic vesicles are the site of oncogenic signaling by $G\alpha_{q/11}$ (31) (Fig 1). Regardless of the exact mechanism of how ARF6 regulates $G\alpha_{q/11}$ signaling, ARF6 provides another potential therapeutic target in UM.

PI3K/AKT Pathway—Phosphatidylinositol (4,5)-bisphosphate 3-kinase (PI3K) catalyzes the formation of phosphatidylinositol (3,4,5)-trisphosphate (PIP3) from PIP2, and PIP3 induces membrane translocation of AKT where it becomes active and signals to promote cell proliferation and survival (18). PI3K signaling is negatively regulated by phosphatase and tensin homolog (PTEN), which reverses PIP2 conversion to PIP3. Complete loss of PTEN is identified in 16% of UM tumors (n=75) and weaker immunostaining of PTEN is found in 42.7% of UM tumors (32). Patients with PTEN-null tumors are associated with a shorter disease-free survival compared to patients with UM tumors expressing normal levels of PTEN (32).

Targeted therapies in UM

Patients with metastatic UM typically die within one year of diagnosis; hence, there is an urgent unmet need to identify novel approaches for treatment which includes targeted therapies as either monotherapy or in combinational strategies.

GPCR Inhibitors—Since activating mutations in either *CYSLTR2*, *GNAQ*, *GNA11* or *PLCB4* are found in ~98% of UMs, this signaling pathway represents a viable therapeutic target for the treatment of UM. One approach to targeting this pathway is the development of specific inhibitors to individual proteins in the pathway. For example, constitutively active GPCRs such as the CysLT₂R-L129Q mutant found in UM (15) could potentially be targeted using a receptor specific inverse agonist, which would bind to and stabilize an inactive conformation of the receptor that should no longer activate $G\alpha_{q/11}$. Unfortunately, CysLT₂R-L129Q is only a viable target in ~4% of UM patients. A more compelling target is the activated G protein, either $G\alpha_q$ or $G\alpha_{11}$, which together are mutated in ~93% of UM patients. In this regard, there are two potent and specific inhibitors that have been reported for $G\alpha_q$, YM-254890 and FR900359 or UBO-QIC. YM-254890 is a cyclic depsipeptide isolated from *Chromobacterium* sp. broth and showed inhibition of $G\alpha_q$ signaling mediated processes such as ADP-induced platelet aggregation and Ca^{2+} mobilization through a $G\alpha_q$ -coupled receptor (33). A crystal structure of the $G\alpha_q$ protein, loaded with GDP, in complex with YM-254890 suggests the inhibitor works by allosterically stabilizing the protein in the GDP bound form, preventing GDP dissociation, and locking it in the inactive state (34). More recently, FR900359, a slightly different cyclic depsipeptide isolated from the *Ardisia crenatasims* plant, has shown similar pharmacological activity and specificity against $G\alpha_q$ (35). Docking models and molecular dynamics studies suggest FR900359 inhibits $G\alpha_q$ in an identical manner to YM-254890 (36). While these compounds appear to be potent and selective inhibitors of $G\alpha_q$ (they have not been tested on $G\alpha_{11}$), it's unclear whether they would be able to inhibit the constitutively active forms of $G\alpha_q$ typically found in UM. For example, while YM-254890 inhibited a $G\alpha_q$ -R183C mutant, it was unable to inhibit $G\alpha_q$ -

Q209L (33). Moreover, one also needs to consider $G\alpha_q$ vs $G\alpha_{11}$ selectivity when inhibiting these proteins since a non-selective $G\alpha_q/11$ inhibitor would likely be toxic, as knockout of both $G\alpha_q$ and $G\alpha_{11}$ in mice leads to death in utero (37). Finally, one could consider targeting PLC β 4 with a selective inhibitor since this is mutated in ~1% of UM and is a downstream effector of $G\alpha_q/11$. However, PLC β 4 is unlikely to be the only downstream target of activated $G\alpha_q/11$ in UM so such an inhibitor might only prove useful in the small number of patients that have a PLC β 4 mutation.

MEK Inhibitors—Due to the high incidence of MEK-ERK1/2 activation in UM, targeting of components of the $G\alpha_q/G\alpha_{11}$ -induced ERK1/2 cascade such as MEK and PKC has been investigated as a therapeutic approach in UM. Inhibition of MEK1/2 either by trametinib, selumetinib or PD0325901, induced growth arrest and apoptosis in *GNAQ/GNA11* mutant UM cell lines and xenograft tumors (38–41). Trametinib is FDA-approved and used in combination with the BRAF inhibitor, dabrafenib for patients with unresectable or metastatic melanoma harboring BRAF V600E and V600K mutations (42). However, reports from clinical studies indicated variable outcomes for MEK inhibition in UM. In a phase I trial involving 16 metastatic UM patients, trametinib was shown to have limited efficacy (43). A phase II trial which enrolled 120 advanced UM patients showed that selumetinib improved the median progression-free survival (PFS) by ~9 weeks compared to chemotherapy (dacarbazine or temozolomide) but only modestly increased median overall survival (44). In the most recent phase III clinical trial (SUMIT), the combination of selumetinib and dacarbazine failed to improve PFS compared to chemotherapy alone and was associated with a low response rate (3–4%) (45). Thus, while pre-clinical studies support MEK inhibitors as part of a therapeutic approach for metastatic UM, it is important to identify agents that in combination enhance the responses of UM to MEK inhibition.

