Published in final edited form as:

Pigment Cell Melanoma Res. 2008 June ; 21(3): 368–378. doi:10.1111/j.1755-148X.2008.00452.x.

Oncogenic activities of metabotropic glutamate receptor 1 (Grm1) in melanocyte transformation

Seung-Shick Shin, Jin Namkoong, Brian A. Wall, Ryan Gleason, Hwa Jin Lee, and Suzie Chen

Department of Chemical Biology, Ernest Mario School of Pharmacy, Susan Lehman Cullman Laboratory for Cancer Research, Rutgers University, Piscataway, NJ, USA

Summary

Previously, we reported a transgenic mouse line, TG-3, that develops spontaneous melanoma with 100% penetrance. We demonstrated that ectopic expression of Grm1 in melanocytes was sufficient to induce melanoma in vivo. In this present study, the transforming properties of Grm1 in two cultured immortalized melanocytes were investigated. We showed that, in contrast to parental melanocytes, these Grm1-clones have lost their requirement of TPA supplement for proliferation and have acquired the ability to form colonies in semi-solid medium. Xenografts of these cells formed robust tumors in both immunodeficient nude and syngeneic mice with a short latency (3–5 days). The malignancy of these cells was demonstrated by angiogenesis and invasion to the muscle and the intestine. The requirement of Grm1 expression for the maintenance of transformation was demonstrated by an inducible siRNA system. Induction of expression of siRNA for Grm1 reduced the number of proliferating/viable cells in vitro and suppressed in vivo xenografted tumor growth in comparison with control. Taken together, these results showed that expression of exogenously introduced Grm1 is sufficient to induce full transformation of immortalized melanocytes.

Keywords

melanoma; metabotropic glutamate receptor; Grm1; glutamate signaling; transformation; inducible siRNA

Introduction

Melanoma is the most aggressive type of skin cancer and is characterized by uncontrolled growth of melanocytes. Despite intense studies by many laboratories, the mechanism of transformation of normal melanocytes into melanoma remains unclear. Earlier, we had reported a transgenic mouse line, TG-3, that displays spontaneous melanoma with 100% penetrance in the absence of any known chemical stimuli or ultraviolet radiation. Subsequently, we identified and confirmed the aberrant expression of metabotropic glutamate receptor 1 (Grm1) in melanocytes is a major driving force in melanomagenesis by producing a new transgenic mouse line, E (Pollock et al., 2003).

Journal Compilation © 2008 Blackwell Munksgaard

*Address correspondence to Dr. Suzie Chen, suziec@rci.rutgers.edu.

There is no conflicting interest in this research among authors.

Supplementary material

The following supplementary material is available for this article online from
<http://www.blackwell-synergy.com/doi/full/10.1111/j.1755-148X.2008.00452.x>.

Grm1 is a member of the seven transmembrane G-protein coupled receptors (GPCRs), which functions in synaptic transmission (Hermans and Challiss, 2001). Grm1 is expressed throughout the central nervous system (CNS) and is implicated in numerous physiological and pathological processes including learning, memory formation, neuro-degeneration, and pain (De Blasi et al., 2001; Hermans and Challiss, 2001; Pin and Duvoisin, 1995). Eight Grms have been identified (De Blasi et al., 2001) and are classified into three groups according to sequence homology and response to agonist. Grm1 and Grm5 belong to Group I. In the CNS, Grm1 is activated by its natural ligand, L-glutamate. Glutamate signaling pathway via Grms, once thought to be restricted in neuronal cells, is now known to be involved in proliferation, migration, and differentiation in a variety of non-neuronal tissues such as bone, testis, pancreas, lung, heart, and the skin (Hinoi et al., 2004; Skerry and Ganev, 2001). Recently, Hoogduijn and co-workers identified the expression of several ionotropic forms of glutamate receptor in normal human melanocytes (Hoogduijn et al., 2006). Moreover, Frati and co-workers showed that Grm5 is functionally expressed in normal human melanocytes but not Grm1 (Frati et al., 2000). These studies are in agreement with our recent report where Grm1-positive melanoma cell lines release excess glutamate suggesting that a functional glutamate signaling pathway exists in melanomas (Namkoong et al., 2007). In addition to our earlier results, further supporting evidences that Grm1 gene could be important in melanoma susceptibility in the human system have been reported (Haas et al., 2007; Ortiz et al., 2007).

Remarkably, constitutive activation of MAPK induced by growth factor receptors (Satyamoorthy et al., 2003) as well as activating mutations of N-RAS (Demunter et al., 2001; Herlyn and Satyamoorthy, 1996) or B-RAF (V600E) (Davies et al., 2002) has been reported to be important in melanomagenesis. In B16 murine melanoma cells, activation of melanocortin 1 receptor (MC1R), a member of GPCR, by α -melanocyte stimulating hormone (α -MSH) induced stimulation of Extracellular signal-Regulated Kinase (ERK) via wild type B-RAF (Busca et al., 2000). These earlier studies showed that GPCRs could also lead to constitutive activation of Mitogen-Activated Protein Kinase (MAPK) resulting in cell transformation and, ultimately, tumor formation. In this report, we show that Grm1 has strong oncogenic activities in two different immortalized melanocyte cultures, melan-a and B10.BR, in the absence of the common activating mutations of N-RAS or B-RAF (V600E).

Results

Isolation of stable Grm1-melanocytic clones

Murine melanocytes, melan-a (Bennett et al., 1987) and B10.BR (Dotto et al., 1989) have been reported by others to exhibit characteristics of normal melanocytes and do not express Grm1 (Marin et al., 2006). These melanocytic cell lines were transfected with Grm1 cDNA (Zhu et al., 1999) or empty vector. Several stable Grm1 clones from melan-a (named to MASS clones) and B10.BR (named to B10SS clones) were isolated. Expression of Grm1 for each clone was confirmed by Western immunoblots (Figure 1). Grm1 expression was only detected in MASS and B10SS clones but not in vector controls. Several vector controls and Grm1 clones were selected for further studies.

