Functional Effects of GRM1 Suppression in Human Melanoma Cells

Janet Wangari-Talbot, Brian A. Wall, James S. Goydos, and Suzie Chen

Abstract

Ectopic expression of a neuronal receptor, metabotropic glutamate receptor 1 (Grm1), in melanocytes has been implicated in melanoma development in mouse models. The human relevance of this receptor’s involvement in melanoma pathogenesis was shown by detecting GRM1 expression in subsets of human melanomas, an observation lacking in benign nevi or normal melanocytes. Grm1-transformed mouse melanocytes and a conditional Grm1 transgenic mouse model confirmed a requirement for sustained expression of Grm1 for the maintenance of transformed phenotypes in vitro and tumorigenicity in vivo. Here, we investigate if continued GRM1 expression is also required in human melanoma cell lines by using two inducible, silencing RNA systems: the ecdysone/Ponasterone A and tetracycline on/off approaches to regulate GRM1 expression in the presence of each inducer. Various in vitro assays were conducted to assess the consequences of a reduction in GRM1 expression on cell proliferation, apoptosis, downstream targeted signaling pathways, and in vivo tumorigenesis. We showed that suppression of GRM1 expression in several human melanoma cell lines resulted in a reduction in the number of viable cells and a decrease in stimulated mitogen-activated protein kinase (MAPK) and PI3K/AKT and suppressed tumor progression in vivo. These results reinforce earlier observations where a reduction in cell growth in vitro and tumorigenesis in vivo were correlated with decreased GRM1 activities by pharmacologic inhibitors of the receptor, supporting the notion that GRM1 plays a role in the maintenance of transformed phenotypes in human melanoma cells in vitro and in vivo and could be a potential therapeutic target for the treatment of melanoma. Mol Cancer Res; 10(11); 1440–50. ©2012 AACR.

Introduction

Malignant melanoma, the most deadly form of skin cancer, which is also the fifth most common cancer in men and the sixth most common cancer in women, poses a substantial clinical burden with more than 76,000 Americans estimated to be diagnosed and 9,000 succumbing to the disease in 2012 (1). Using a transgenic mouse model (2), our group identified that the ectopic expression of metabotropic glutamate receptor 1 (Grm1), in melanocytes was sufficient to induce spontaneous melanoma development (3). Grm1 is a G-protein–coupled receptor (GPCR) whose expression is normally localized to the central and peripheral nervous system and has functional implications on learning and memory formation (4). Analysis of human melanoma cell lines and biopsy samples has detected GRM1 expression in 80% of cell lines and 65% of primary to metastatic biopsy samples but not in normal melanocytes or benign nevi (5, 6). In addition, we showed that stable mouse melanocytic clones with exogenously transfected Grm1 exhibited transformed phenotypes in vitro and were tumorigenic in vivo (7). Furthermore, a conditional Grm1 transgenic mouse model confirmed the requirement for continuous expression of Grm1 in murine melanocytes for initial tumor formation and progression in vivo (8).

The metabotropic glutamate receptor family encompasses 8 receptors, which are divided into 3 groups according to agonist pharmacology, sequence homology, and transduction mechanisms via coupling to second messenger systems (4, 9–11). Group I GRM1 and GRM5 receptors are coupled to the activation of phospholipase C through Gq proteins; group II GRM2 and GRM3 receptors and group III GRM4, GRM6, GRM7, and GRM8 receptors are negatively coupled to adenyl cyclase through Gi/0 proteins in heterologous expression systems (4, 9–11). In addition to GRM1, 2 other GRMs have been shown to have roles in melanoma development. It was recently shown that overexpression of Grm5 in mouse melanocytes can induce melanoma development in a transgenic mouse model (12). This overexpression of Grm5 was found to result in the activation of the mitogen-activated protein kinase (MAPK) pathway (12). Another group conducted a large-scale mutational analysis of GPCRs and identified mutations in GRM3 in approximately
17% of melanoma tumor samples (13). Some of these tumors were found to confer a growth advantage for the tumors through the action of MEK1/2, a kinase in the MAPK signaling pathway. In addition, GRM3 mutant melanoma cells were found to be more responsive to MEK inhibition with AZD-6244 than wild-type cells, especially when they also harbored BRAFV600E mutations (13).