The poor clinical response of metastatic UM to MEK inhibitors may be in part due to factors produced by the tumor microenvironment. Hepatocyte growth factor (HGF) provided resistance to trametinib growth inhibitory effects in metastatic UM cell lines and targeting of cMET (the receptor for HGF) enhanced the effects of trametinib in metastatic UM (40, 46). HGF-cMET signaling induced downregulation of BH3 pro-apoptotic proteins, BIM and BMF, which correlated with HGF-mediated inhibition of apoptosis in trametinib-treated cultures (46). Importantly, HGF is secreted by quiescent hepatic stellate cells that can be found in the liver microenvironment and phosphorylated/activated cMET is detected in the majority of UM liver metastases (40, 46). HGF promotes activation of the PI3K/AKT pathway as a mechanism of resistance to trametinib and the increase in phosphorylation of AKT is mediated by PI3K isoforms α , δ and γ . Use of a β -sparing PI3K inhibitor, GDC0032, reversed HGF-induced resistance to trametinib (46). These findings support an earlier study which reported the combination of MEK and PI3K inhibitors for UM and these agents induce marked early apoptosis in *GNAQ* mutant UM cell lines compared to the inhibitors alone which have moderate effects on apoptosis (40, 41).

MEK inhibitors have also been tested in combination with PKC inhibitors for UM. Inhibition of PKC alone such as by AEB071 or sotrastaurin induced cell cycle G1 phase arrest and suppressed PKC and ERK1/2 signaling (39). However, these effects were not durable, whereas in combination with MEK inhibitors, PD0325901 or MEK162, sustained

inhibition of the ERK1/2 pathway was observed (39). Furthermore, the inhibitors caused a strong synergistic growth inhibitory effect on UM cell lines and marked tumor regression in UM xenograft models. Recently, screening of drug combinations involving AEB701 in a large panel of UM patient-derived xenografts (PDX) also determined two non MEK inhibitors that could potentially be used in combination with PKC inhibitors as therapeutic strategies for metastatic UM (47). These were the p53-MDM2 inhibitor, CGM097 and the mTORC1 inhibitor, RAD001. AEB701 and CGM097 or AEB701 and RAD001 markedly reduced tumor growth and induced tumor regression (47). These studies provide evidence for a number of targeted therapies that may be evaluated in combination with MEK inhibitors or PKC inhibitors in clinical studies to improve the survival of patients with metastatic UM.

Conclusions

There is a lack of effective treatment options for advanced UM despite success in the treatment of primary UM tumors. Whilst identification of driver mutations in UM such as *GNAQ* and *GNAI1* has shed light to potential therapeutic targets, alterations in GPCR signaling occur early in disease progression. Other mutations and their impact on responses of UM to GPCR targeted therapies will need to be explored (48). For example, inactivating mutations of *BAP1* have been found in ~80% of aggressive UM (49) while *SF3B1* and *EIF1AX* were shown to correlate with a favorable prognosis (50). In addition, ongoing investigations will be needed to devise strategies that elicit favorable treatment outcomes using therapies targeting GPCR signaling. As in other forms of advanced-stage melanoma, it is likely that combination approaches will be key to elicit high response rates and the most effective combinations and scheduling of inhibitors will need to be identified.

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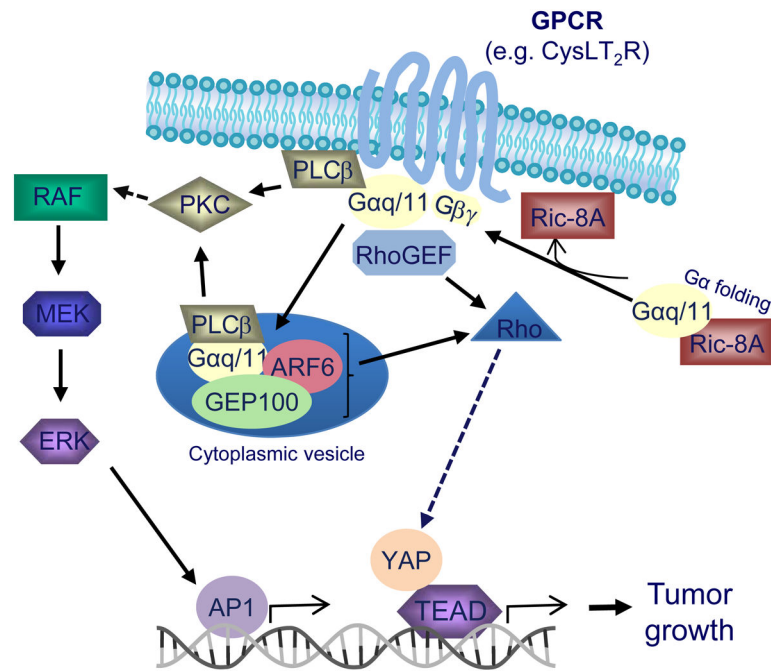


Fig 1.

Alterations in GPCR signaling in UM. GPCRs (e.g. CysLT₂R) activate the G α subunit proteins, G α_q and G α_{11} , which stimulate downstream effector pathways including ERK1/2 and YAP. Ric-8A chaperones participate in folding of G proteins which is important for membrane localization of the G proteins. ARF6 promotes localization of G $\alpha_q/11$ to cytoplasmic vesicles where oncogenic G $\alpha_q/11$ signaling can be active. In UM, mutations in *GNAQ*, *GNA11*, *CYSLTR2* or *PLCB4* lead to constitutive activation of pathways downstream of G α_q and G α_{11} proteins.