Grm1 is functional in MASS and B10SS clones

Earlier, we demonstrated that, in cell lines derived from TG-3 tumors, Grm1 signaling was coupled to $G_{\alpha_q/11}$ which activates phospholipase C (PLC). PLC hydrolyzes phosphatidylinositol (PIP2) into two individual second messengers; inositol-1,4,5-triphosphate (IP3) and diacylglycerol (Marin et al., 2006). To examine the functionality of Grm1 in MASS and B10SS clones, levels of accumulated IP3 were assessed after stimulation with a Grm1-agonist, L-quisqualate (Q). Similar levels of Q-induced IP3 accumulation were detected in all Grm1-stable clones examined; a representative result is shown (Figure 2A). Stimulation of these cells with

Q for 5 min resulted in a significant increase (3.5-fold) of accumulated IP3 compared with no treatment (NT). The specificity of increased IP3 by Q was demonstrated by down-regulated IP3 level when these cells were preincubated with a non-competitive Grm1-antagonist, BAY 36-7620 (BAY) followed by induction with Q (Figure 2A). Some non-competitive antagonists such as BAY also have 'inverse agonist' activity, which is defined as one that also could inhibit basal activities of a given receptor (Carroll et al., 2001). As shown in Figure 2(A), pretreatment of these cells with BAY followed by Q reduced IP3 accumulation which is lower than the basal level of IP3 (NT). Therefore, these results indicate that Grm1 in MASS and B10SS stable clones is functional and responsive to stimulation/inhibition by its agonist/antagonist.

MAPK is activated in Grm1-stable clones by Grm1-agonist stimulation

We examined if stimulation of Grm1 by Q in MASS/B10SS clones led to activation of MAPK (ERK1/2). Strong activation of ERK1/2 was induced in all MASS/B10SS clones tested by treatment with Grm1-agonist, Q (Total four independent clones were examined. See Figure S2 in supplement data). As an example, MASS 20 was shown in Figure 2(B). The specificity of the ERK1/2 activation by Q was demonstrated by pretreatment of MASS/B10.BR clones with the non-competitive antagonist of Grm1, BAY, where nearly all activated ERK 1/2 was abolished (Figure 2B). In contrast, activation of ERK1/2 in vector control clones was not modulated by BAY, because of the absence of a functional Grm1 (Figure 2B). The steady state levels of phospho-ERK1/2 in the vector control clones were higher than MASS/B10BR clones, possibly contributed by the requirement of 12-*O*-tetradecanoylphorbol-13-acetate (TPA) supplement for growth as shown for untransformed melanocytes (Bennett et al., 1987; Wellbrock et al., 2004). Activation of either c-Jun N terminal kinase (JNK) or p38 was not induced by Q in MASS/B10SS clones (data not shown).

Cell cycle analysis for MASS clones treated with BAY showed a significant accumulation of cells in the sub-G1 phase indicative of apoptotic cells. MASS20 showed a 15-fold increase in the sub-G1 phase (30.52%) in comparison with NT (1.92%) after BAY treatment for 48 h (Figure 2C, top panels). BAY-treated MASS29 cells also showed nearly a 10-fold increase in the sub-G1 phase (Figure 2C, middle panels). In contrast, vector clones did not show any significant changes in the sub-G1 population after 48 h of BAY treatment (Figure 2C, bottom panels). Western immunoblots showed the cleaved form of Poly (ADP-ribose) polymerase (PARP) supporting the apoptotic response of MASS clones by BAY treatment (Figure 2D). Taken together, these results suggest that Grm1-specific antagonist, BAY, induces apoptosis in MASS clones only.

MASS and B10SS clones are TPA- and anchorage-independent

We first evaluated the requirement of TPA for growth of MASS/B10SS clones. It is known that the dependency on TPA for growth between normal and transformed melanocytes is quite different. For example, normal melanocytes including melan-a and B10.BR cells require the supplement of TPA or cholera toxin for optimal growth (Bennett et al., 1987; Halaban, 2000), while melanocytes transformed with known oncogenes or melanoma cells no longer require TPA for growth (Dotto et al., 1989; Wellbrock et al., 2004). All Grm1 stable clones derived from either melan-a or B10.BR grew well regardless whether TPA is present. In contrast, vector controls could not proliferate in the absence of TPA (Figure 3A). Taken together, results from these in vitro cell growth assays showed that Grm1 stable clones, unlike the vector control cells, grew equally well with or without TPA supplement.

However, TPA-independent growth of melanocytes does not always translate to fully transformed phenotype. For example, Dotto and co-workers showed that stable bFGF-B10.BR melanocytes were TPA independent but these cells were not tumorigenic when inoculated into immunodeficient mice (Dotto et al., 1989). We performed two different assays to test if these

clones were anchorage independent. Anchorage independence often is used as a preliminary indicator for tumorigenicity of cells in vivo (Shin et al., 1975). The first assay is the AlamarBlue™ reduction method, which is a rapid assay that allows a real time monitoring of cell growth in soft agar (Ke et al., 2004). The metabolism of MASS clones in the semi-solid condition was very active in comparison with the vector control (Figure 3B). We also performed the traditional anchorage assays for the detection of colony formation in soft agar. Several Grm1 clones were tested and they all formed colonies in soft agar whereas vector control did not (Figure 3C). Taken together, we concluded that ectopic expression of Grm1 in normal melanocytes confers them to be TPA and anchorage independent.

Excess glutamate is released by Grm1-stable clones

Recently, our laboratory reported that Grm1-positive human melanoma cells released excess amount of glutamate (Namkoong et al., 2007). In agreement with this observation, we also detected excess glutamate in stable-Grm1 clones suggesting that ectopic expression of Grm1 in melanocytes could lead to extracellular release of glutamate. In MASS clones, more than threefold of glutamate was released to the growth medium compared with vector control (Figure 3D top panel). MTT assays were performed in parallel to be sure that the cells were proliferating and the excess released glutamate was not because of cell death (Figure 3D, bottom panel). These results suggest possible presence of autocrine loop utilizing released glutamate for cell proliferation in these Grm1 stable clones.