Taken together, these reports suggest that glutamate receptors and glutamatergic signaling may play greater roles than previously thought in melanoma biology. Given that the large percentages of human melanomas examined showed GRM1 expression, we were interested to know if suppression of GRM1 expression may modulate the growth of human melanoma cells in vitro and stimulation of downstream signaling cascades as well as tumorigenic potentials in vivo. Here, we showed that siRNA targeted to GRM1 suppressed expression of the receptor and led to decreased levels of activated mitogenic MAPK as well as anti-apoptotic PI3/AKT pathways resulting in reduced cell proliferation and increased apoptosis in vitro and in vivo.

**Materials and Methods**

**Antibodies and reagents**

Antibodies against GRM1 (Novus Biologics); phospho-AKT (S473), total-AKT, phospho-ERK1/2, total-ERK1/2, PARP, caspase-3, and cleaved caspase-3 (Cell Signaling); phospho-AKT 2 (S474; Genscript); α-tubulin (Sigma); anti-VgXR-15C3 and 9B9 antibodies were a gift from the Developmental Studies Hybridoma Bank, University of Iowa (Iowa City, IA); Zecin, G418, blasticidin, hygromycin, Lipofectamine 2000, Plus Reagent, ViraPower Lentiviral Packaging mix (Invitrogen); doxycycline (Sigma); FACS (Enzo Life Sciences); sucrose (Fisher Scientific); DOTAP (Roche Applied Science); polybrene (Chemicon).

**Cell culture**

UACC903, 1205Lu, and C8161 were provided by Drs. Jeffry Trent (The Translational Genomics Research Center, Phoenix, AZ), Meenhard Herlyn (Wistar Institute, Philadelphia, PA), and Mary Hendrix (Children’s Memorial Research Center, Chicago, IL). The cells were maintained in RPMI-1640 plus 10% FBS. HEK293T cells and H1299 cells were provided by Drs. Renping Zhou and C. S. Yang (Children's Memorial Research Center, Chicago, IL). The cells were maintained in Dulbecco's Modified Eagle's Medium plus 10% FBS. HEK293T cells and H1299 cells were maintained in Dulbecco’s Modified Eagle’s Medium plus 10% FBS.

**Lentivirus production**

Tet receptor (TetR) cloned in lentiviral vector PLenti6L was a generous gift provided by Dr. Andrew Aplin (Kimmel Cancer Center, Philadelphia, PA). Lentivirus production was done using the ViraPower Packaging Mix according to the manufacturer’s instruction.

**DNA transfection and lentivirus transduction**

**Ponasterone-inducible system.** C8161 cells were transfected with 4 μg of pVGXRX plasmid DNA (a gift from Dr. Danny Rangasamy, The Australia National University, Canberra, Australia) with Lipofectamine 2000 reagent as per the manufacturer’s protocol. Stable clones expressing pVGXRX receptor were selected using 5 μg/mL of zeocin in RPMI plus 10% FBS. Receptor expression was confirmed by Western blotting. C8161 pVGXRX cells were transfected with 4 μg inducible siGRM1 plasmid DNA with the siRNA sequence 5’-GATGTACATCTATTGCGC-3’ (7), cloned into the pIND vector containing 5 repeats of ecdysone response elements (14) using Lipofectamine 2000. Stable C8161 pVGXRX siGRM1 clones were generated by selection with 5 μg/mL zeocin and 300 μg/mL G418. Induction of siGRM1 was achieved by treating the cells with 1 to 10 μmol/L of ponasterone A (PonA) for 7 days as described (15, 16).

**Western blotting**

Protein lysates were extracted from C8161 pVGXRX, C8161 pVGXRX-siGRM1 A10, or C8161 pVGXRX-siGRM1 A13 treated with plain media, vehicle control (dimethyl sulfoxide; DMSO), or 10 μmol/L PonA as described previously (6) and assayed for the expression of GRM1, phosphorylated and total ERK1/2, tubulin, caspase-3, and cleaved caspase-3 with the corresponding antibodies. Similarly, protein extracts from UACC903 TetR, UACC903 TetR siGRM1-8, UACC903 TetR siGRM1-11, 1205 Lu TetR, 1205LuTetR siGRM1-1, or 1205 Lu TetR siGRM1-9 treated with either plain media or 10 ng/mL doxycycline for 7 days and assayed for GRM1, phosphorylated and total ERK1/2, phosphorylated AKT (S473), phosphorylated AKT2, and α-tubulin expression. The blots were scanned and the intensity of the protein bands analyzed with OptiQuant Software (PerkinElmer Life Sciences).

