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Metabotropic Glutamate Receptor 1 (Grm1) Is An Oncogene In Epithelial Cells

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Abstract

Non-neuronal expression of components of the glutamatergic system has been increasingly observed, and our laboratory previously had demonstrated the etiological role of ectopically expressed metabotropic glutamate receptor 1 (Grm1/mGluR1) in mouse models of melanoma. We hypothesize that inappropriate glutamatergic signaling in other cell types can dysregulate growth leading to transformation and tumorigenesis. As most cancers are carcinomas, we selected an immortalized primary baby mouse kidney (iBMK) cell model to assess whether Grm1 can transform epithelial cells. These iBMK cells, engineered to be immortal yet non-tumorigenic and retaining normal epithelial characteristics, were used as recipients for exogenous Grm1 cDNA. Several stable Grm1 expressing clones were isolated and the Grm1-receptors were shown to be functional, as evidenced by the accumulation of second messengers in response to Grm1 agonist. Additionally activated by agonist were MAPK and AKT signaling cascades, major intracellular pathways shown by many investigators to be critical in melanomagenesis and other neoplasms. These Grm1-iBMK cells exhibited enhanced cell proliferation in *in vitro* MTT assays and significant tumorigenicity in *in vivo* allografts. Persistent Grm1 expression was required for the maintenance of the *in vivo* tumorigenic phenotype as demonstrated by an inducible Grm1-silencing RNA. These are the first results that indicate Grm1 can be an oncogene in epithelial cells. Additionally, relevance to human disease in the corresponding tumor type of renal cell carcinoma (RCC) may be suggested by observed expression of GRM1/mGluR1 in a number of RCC tumor biopsy samples and cell lines, and the effects of GRM1 modulation on tumorigenicity therein. Moreover RCC cell lines exhibited elevated levels of extracellular glutamate, and some

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Conflict of interest

The authors declare no conflict of interest.

lines responded to drugs which modulate the glutamatergic system. These findings imply a possible role for glutamate signaling apparatus in RCC cell growth, and that the glutamatergic system may be a therapeutic target in renal cell carcinoma.

Keywords

Grm1; Glutamatergic Signaling; Oncogene; Cell Transformation; Kidney Epithelial Cells; Carcinoma

Introduction

Although recognition of the glutamatergic system has been restricted to customary roles in the central nervous system (CNS) and linked neuropathologies, expression in a variety of tissues has come to be acknowledged [1–9]. Among such varied functions as in metabolism and structure, L-glutamate also is an evolutionarily conserved cell signaling molecule [10]. It thus becomes likely that dysfunction of the glutamatergic system can lead to diverse disease types in humans including those of cell growth, or cancer.

In fact our laboratory was first to implicate an aberrantly expressed metabotropic glutamate receptor, Grm1, in the etiology of a neoplasm - melanoma - and subsequently demonstrated its significance in the progression of human malignancy [11–13]. Shin et al., offered corroboration in a report that introduction of Grm1 into immortalized non-tumorigenic mouse melanocytic cell lines can remove their requirement for TPA for *in vitro* growth, and render the cells tumorigenic *in vivo* [14]. More recent work has developed a sound rationale for targeting this receptor for therapy in melanocytic disease [15, 16]. Recently additional members of the metabotropic glutamate receptor family, Grm5 and GRM3, were shown to be important in melanoma pathogenesis [17, 18]. Results from these studies further associate glutamate signaling and melanoma.

Despite that melanocytes and neurons may have in common developmental origins from the embryonic neural crest, the intracellular signaling pathways accessible by these metabotropic glutamate receptors (mGlu) are distributed generally among diverse cell types. In experimental systems designed to study synaptic function, for example, it has been shown that ectopically expressed mGlu including Grm1 can successfully link to endogenous signaling apparatus [19]. It is also noteworthy that concentrations of glutamate, the major excitatory neurotransmitter in the CNS and natural ligand for these receptors, can be orders of magnitude higher outside of the closely controlled microenvironment of the synapse [20], and that activated mGlu receptors can couple indirectly to glutamate release, invoking an autocrine-like loop [14, 21]. Additionally, various G protein-coupled receptors (GPCRs) including the glutamate receptors may exhibit constitutive basal activity without the need for agonists [22].

We suggest that activity of an otherwise normal glutamate receptor in an ectopic cellular milieu can activate signaling pathways which dysregulate cell growth and ultimately lead to tumor formation. As most human cancers are of epithelial origin (carcinomas), we propose a model system to investigate whether Grm1 can transform epithelial cells. As part of a design

to interrogate mechanisms of epithelial tumor progression, primary mouse epithelial cells underwent genetically defined immortalization that permitted the retention of normal epithelial characteristics, including a lack of innate tumorigenicity [23, 24]. The resulting W2 baby mouse kidney (iBMK) cells facilitate *in vivo* screening for elements that enable tumorigenesis, and cells of the sibling D3 line, additionally engineered for apoptosis-impairment, allow for examination of factors that promote tumor growth.