Grm1-stable clones are tumorigenic in both nude and syngeneic mice

One of the most stringent criteria for cell transformation is the assessment of tumorigenic potential in vivo. We performed tumorigenicity assays by inoculating MASS/B10SS clones into the flanks of immunodeficient nude mice. A total of six MASS clones and three B10SS clones were tested. All the Grm1-stable clones began to form detectable nodules by day 3–5 after inoculation of 10^6 cells/site (Figure 4A, B); the appearance of the pigment is because of the melanin content in these melanocytes. The majority of the mice were killed after 3 weeks because of tumor burden. Both MASS and B10SS clones were tumorigenic; the only difference was that in B10SS cells the latency for detectable growth was slightly longer than MASS clones (10 days versus 5 days, respectively). If we reduced the number of cells injected from 10^6 to 10^5 /site, there was no difference in the tumorigenic potential except for a longer latency (5 days versus 7 days). In contrast, mice that were injected with vector control clones showed complete absence of tumor growth (Figure 4A, B). The ability of these Grm1-stable MASS clones to form tumors in syngeneic mice (C57/BL6) was also assessed. Very similar pattern of tumorigenesis, as observed for immunodeficient nude mice, was detected in these syngeneic mice (Figure 4C). Again no growth was detected in C57/BL6 mice inoculated with vector control cells (Figure 4C). Visible lesions of MASS clones in nude mice were detected approximately after 3–5 days of inoculation and the size of tumors reached 600 mm^3 after 15 days (Figure 4D). Tumorigenesis is a multi-step process that involves several discrete steps including independence of growth factors, evading apoptosis, increase in angiogenesis, and ability to metastasize (Hanahan and Weinberg, 2000). Tumor xenografts of MASS clones showed strong angiogenic activities (Figure 5A). The invasiveness of these Grm1 clones was demonstrated by the invasion of these tumor cells to the muscle and the intestine after 2 and 4 weeks, respectively (Figure 5B). Taken together, ectopic expression of Grm1 in normal melanocytes resulted in cellular transformation and induced robust tumor formation with malignant phenotypes in vivo.

Grm1 is required for the maintenance of tumorigenicity in Grm1-stable clones

To investigate if continuous Grm1 expression is required for the maintenance of the transformed phenotype in these Grm1 clones, we utilized the tetracycline-inducible siRNA

system (Matsukura et al., 2003). Briefly, a plasmid encoding the Tet^R was first transfected into MASS clones. Several stable clones were isolated and expression of Tet^R was confirmed by RT-PCR and Western immunoblots (data not shown). siGrm1-Tet^O or control siGFP-Tet^O recombinant DNA was transfected into stable Tet^R-MASS clones. Several inducible siGrm1-MASS or siGFP-MASS stable clones were isolated. Suppression of Grm1 expression induced by doxycycline (an analog of tetracycline) was assessed by Western immunoblots (data not shown). Cellular responses by suppression of Grm1 expression in these stable inducible siGrm1-MASS clones were evaluated by MTT growth assays in the presence or absence of doxycycline. Inclusion of doxycycline (2 ug/ml) resulted in approximately 50% of growth inhibition after 7 days while siGFP controls did not show any inhibition of cell growth under the same conditions (Figure 6A).

Next, we examined the tumorigenic potential of these inducible siGrm1- or siGFP-MASS clones in vivo. Inducible siGrm1- or siGFP-MASS clones were inoculated into immunodeficient nude mice. Doxycycline (0.1%, w/v) was added to the drinking water. Tumor volumes were measured twice a week with a vernier caliper. In comparison with the control group (no doxycycline), a decrease of approximately 60% in tumor volume was observed in the treatment groups at day 20 (Figure 6B). No significant change in tumor volumes was detected in siGFP group with or without doxycycline (Figure 6B). To verify that the suppression of tumorigenesis was indeed induced by siGrm1, we removed doxycycline from the drinking water at day 20. As predicted, tumor growth resumed in those animals and tumor volume approached that of the control group (Figure 6C). These data suggested that Grm1 expression is necessary for the maintenance of transformed and tumorigenic phenotypes in Grm1 melanocytic clones.

Discussion

In the current study, we characterized the oncogenic properties of Grm1 in two different spontaneously immortalized murine melanocytes. These stable Grm1-melanocytes were transformed as evident by various cell-based assays and robust tumor formation in both immunodeficient and syngeneic mice. Although the role of Grm1 has been intensively studied in neuronal cells, the transformation potential of Grm1 in cultured melanocytes originated from neural crest cells is first assessed in this study.

Earlier report by others showed that elevated levels of activating B-RAF (V600E) were toxic for melanocytes (Wellbrock et al., 2004). In both MASS (melan-a-Grm1) and B10SS (B10.BR-Grm1) clones, various levels of Grm1 expression were detected by Western immunoblots and the growth patterns of these clones were not compromised by ectopic expression of Grm1 suggesting the introduction of Grm1 did not alter the cellular homeostasis of melanocytes. It has been shown by others and confirmed by our lab that expression of p16^{Ink4a}/p19^{Arf} is absent in melan-a cells [(Sviderskaya et al., 2002); data not shown]. Therefore, lack of p16^{Ink4a}/p19^{Arf} could be considered as the initiation of genetic hit, subsequent ectopic expression of Grm1 induces a fully transformed phenotype in melan-a cells. Previously, we demonstrated that introduction of murine Grm1 cDNA regulated by a melanocyte-specific promoter, DCT, alone is sufficient to induce melanoma development in vivo (Pollock et al., 2003). Cell cycle regulators in addition to p16^{Ink4a}/p19^{Arf} may also be involved in Grm1-induced melanomagenesis in vivo cannot be ruled out.