**MTT assays**

MTT assays were conducted as described (6) with 1 × 10^3 cells per well of C8161 pVGXRX siGRM1 clones A10, and C8161 pVGXRX siGRM1 A13, or C8161 pVGXRX. The conditions were no treatment (NT), vehicle (DMSO) or 1, 5, and 10 μmol/L of PonA for 7 days. Absorbance was determined on days 0, 3, and 7 using a Tecan Plate reader (Infinite 200 Tecxan USA).
In vivo xenografts

**Ponasterone-inducible system.** C8161 pVgRXR siGRM1 A13 cells and vector control C8161 pVgRXR cells were injected at 10^6 cells per site in the dorsal flanks of 5- to 6-week-old athymic nude mice. When the tumor volumes reached approximately 10 to 20 mm³ as measured with a vernier caliper, mice were divided into 2 groups with similar tumor volumes with 6 mice per group. One group was treated with vehicle (olive oil; Veh) and the other one with PonA (10 mg/kg). Mice were treated twice a week with vehicle or PonA administered via intraperitoneal injection as described [16]. All tumor-bearing mice were euthanized after 37 to 42 days due to tumor burden in the control groups.

**Tetracycline/doxycycline-inducible system.** 1205 Lu TetR siGRM1-9, 1205 Lu TetR siGRM1-1, or UACC903 TetR siGRM1-8 and UACC903 TetR siGRM1-11 cells were injected as 10^6 cells per site in the dorsal flanks of 5- to 6-week-old athymic nude mice. When the tumor volumes reached approximately 10 to 20 mm³ as measured with a vernier caliper, mice were divided into no treatment (NT) or doxycycline (Dox) groups with similar tumor volumes in each group with 6 mice per group. A 0.1% doxycycline and 1% w/v sucrose solution was provided to the animals and replaced twice weekly in the Dox treatment groups as previously described [7]. The tumor volume was measured once a week, and all tumor-bearing mice euthanized when the tumor burden in the control groups approached maximum permitted levels.

**Immunohistochemistry**

Immunohistochemical staining on excised tumor xenografts from C8161 pVgRXR and C8161 pVgRXR siGRM1 A13 PonA or vehicle-treated controls, 1205 Lu TetR and 1205 Lu TetR siGRM1-9 doxycycline-treated and not-treated controls to detect changes in the number of apoptotic and proliferating cells (cleaved caspase-3 and Ki-67, respectively).
was conducted by Tissue Analytical Services at the Cancer Institute of New Jersey (New Brunswick, NJ). The number of stained cells was quantified with a digital Aperio ScanScopeGL system and ImageScope software (v 10.1.3.2028; Aperio Technologies Inc.) according to the manufacturer’s protocol with modifications as described (18).

Statistics

Statistics analysis was calculated using Microsoft Excel with 2-sample t tests with statistical significance set at \( P < 0.05 \).

Results

Ponasterone A and doxycycline-inducible suppression of GRM1 expression

GRM1-positive C8161 human melanoma cells were transfected with a plasmid construct encoding a heterodimeric receptor consisting of the ecdysone receptor (EcR) and the mammalian homologue of the ultraspiracle protein and the retinoid X receptor (RXR; pVgRXR). Cells expressing the receptor were then transfected with a plasmid encoding GRM1 siRNA (siGRM1). This construct also contains 5 copies of the ecdysone response element (EcRE) which are upstream of a minimal promoter and which can drive expression of siGRM1 in the presence of the inducer, an analogue of ecdysone, PonA (14, 15). We showed that in the presence of the inducer, 10 \( \mu \)mol/L PonA, GRM1 expression was diminished in several clones with examples of 2 such clones shown (Fig. 1A), whereas cells containing the RXR receptor but lacking the siGRM1-RNA, C8161 pVgRXR did not show any decrease in levels of GRM1 expression (Fig. 1A). Quantitation of the intensity of the Western blots indicate an approximately 40% decrease in C8161 pVgRXR siGRM1 A13 and an approximately 50% decrease in C8161 pVgRXR siGRM1 A10 (Fig. 1B).