Normal murine melanocytes have been widely used to assess the potential of cellular transformation. For example, melan-a cells were used to assess the oncogenic potential of activating mutation of B-RAF (Wellbrock et al., 2004) and the transforming ability of melanoma growth stimulatory activity/growth-regulated cytokines (MGSA/GRO) (Owen et al., 1997). B10.BR cells were used to evaluate the transformed behaviors of MAP kinase kinase

(MEK) (Govindarajan et al., 2003) and the influence of bFGF on the transformation of melanocytes (Dotto et al., 1989). In these studies, the onset of tumor induced by stimulation of MAPK pathways is at least 20 days. For example, the appearance of tumor in ^{V600E}B-RAF-melan-a clones (10^7 cells/site) was approximately 20 days (Wellbrock et al., 2004). In MGSA/GRO melan-a clones (5×10^6 cells/site) which activates RAS, the appearance of tumors was 20–30 days (Owen et al., 1997; Wang et al., 2000). Activating mutation of N-, H-, or K-RAS (G12V) when transfected into D6-Mel (10^7 cells/site; similar genetic background as melan-a) induced tumor formation after approximately 25, 40, and 50 days, respectively (Whitwam et al., 2007). We showed in the current studies that the onset of tumor formation of MASS clones (10^6 cells/site) was approximately 3–5 days with the size of tumor reaching 600 mm³ after 15 days (Figure 4D). The short latency, in addition to the vigorous tumor formation in xenografts of MASS clones, suggests that another cellular signaling cascade may confer a synergy effect with activated MAPK pathway. One of the candidates is AKT/Protein Kinase B (PKB). The synergy effect of MAPK and AKT pathways toward aggressive behaviors of melanomas is not new and has been proposed by others. Activation of AKT/PKB together with the constitutively activated MAPK (ERK) pathway induced by activating mutation of RAS (G12V-KRAS) shortened the onset of tumors and resulted in more aggressive phenotypes of D6-mel-RAS xenografts (Whitwam et al., 2007).

The malignancy of MASS cells is also verified by the apparent signs of angiogenesis as well as invasion to other tissues by the xenografts. We have examined for possible upregulation of angiogenic inducers such as vascular endothelial growth factor (VEGF) or basic fibroblast growth factor (bFGF) in excised xenografts, preliminary results showed little or no modulation of either one, suggesting that other angiogenic switches may be activated in our system. Invasiveness of MASS clones is demonstrated by the appearance of secondary tumors in the muscle and the intestine. These observations are consistent with the earlier result where tumor-bearing TG-3 mice showed metastases to the muscle at later stages of tumor development (Zhu et al., 1998). Clinically, the intestinal metastases are very rare in other cancers, however, Gupta and co-workers reported that tumors from transformed human melanocytes induced with SV40ER, hTERT, and oncogenic RAS (G12V) showed metastasis to the intestine (Gupta et al., 2005). The malignancy of MASS clones is also demonstrated by the formation of tumors in syngeneic C57/BL6 mice suggesting that the intact immune systems in these mice did not impair the potential of Grm1-induced tumorigenesis. It has been reported by others that transfection of oncogenic E1a or myc into B10.BR cells resulted in tumor formation in immunodeficient nude mice but not in immunocompetent syngeneic mice. In contrast, oncogenic H-RAS transformants resulted in tumor formation in both nude and syngeneic mice (Dotto et al., 1989). Similar to H-RAS transformants, MASS clones also were very tumorigenic in both nude and syngeneic mice, suggesting more severe alterations might occur in melanocytes transformed by H-RAS and Grm1 than E1a or myc oncogene.

We investigated possible involvement of other growth factor receptors (FGFR1, PDGFR β , EGFR1, and IGF1R) in Grm1-induced melanomagenesis. However, none of the receptors/ligands examined so far showed any evidence of cooperation with Grm1 in melanocyte transformation. Although we could not identify if one or more of receptor tyrosine kinases (RTKs) is involved in Grm1-induced transformation in MASS clones, the excess glutamate released in these MASS clones and not in the parent melan-a cells, suggesting the existence of an autocrine activation loop by Grm1 ligand, glutamate. The critical role of glutamate signaling in several other human cancers has been suggested by others. For example, glioma cells releasing excess amount of glutamate showed more aggressive growth than parental glioma cells (Takano et al., 2001). Suppression of glutamate signaling by antagonists of ionotropic glutamate receptors inhibited the growth of human tumor cell lines including neuroblastoma, rhabdomyosarcoma, brain astrocytoma, thyroid carcinoma, lung carcinoma, colon adenocarcinoma, and breast carcinoma. No inhibitory effect was observed in normal human

skin fibro-blasts under similar conditions (Rzeski et al., 2001). These publications support the data from our current study on MASS clones and our recent report where all human melanoma cells tested released excess amount of glutamate but not normal melanocytes (Namkoong et al., 2007). These observations suggest that, in normal melanocytes, glutamate release is tightly regulated by paracrine mechanism, and if transformed, melanoma cells release glutamate by autocrine activation mechanism to activate glutamate receptors and promote cell proliferation. Taken together, these studies suggest that there is a correlation between glutamate release and the malignancy of human cancers. In this report, we provided convincing evidences that ectopic expression of Grm1 leads to melanocyte transformation through activation of MAPK (ERK) which is a pivotal regulator in melanomagenesis. Constant release of glutamate, the natural ligand of Grm1, provided one of the mechanisms for the activation of ERK and proliferation.

Methods

Antibodies and reagents

Anti-phosphorylated ERK1/2, anti-ERK1/2, anti-PARP were purchased from Cell Signaling (Danvers, MA, USA); Anti-Grm1 was purchased from BD Biosciences (Franklin Lakes, NJ, USA); and α -tubulin antibody, myo-inositol, and TPA were obtained from Sigma (St Louis, MO, USA). ι -quisqualate [(L)-(+)- α -amino-3,5-dioxo-1,2,4-oxadiazolidine-2-propanoic acid] and BAY 36-7620 [(3aS,6aS)-6-anaphtalen-2-ylmethyl-5-methyliden-hexahydro- cyclopental [c]-furan-1-on] were purchased from Tocris (Ellisville, MO, USA).

Culture conditions and DNA transfection

Melan-a cells were provided by Dr. Dorothy Bennett (St. George's University of London, UK) and maintained in RPMI (Gibco BRL Laboratories, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Sigma), 100 U/ml of penicillin/streptomycin (Gibco BRL Laboratories), and 200 nM TPA (Sigma). Another immortalized melanocyte cell line, B10.BR, was provided by Dr. Ruth Halaban (Yale University, New Haven, CT, USA). B10.BR cells were grown in Ham's F10 (Gibco BRL Laboratories) containing 10% horse serum (Gibco BRL Laboratories), 100 U/ml of penicillin/streptomycin and supplemented with 85 nM TPA. Cloning of full-length Grm1 cDNA from a mouse brain cDNA library was reported previously (Zhu et al., 1999). Subsequently, Grm1 cDNA was subcloned into a modified PCI-neo vector with Dopachrome tautomerase (DCT) promoter. Grm1 cDNA or empty vector (2.5 lg) was transfected into melan-a or B10.BR cells (4×10^5 cells per 60-mm plate) using DOTAP transfection reagent (Roche, Basel, Switzerland) following the manufacturer's instruction. Stable Grm1 clones were selected by 400 μ g/ml of neomycin (Gibco BRL Laboratories). For the measurement of glutamate release, inositol-1,4,5-triphosphate (IP3) and induction experiments by Grm1-agonist/antagonist, cells were grown in glutamate and glutamine-free RPMI plus 10% dialyzed FBS (Gibco BRL Laboratories) with the supplement of GlutaMax™ (Gibco BRL Laboratories) to minimize the presence of glutamate, the natural ligand of Grm1.

Inositol-1,4,5-triphosphate measurement

Inositol-1,4,5-triphosphate accumulation was measured as described previously (Marin et al., 2006; Namkoong et al., 2007). Briefly, cells were grown in 24-well plates with sequential media changes. After overnight incubation in the presence of 3 μ Ci of myo-[3 H]inositol (3.22TBq/mmol; GE Healthcare, Piscataway, NJ, USA), cells were incubated in fresh glutamate/inositol/serum-free RPMI with LiCl (10 mM) for 15 min in the presence or absence of BAY 36-7620 (0.1 μ M) before stimulation with ι -quisqualate (10 μ M) for 5 min. The reactions were terminated and samples were washed twice with water-saturated diethyl ether (Sigma). Levels of incorporated [3 H] inositol in IP3 were measured by a scintillation counter (Beckman Coulter, Inc., Fullerton, CA, USA).

MTT cell proliferation assay

MTT assays were performed according to the manufacturer's protocol (Roche, Basel, Switzerland). Briefly, 2×10^3 cells/well was plated in a 96-well plate with reagents as indicated. Experiments were performed in quadruplicates and a representative graph of three independent experiments was shown. Absorbance was measured at 550 nm with the reference at 750 nm by GENios plate reader (Tecan, Durham, NC, USA) for the indicated time points.

Anchorage growth assays

AlamarBlue™ anchorage assays were performed as reported previously with minor modifications (Ke et al., 2004). In short, cell suspensions ($100 \mu\text{l}$) containing 2×10^3 cells with 0.33% (final concentration) of agar were plated into each well of a 96-well plate. The cells were allowed to grow for 3 days and stained with AlamarBlue™ (1:10 volume reagent; Biosource International, Camarillo, CA, USA). Cell growth was measured with a CytoFluor® Series 4000 Multi-Well Plate Reader (PerSeptive Biosystems, Framingham, MA, USA) at 530 nm (excitation) and at 590 nm (emission). For the conventional anchorage assays, bottom soft agar layer for each 60-mm plate contained RPMI plus 10% FBS with final concentration of 0.5% of SeaPlaque® GTG® agarose (Cambrex, Rockland, ME, USA) and overlay with cell suspension (10^6 cells) mixed with RPMI plus 10% FBS containing 0.33% of SeaPlaque® GTG® agarose. Cells were fed twice a week with 1 ml of RPMI plus 10% FBS with 0.33% SeaPlaque® GTG® agarose.

Tumorigenicity assay

All animal studies were approved by the Institutional Review Board for the Animal Care and Facilities Committee of Rutgers University. Nude mice were purchased from Taconic (Hudson, NY, USA). To assess the tumorigenicity of Grm1-transfectants, 10^6 or 10^5 cells/site were injected subcutaneously into 3- to 4-week-old male immunodeficient nude or syngeneic (C57/BL6) mice (The Jackson Laboratory, Bar Harbor, ME, USA). Tumor volumes were measured twice a week with a vernier caliper and calculated by the formula ($d^2 \times D/2$) as described (Stepulak et al., 2005).

Inducible RNA interference

The siRNA sequence for Grm1 (siGrm1) was 5'ATGTACATCATTATTGCC3' and cloned into pRNATin-H1.2/Hygro vector (GenScript, Piscataway, NJ, USA). A plasmid coding tetracycline repressor (Tet^R) was obtained from Dr. Daiya Takai (University of Southern California, Los Angeles, CA, USA). A total of 2.5 μg of Tet^R plasmid DNA and 0.5 μg of puromycin plasmid DNA were co-transfected into one of the stable Grm1-melanocytic clones, MASS20. Stable MASS20-Tet^R clones were selected with 0.8 $\mu\text{g}/\text{ml}$ of puromycin (Sigma). Expression of Tet^R was confirmed by reverse transcription PCR (RT-PCR) and Western immunoblots with rabbit polyclonal anti-Tet^R (Abcam Inc., Cambridge, MA, USA). Subsequently, MASS20-Tet^R clones were transfected with the siGrm1 plasmid and selected with 100 $\mu\text{g}/\text{ml}$ of hygromycin.

Measurement of glutamate release

Amplex Red Glutamic Acid/Glutamate Oxidase assay kit (Invitrogen-Molecular Probes, Eugene, OR, USA) was used to measure the amount of glutamate released in the medium. Cells were plated at 10^3 cells/well with 200 μl of medium in 96-well plates and grown for 3 days in glutamate/glutamine deficient RPMI supplement with 2 mM of GlutaMax™ and 10% dialyzed-FBS as described above. After each specified time, 100 μl of medium were collected from each well for the measurement of glutamate released according to the manufacturer's protocol. Cells left with the remaining 100 μl of medium in the wells were used for MTT cell proliferation assays to confirm the viability of cells.