For the tetracycline-inducible system, GRM1-positive UACC903 and 1205 human melanoma cells were infected with a lentiviral construct to ensure high expression levels of the TetR. The TetR-positive cells were then transfected with si-GRM1-RNA and treated with 10 ng/mL doxycycline to induce expression of siGRM1. Several independent clones were isolated and examples of some of the clones are shown. 1205 Lu TetR siGRM1 clones 1 and 9 and UACC903 TetR clones 8 and 11 exhibited decreased GRM1 expression compared with 1205 Lu TetR and UACC903 TetR cells that did not display any decrease in GRM1 expression after treatment with doxycycline (Fig. 1C). Quantitation of the intensity of the no treatment and doxycycline-treated samples showed that in the 1205 Lu TetR siGRM1 clones 1 and 9 and UACC903 TetR clones 8 and 11 exhibited decreased GRM1 expression compared with 1205 Lu TetR and UACC903 TetR cells that did not display any decrease in GRM1 expression after treatment with doxycycline (Fig. 1C). Quantitation of the intensity of the no treatment and doxycycline-treated samples showed that in the 1205 Lu TetR siGRM1 clones 1 and 9 and UACC903 TetR clones 8 and 11 exhibited decreased GRM1 expression, whereas (Fig. 1D, left) UACC903 TetR siGRM1 clones 8 and 11 exhibited an approximately 90% and 80% decrease in GRM1 expression, respectively (Fig. 1D, right).

Reduced GRM1 expression led to suppression in cell growth and enhanced apoptosis in vitro

We have shown previously that when the GRM1 receptor is rendered nonfunctional by treatment with a competitive or noncompetitive GRM1 antagonist, it results in suppression of the proliferation in vitro, in several GRM1-expressing human melanoma cell lines (6, 19). We conducted MTT cell...
viability/proliferation assays on clones from the ecdysone-regulated siRNA expression system; we showed that treatment with the inducer, PonA, resulted in a 15% to 30% decrease in C8161 pVgRXR siGRM1 cells, whereas control C8161 pVgRXR cells did not show any modulation in cell growth (Fig. 2A). Furthermore, we also showed that a reduction in viable cell number, at least in part, could be attributed to an increase in the apoptotic cell population as assessed by a rise in the levels of an apoptotic marker, the cleaved form of PARP (Fig. 2B).

MAPK signaling suppression by GRM1 siRNA

Possible alterations in MAPK signaling cascade by suppression of GRM1 in human melanoma cells were assessed. Treatment of C8161 pVgRXR siGRM1 A13 with the inducer, PonA, resulted in a decrease in the levels of activated/phosphorylated ERK1/2 in comparison with vector control C8161 pVgRXR cells (Fig. 3A). Quantification of the intensities of the phosphorylated ERK1/2 and total ERK1/2 (T-ERK1/2) showed a 40% decrease in the levels of the activated ERK1/2 in C8161 pVgRXR siGRM1 A13 lysates (Fig. 3B). Using the doxycycline-inducible system, we also observed a reduction in ERK1/2 phosphorylation in UACC903 TetR siGRM1 clones induced with 10 ng/mL doxycycline, which was not observed in UACC903 TetR controls under similar conditions (Fig. 3C). Quantitation of the Western blots comparing phosphorylated ERK1/2 with total ERK1/2 showed an approximately 50% and 75% reduction in intensity in UACC903 TetR siGRM1 clones 8 and 11, respectively (Fig. 3D). In 1205 Lu TetR siGRM1 clones, we also observed a reduction in phosphorylated ERK1/2 with clone 9 showing greater suppression than clone 1 (Fig. 3E). Quantitation of the Western blots comparing phosphorylated ERK1/2 with clone 9 showed an approximately 27% and 64% reduction in intensity in the 1205 Lu TetR siGRM1 clones 1 and 9, respectively (Fig. 3F).