Cell cycle analysis

Cells were plated at 2×10^6 per 100-mm culture plate and treated with BAY 36-7620 (50 μ M). After 24 or 48 h, cells were collected and washed twice with ice-cold phosphate-buffered saline (PBS). Cell pellets were fixed by drop wise addition of ice-cold 70% ethanol and incubated for 20 min at 4°C. Fixed cells were washed twice with ice-cold PBS and resuspended in 500 μ l of PBS. Cells were treated with RNase A solution (20 μ g/ml) (Sigma), and labeled with propidium iodide (50 μ g/ml) (Sigma) for 30 min. Cell cycle analysis was performed by the Flow Cytometry Facility Core at Rutgers University using a Beckman Coulter system (EPICS XL-MCL model).

Western immunoblots

Protein lysates were prepared as described previously (Cohen-Solal et al., 2002). Briefly, media was removed and cells were washed once with ice-cold PBS. After removal of PBS, the extraction buffer was added directly to the plates and cells were collected with a cell scraper. Cells were incubated on ice for 20 min. Cell debris was removed by centrifugation at 10 000 g at 4°C for 20 min and supernatant was taken for Western immunoblot analysis. For the detection of the cleaved form of PARP, cells were plated at 2×10^5 per 60-mm plate and treated with 50 μ M of BAY or dimethyl sulfoxide (DMSO, vehicle) for 24 h. Cells were harvested and lysed for Western immunoblot.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work has been supported by the following grants: National Cancer Institute grant R01CA108720, National Institute for Environmental Health Services grant ES05022, Melanoma Research Foundation grant GM55145, and New Jersey Commission for Cancer Research-NJCCR 07-1064-CCR-E0. We wish to thank Dr. Dorothy Bennett for melan-a cells, Dr. Ruth Halaban for B10.BR cells, Dr. Daiya Takai for providing inducible Tet^R plasmid and Dr. Alice Liu for AlamarBlue™ assays.

References

- Bennett DC, Cooper PJ, Hart IR. A line of nontumorigenic mouse melanocytes, syngeneic with the B16 melanoma and requiring a tumour promoter for growth. *Int. J. Cancer* 1987;39:414–418. [PubMed: 3102392]
- Busca R, Abbe P, Mantoux F, Aberdam E, Peyssonnaud C, Ballotti R. Ras mediates the cAMP-dependent activation of extracellular signal-regulated kinases (ERKs) in melanocytes. *EMBO J* 2000;19:2900–2910. [PubMed: 10856235]
- Carroll FY, Stolle A, Beart PM, et al. BAY36-7620: a potent non-competitive mGlu1 receptor antagonist with inverse agonist activity. *Mol. Pharmacol* 2001;59:965–973. [PubMed: 11306677]
- Cohen-Solal KA, Crespo-Carbone SM, Namkoong J, Mackason KR, Roberts KG, Reuhl KR, Chen S. Progressive appearance of pigmentation in amelanotic melanoma lesions. *Pigment Cell Res* 2002;15:282–289. [PubMed: 12100494]
- Davies H, Bignell GR, Cox C, et al. Mutations of the BRAF gene in human cancer. *Nature* 2002;417:949–954. [PubMed: 12068308]
- De Blasi A, Conn PJ, Pin J, Nicoletti F. Molecular determinants of metabotropic glutamate receptor signaling. *Trends Pharmacol. Sci* 2001;22:114–120. [PubMed: 11239574]
- Demunter A, Stas M, Degreef H, De Wolf-Peeters C, van den Oord JJ. Analysis of N- and K-ras mutations in the distinctive tumor progression phases of melanoma. *J. Invest. Dermatol* 2001;117:1483–1489. [PubMed: 11886512]
- Dotto GP, Moellmann G, Ghosh S, Edwards M, Halaban R. Transformation of murine melanocytes by basic fibro-blast growth factor cDNA and oncogenes and selective suppression of the transformed

- phenotype in a reconstituted cutaneous environment. *J. Cell Biol* 1989;109:3115–3128. [PubMed: 2556408]
- Fрати C, Marchese C, Fisichella G, Copani A, Nasca MR, Storto M, Nicoletti F. Expression of functional mGlu5 metabotropic glutamate receptors in human melanocytes. *J. Cell. Physiol* 2000;183:364–372. [PubMed: 10797311]
- Govindarajan B, Bai X, Cohen C, Zhong H, Kilroy S, Louis G, Moses M, Arbiser JL. Malignant transformation of melanocytes to melanoma by constitutive activation of mitogen-activated protein kinase kinase (MAPKK) signaling. *J. Biol. Chem* 2003;278:9790–9795. [PubMed: 12514183]
- Gupta PB, Kuperwasser C, Brunet JP, Ramaswamy S, Kuo WL, Gray JW, Naber SP, Weinberg RA. The melanocyte differentiation program predisposes to metastasis after neoplastic transformation. *Nat. Genet* 2005;37:1047–1054. [PubMed: 16142232]
- Haas HS, Pfragner R, Siegl V, Ingolic E, Heintz E, Schraml E, Schauenstein K. The non-competitive metabotropic glutamate receptor-1 antagonist CPCCOEt inhibits the in vitro growth of human melanoma. *Oncol. Rep* 2007;17:1399–1404. [PubMed: 17487397]
- Halaban R. The regulation of normal melanocyte proliferation. *Pigment Cell Res* 2000;13:4–14. [PubMed: 10761990]
- Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell* 2000;100:57–70. [PubMed: 10647931]
- Herlyn M, Satyamoorthy K. Activated ras. Yet another player in melanoma? *Am. J. Pathol* 1996;149:739–744. [PubMed: 8780377]
- Hermans E, Challiss RA. Structural, signalling and regulatory properties of the group I metabotropic glutamate receptors: prototypic family C G-protein-coupled receptors. *Biochem. J* 2001;359:465–484. [PubMed: 11672421]
- Hinoi E, Takarada T, Ueshima T, Tsuchihashi Y, Yoneda Y. Glutamate signaling in peripheral tissues. *Eur. J. Biochem* 2004;271:1–13. [PubMed: 14686914]
- Hoogduijn MJ, Hitchcock IS, Smit NP, Gillbro JM, Schallreuter KU, Genever PG. Glutamate receptors on human melanocytes regulate the expression of MiTF. *Pigment Cell Res* 2006;19:58–67. [PubMed: 16420247]
- Ke N, Albers A, Claassen G, Yu DH, Chatterton JE, Hu X, Meyhack B, Wong-Staal F, Li QX. One-week 96-well soft agar growth assay for cancer target validation. *Biotechniques* 2004;36:826–828. 830, 832–833. [PubMed: 15152603]
- Marin YE, Namkoong J, Cohen-Solal K, Shin SS, Martino JJ, Oka M, Chen S. Stimulation of oncogenic metabotropic glutamate receptor 1 in melanoma cells activates ERK1/2 via PKCepsilon. *Cell. Signal* 2006;18:1279–1286. [PubMed: 16305822]
- Matsukura S, Jones PA, Takai D. Establishment of conditional vectors for hairpin siRNA knockdowns. *Nucleic Acids Res* 2003;31:e77. [PubMed: 12888529]
- Namkoong J, Shin SS, Lee HJ, Marin YE, Wall BA, Goydos JS, Chen S. Metabotropic glutamate receptor 1 and glutamate signaling in human melanoma. *Cancer Res* 2007;67:2298–2305. [PubMed: 17332361]
- Ortiz P, Vanaclocha F, Lopez-Bran E, et al. Genetic analysis of the GRM1 gene in human melanoma susceptibility. *Eur. J. Hum. Genet* 2007;15:1176–1182. [PubMed: 17609672]
- Owen JD, Strieter R, Burdick M, Haghnegahdar H, Nanney L, Shattuck-Brandt R, Richmond A. Enhanced tumor-forming capacity for immortalized melanocytes expressing melanoma growth stimulatory activity/growth-regulated cytokine beta and gamma proteins. *Int. J. Cancer* 1997;73:94–103. [PubMed: 9334815]
- Pin J-P, Duvoisin R. The metabotropic glutamate receptors: structure and functions. *Neuropharmacology* 1995;34:1–26.
- Pollock PM, Cohen-Solal K, Sood R, et al. Melanoma mouse model implicates metabotropic glutamate signaling in melanocytic neoplasia. *Nat. Genet* 2003;34:108–112. [PubMed: 12704387]
- Rzeski W, Turski L, Ikonomidou C. Glutamate antagonists limit tumor growth. *Proc. Natl. Acad. Sci. USA* 2001;98:6372–6377. [PubMed: 11331750]
- Satyamoorthy K, Li G, Gerrero MR, Brose MS, Volpe P, Weber BL, Van Belle P, Elder DE, Herlyn M. Constitutive mitogen-activated protein kinase activation in melanoma is mediated by both BRAF mutations and autocrine growth factor stimulation. *Cancer Res* 2003;63:756–759. [PubMed: 12591721]

- Shin SI, Freedman VH, Risser R, Pollack R. Tumorigenicity of virus-transformed cells in nude mice is correlated specifically with anchorage independent growth in vitro. *Proc. Natl. Acad. Sci. USA* 1975;72:4435–4439. [PubMed: 172908]
- Skerry TM, Genever PG. Glutamate signalling in non-neuronal tissues. *Trends Pharmacol. Sci* 2001;22:174–181. [PubMed: 11282417]
- Stepulak A, Sifringer M, Rzeski W, et al. NMDA antagonist inhibits the extracellular signal-regulated kinase pathway and suppresses cancer growth. *Proc. Natl. Acad. Sci. USA* 2005;102:15605–15610. [PubMed: 16230611]
- Sviderskaya EV, Hill SP, Evans-Whipp TJ, Chin L, Orlow SJ, Easty DJ, Cheong SC, Beach D, DePinho RA, Bennett DC. p16(Ink4a) in melanocyte senescence and differentiation. *J. Natl. Cancer Inst* 2002;94:446–454. [PubMed: 11904317]
- Takano T, Lin JH, Arcuino G, Gao Q, Yang J, Nedergaard M. Glutamate release promotes growth of malignant gliomas. *Nat. Med* 2001;7:1010–1015. [PubMed: 11533703]
- Wang D, Yang W, Du J, Devalaraja MN, Liang P, Matsumoto K, Tsubakimoto K, Endo T, Richmond A. MGSA/GRO-mediated melanocyte transformation involves induction of Ras expression. *Oncogene* 2000;19:4647–4659. [PubMed: 11030154]
- Wellbrock C, Ogilvie L, Hedley D, Karasarides M, Martin J, Niculescu-Duvaz D, Springer CJ, Marais R. V599EB-RAF is an oncogene in melanocytes. *Cancer Res* 2004;64:2338–2342. [PubMed: 15059882]
- Whitwam T, Vanbrocklin MW, Russo ME, Haak PT, Bilgili D, Resau JH, Koo HM, Holmen SL. Differential oncogenic potential of activated RAS isoforms in melanocytes. *Oncogene* 2007;26:4563–4570. [PubMed: 17297468]
- Zhu H, Reuhl K, Zhang X, Botha R, Ryan K, Wei J, Chen S. Development of heritable melanoma in transgenic mice. *J. Invest. Dermatol* 1998;110:247–252. [PubMed: 9506443]
- Zhu H, Ryan K, Chen S. Cloning of novel splice variants of mouse mGluR1. *Brain Res. Mol. Brain Res* 1999;73:93–103. [PubMed: 10581402]

Figure 1.

Stable Grm1 melanocytic clones were selected. Spontaneously immortalized melanocytes, melan-a and B10.BR were transfected with empty vector or Grm1 cDNA. Stable clones expressing Grm1 were selected with neomycin. Mouse brain lysate was loaded as a positive control. Various levels of Grm1 expression at ~150 kD were detected. Total ERK (ERK1/2) was used as a protein loading control. (MASS and B10SS clones represent melan-a and B10.BR cells expressing Grm1, respectively).