Inhibition of AKT phosphorylation by GRM1 siRNA

We have previously shown that the PI3K/AKT pathway functions downstream of Grm1 and also identified the Akt2 isoform to be the mediator of Akt phosphorylation in our system (20). Here, we investigated whether there are changes in AKT phosphorylation with siRNA-mediated suppression of GRM1 expression. Our results indicate that suppression of GRM1 expression by siRNA can inhibit the
phosphorylation and activation of AKT in UACC903 human melanoma cells (Fig. 4A). Similar results were observed in siGRM1-1205 Lu human melanoma cell lines (Fig. 4C). Furthermore, we showed that AKT phosphorylation is mediated by the AKT2 isoform (Fig. 4A and C). Quantitation of the Western blots comparing phosphorylated AKT with total AKT showed an approximately 40% and 50% decrease in clones 1 and 9, respectively (Fig. 4D, left). Comparison of AKT2 phosphorylation normalized to total AKT with AKT2 showed an approximately 30% and 40% decrease in clones 1 and 9, respectively (Fig. 4D, left). Comparison of AKT2 phosphorylation normalized to total AKT showed an approximately 40% and 50% decrease in clones 1 and 9, respectively (Fig. 4D, left). Comparison of AKT2 phosphorylation normalized to total AKT showed an approximately 40% and 50% decrease in clones 1 and 9, respectively (Fig. 4D, left).

**Suppression of GRM1 expression by siRNA reduces in vivo tumorigenicity**

Next, we conducted in vivo xenograft experiments with vector control C8161 pVgRXR and C8161 pVgRXR siGRM1 A13 cells (Fig. 5A and B). The cells were inoculated into the dorsal flanks of nude mice and when the tumor volumes reached approximately 10 to 20 mm³, the mice were randomly divided into 2 groups, either treated with vehicle (olive oil) or 10 mg/kg of PonA in olive oil by intraperitoneal injections twice weekly as described (16, 21). We showed that in the C8161 pVgRXR siGRM1 A13 xenografts treated with PonA, the tumors appeared to progress at a slower rate compared with those treated with the vehicle and resulted in an approximately 45% ($P < 0.005$) decrease in tumor volume. In contrast, in the control C8161 pVgRXR, there was no suppression of tumor growth when treated with PonA; in fact, we observed slightly faster growth in comparison with vehicle-treated ones (Figs. 5A and B). We also conducted xenograft studies with cells harboring tetracycline-inducible siRNA. 1205 Lu TetR siGRM1 clones 1 and 9 or UACC903 TetR siGRM1 clones and not in 1205 Lu TetR controls after doxycycline (10 ng/mL) treatment. D, quantification of the intensities on the immunoblots from (C) with OptiQuant software. Total AKT was used to show equal loading. Dox, doxycycline; NT, no treatment.

Figure 4. Modulation of PI3K/AKT signaling by GRM1 siRNA. A, suppression of total AKT (serine 473) and AKT2 phosphorylation in UACC903 TetR siGRM1 clones 8 and 11, but not in UACC903 TetR after doxycycline (10 ng/mL) treatment. B, quantification of the intensities on the immunoblots from (A) with OptiQuant software. C, suppression of AKT (serine 473) and AKT2 phosphorylation in 1205 Lu TetR siGRM1 clones and not in 1205 Lu TetR controls after doxycycline (10 ng/mL) treatment. D, quantification of the intensities on the immunoblots from (C) with OptiQuant software. Total AKT was used to show equal loading. Dox, doxycycline; NT, no treatment.
Doxycycline treatment led to a statistically significant increase in cleaved caspase-3 expression and a decrease in Ki-67 expression in the PonA-treated samples over the not-treated controls with PonA exhibiting a statistically significant increase in cleaved caspase-3 over vehicle-treated samples. Analysis of the same samples showed a statistically significant decrease in Ki-67 expression in the PonA-treated samples compared to vehicle-treated samples. Using the doxycycline-regulated siRNA system, no differences were observed in cleaved caspase-3 and Ki-67 in the 1205 Lu TetR vector controls after doxycycline treatment. In the 1205 Lu TetR siGRM1-9–derived tumors, treatment with doxycycline led to a statistically significant increase in cells positive for cleaved caspase-3 over the not-treated controls with P < 0.05 (Fig. 6D, left). Meanwhile, a statistically significant decrease in Ki-67 cells was detected in the doxycycline-treated tumors, treatment with doxycycline led to a statistically significant increase in cells positive for cleaved caspase-3 over the not-treated controls with P < 0.05 (Fig. 6B, left). These results showed that in vivo suppression of GRM1 expression in these human melanoma cells has both antiproliferative and proapoptotic effects.
Discussion