Figure 2.

(A) The functionality of Grm1 was assessed by measuring levels of accumulated IP3. MASS clones were stimulated with Grm1-agonist, L-quisqualate (Q, 10 μM), for 5 min, or preincubated with Grm1-antagonist, BAY 36-7620 (BAY, 0.1 μM), for 15 min followed by stimulation with Q for 5 min. Data were expressed relative to no treatments (NT). Fetal bovine serum (10%) was used as a positive control (S). An average of three independent experiments of a representative cell line (MASS20) is shown; bars, standard deviations; *P < 0.05 compared with NT (t test); **P < 0.05 compared with Q (t test). Statistically significant elevated levels of accumulated IP3 were detected in Q-treated cells. Pretreatment of these cells with BAY abolished Q-induced IP3 accumulation. (B) Stimulation of Grm1 in MASS20 cells led to the activation of ERK. Treatments of these cells with Q (10 μM) resulted in ERK activation. Pretreatment of these cells with BAY (10 μM) for 30 min prior to Q induction resulted in no activation of ERK. In contrast, no modulation in levels of p-ERK in the vector control was observed. Same membrane was stripped and reprobed with total ERK. (C) Cell cycle analysis of MASS and vector control clones treated by BAY 36-7620. Cell cycle analysis was performed with MASS20, 29 and vector control cells treated with BAY 36-7620 for 48 h. Treatment of MASS20 or MASS 29 cells with BAY led to accumulation in the sub-G1 phase, an indicator of apoptotic response. DMSO treatment (vehicle) showed little or no effects. The percentage of cells in each phase was given (C = sub-G1; D = G1; E = S; and F = G2/M phase). (D) Levels of the cleaved forms of PARP were examined by Western immunoblots in MASS clones treated with 50 μM of BAY36-7620 for 24 h. Elevated levels of cleaved PARP were only observed in MASS clones treated with BAY compared with vehicle (DMSO) treated ones. The same blot was stripped and reprobed with ERK1/2 to show equal loading.

Figure 3.

Grm1-stable clones are TPA- and anchorage-independent. (A) The requirement of TPA-supplement on the proliferation of Grm1-stable clones was assessed by MTT cell growth assays. MASS, B10SS, and vector controls (2×10^3 cells/well) were grown in 96-well plates in the presence (white bar) or absence (black bar) of TPA up to 4 days. MTT assays were performed in quadruplicate and mean values of absorbance at day 4 are shown. Similar results were obtained from three independent experiments. Bar, SD; * and **p < 0.001 compared with melan-a (Mel Vec1) or B10.BR vector control (B10 Vec1), respectively. (B) Anchorage growth assays were performed in a 96-well plate. 100 μ l of soft agar layer containing 2×10^3 cells were plated in each well. The cells were allowed to grow for 3 days and the cell growth was measured using AlamarBlue™ staining. Fluorescence changes induced by reduction of AlamarBlue™ were measured at the excitation at 530 nm and the emission at 590 nm. Experiment was performed in quadruplicate and mean values are shown. Similar results were obtained from three experiments. (C) Conventional colony formation assays in soft agar were evaluated for MASS clones. 10^6 cells of MASS or vector control clones were plated in 60 mm plates. Colonies (colony > 100 cells) were counted after 3 weeks in four randomly selected areas with a dissecting microscope (Wild TYP 352873, Heerbrugg, Switzerland) at 20 $\times$ magnification. Two independent sets of experiments showed similar results. (D) MASS clones and vector controls were examined for levels of released glutamate. Cells were plated in 96-well plates at 2×10^3 cells/well and half of the media (100 μ l) were collected to measure the amount of glutamate at time points indicated (top panel). Cells in the remaining half of the media (100 μ l) were used for MTT cell growth assays (bottom panel). Media without cells were used as the media control in each experiment. At least three independent experiments

were done. All the stable Grm1 clones released excess amounts of extracellular glutamate in comparison with the vector control (melan-a vec1).

Figure 4.

Grm1 stable clones formed tumors in both syngeneic and nude mice. 10⁶ cells of each MASS clone (A) and B10SS clone (B) were inoculated subcutaneously into immunodeficient nude mice. Examples of tumors of MASS20 and B10SS17 are shown. Tumorigenicity of Grm1 stable clones were not altered by decreasing the inoculated cell numbers to 10⁵ cells. (C) Several MASS clones (10⁶ cells/site) were also injected into immunocompetent syngeneic mice, C57/BL6. An example is shown (MASS20). (D) Volumes of MASS20 tumors (10⁶ cells/site) in nude mice were measured at time points indicated. Black lesions were visible at day 3 after inoculation.

Figure 5.

Grm1-stable clones showed malignant phenotype in nude mice. (A) Formation of new blood vessels (angiogenesis). All the MASS clones tested showed strong angiogenic activities. An example of angiogenesis from MASS20 tumors is shown. (B) Invasion of MASS clones was detected in the muscle (top picture) and the intestine (bottom picture) after 2 weeks and 4 weeks, respectively.

Figure 6.

Inducible siRNA for Grm1 (siGrm1) suppressed proliferation of Grm1 stable clones. (A) MTT cell proliferation assays were performed for 7 days with siGrm1 B20 (one of stable MASS20 clones with inducible siGrm1). Addition of an inducer, doxycycline, to the media resulted in growth inhibition of these clones, but not control clones transfected with siRNA for the green fluorescence protein (GFP). Data are shown for one of the clones, siGFP7. (B) Tumorigenicity of siGrm1 B1, siGrm1 B3 and siGFP7 was assessed by injecting 10^6 cells/site subcutaneously into immunodeficient nude mice. Doxycycline (final concentration: 0.1%, w/v) was added to the drinking water for 20 days. Tumor formation was suppressed only in inducible siGrm1 clones (siGrm1 B1 and B3), but not siGFP7. Differences of tumor sizes at day 20 were shown.

(C) Removal of doxycycline from the drinking water at day 20 resulted in resumption of tumor growth of inducible siGrm1 clones. Bar, SD; * and ** $P < 0.01$ compared with (–) Dox (t test).