We have previously reported that targeted Grm1 expression to melanocytes was sufficient to induce spontaneous melanoma development in vivo; subsequently, we also detected GRM1 expression in a subset of human melanoma cell lines and biopsies suggesting that GRM1 may be involved in melanomagenesis (2, 3, 6). In addition, 2 other metabotropic glutamate receptors have recently been documented to be involved in melanoma pathogenesis. Overexpression of Grm5 (12) and mutations in GRM3 (13) make...
this particular group of receptors and glutamate signaling attractive candidates for understanding the onset and progression of this deadly disease.

Gene silencing by siRNAs (22) has been used to silence the expression of specific genes to study their roles in different cell types and in various organisms. Inducible gene expression systems have also been developed to regulate expression in a temporal and quantitative manner to aid in the study of gene function (21). These regulatable expression systems rely on small molecules that serve as inducers to modify synthetic transcription factors, which regulate the expression of a target gene (21). The tetracycline operon–based, tetracycline-inducible system (7, 23, 24) and the nonmammalian steroid–based, ecdysone-inducible system (15, 16, 25) have been shown to be both reversible and efficient in regulating the expression of various mammalian genes. These inducible systems are especially critically important when siRNAs are used in inhibiting expression of genes crucial for cell or organism survival, as silencing occurs only in the presence of the inducer (14, 26). We and others have shown that the sustained expression of the inducer (14, 26). We and others have shown that the organism survival, as silencing occurs only in the presence of the inducer (14, 26).

It has been shown that GRM1 activation is coupled to the extracellular signal-regulated kinase (ERK1/2), a component of the classical MAPK pathway through G-protein and kinase-dependent mechanisms in the neuronal cells (27). Activation of ERK1/2 by phosphorylation on tyrosine and threonine residues has a pivotal role in intracellular signaling and can mediate cellular processes such as cell proliferation, invasion, metastasis, survival angiogenesis, and apoptosis (28, 29). Our group has shown that similar to the neuronal cells, MAPK activation can also result from upstream receptor-mediated activation events such as the activation of GPCRs including GRM1.

Despite the lack of specific GRM1 antagonists with clinical applications, our group has used Rilutek (riluzole), an U.S. Food and Drug Administration (FDA)-approved drug for the treatment of amyotrophic lateral sclerosis (36, 37) in clinical trials for patients with melanoma (38, 39). Riluzole inhibits the release of glutamate from intracellular to extracellular environments (40, 41). GPCRs with oncogenic activity are known to create autocrine/paracrine loops to maintain receptor activation (42–44). Inhibition of glutamate release by riluzole in GRM1-expressing cells disrupts glutamate receptor signaling and thus functions as an antagonist to the receptor (6). Results from our phase 0 and II trials strongly suggest that riluzole monotherapy has modest antitumor activity (38, 39); however, combining it with other anticancer agents may lead to additive or synergistic responses as shown in our preclinical studies (45). On the basis of these results, perhaps, it is not surprising that modulation of GRM1 expression results in concurrent suppression of activities of 2 major signaling pathways known to be critical in melanogenesis.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: J. Wangari-Talbot, J.S. Goydos, S. Chen
Development of methodology: J. Wangari-Talbot, S. Chen
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J. Wangari-Talbot, B.A. Wall, J.S. Goydos, S. Chen
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J. Wangari-Talbot, B.A. Wall, J.S. Goydos, S. Chen
Writing, review, and/or revision of the manuscript: J. Wangari-Talbot, B.A. Wall, J.S. Goydos, S. Chen
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