In this report we demonstrate that full-length wild-type Grm1 is tumorigenic when ectopically expressed in epithelial cells, and also couples to MAPK and AKT signaling, two of the hallmark activated signaling pathways fundamental to growth, proliferation, and survival in cancer cells, including melanoma. In addition, a role for glutamate signaling apparatus in Renal Cell Carcinoma tumor cell growth is explored.

Results

Stable expression of exogenous Grm1 in W2- and D3 iBMK epithelial cells

Coding sequence for full-length form of the Grm1 receptor was subcloned into standard mammalian expression vector pCI-neo (Promega). This expression construct, or an empty-vector control, was transfected into W2- and D3 iBMK epithelial cells. Individual drug-resistant clones were isolated, expanded, and screened by Western immunoblot for Grm1 expression, which was detected at various levels in several stable Grm1 clones, but not in empty-vector controls or parental W2 and D3 cell lines (Fig. 1a). A number of Grm1-expressing and empty-vector control lines were selected for more detailed analysis.

Exogenous Grm1 receptor is functional in W2- and D3-Grm1 cells

Accumulation of second messenger IP₃ in W2- and D3-Grm1 clones is specifically induced by Grm1-agonist—Guided by previous studies of G-protein coupled metabotropic glutamate receptor signaling, both in the CNS and in melanoma derived tumor cells [15, 25, 26], functionality of the exogenous receptor was assessed in W2- and D3-Grm1 clones by the accumulation of the phospholipase C second messenger inositol-1,4,5-triphosphate (IP₃) following challenge with Grm1-agonist. Results from a typical experiment for each cell type are shown (Fig. 1b). Whereas stimulation by the pharmacological agonist, L-quisqualic acid (Q), led to a significant increase in IP₃ levels over vehicle-only treated W2- and D3-Grm1 cells, in all such clones examined the increase in IP₃ was abrogated by pre-incubation of the cells with the Grm1-receptor specific non-competitive antagonist BAY 36-7620 (BAY). Fetal bovine serum, as a positive control for the system, led to an increase in IP₃ similar to that produced by the agonist Q (data not shown). Empty-vector transfectants (W2- and D3-vec) did not exhibit a Grm1-specific response. The IP₃ results are thus indicative of both the functionality and specificity of the exogenous Grm1-encoded cell surface receptor introduced into the cells.

MAPK/ERK is specifically activated by Grm1 agonist in both W2- and D3-Grm1 cells—Over-activation of the MAPK/ERK signaling pathways is a hallmark of many neoplasms, including melanoma. Phosphorylation of ERK 1/2 (MAPK3 and MAPK1), as assessed by immunoblot, was the read-out used to report MAPK pathway activation by

stimulation of exogenous Grm1-receptor. A maximal activation was seen within five minutes of induction by the agonist Q, followed by a slower diminution of signal. Typical results at the maximum are seen in Fig. 2a and 2b. Once more, specificity of signaling was demonstrated by pre-incubation with the Grm1-receptor specific antagonist BAY; under this condition ERK/MAPK cannot be activated by Grm1-agonist.

AKT is specifically activated by Grm1 agonist in both W2- and D3-Grm1 cells

—Recently studies in our laboratory indicated that the Grm1-receptor could additionally signal to the AKT pathway in melanoma cells [27]. This pathway is known for roles in both mitogenic signaling and cell survival, and has increasingly become a focus in multiple malignancies. In experiments exactly parallel to those for ERK 1/2, AKT was also specifically stimulated or inhibited by Grm1-agonist or antagonist, respectively, again demonstrating a functioning receptor. A time-dependent activation in pAKT again paralleled that of pERK, with a slight lag, i.e. a maximal stimulation closer to 10 minutes. A typical experiment is shown (Fig. 2c).

***In vitro* growth properties of W2- and D3-Grm1 cells**

W2-Grm1 clones uniformly exhibited an increased growth rate *in vitro* compared to W2-vec controls as measured by standard MTT cell proliferation/viability assays. D3-Grm1 clones also displayed a consistent, but somewhat lesser increase in growth relative to D3-vec cells, as might be expected given that their D3 parental cells are already partially transformed/tumorigenic by engineered apoptosis-defect (Supp. Fig. 1).

W2- and D3-Grm1 clones are tumorigenic *in vivo*

We were however much more interested in the definitive measurement of neoplastic transformation: *in vivo* tumorigenicity. Several independent stable W2-Grm1 and D3-Grm1 iBMK clones were inoculated subcutaneously into the dorsal flanks of nude mice to establish allografts (n=20 each). As they developed, tumor dimensions were measured by vernier caliper twice-weekly. Each study was terminated when tumor burden grew too great as per institutional guidelines. W2-Grm1 cells were permitted 13 weeks of growth after inoculation before termination. Depending on the independent W2-Grm1 clone in question, tumor volume averaged more than 500 – 800 mm³. No measurable tumors whatsoever were observed from empty-vector transfected W2-vec cells at this endpoint (Fig 3a). D3-Grm1 tumors became palpable one week post-injection. When D3-Grm1 line studies were ended after 8 weeks, tumors of D3-Grm1 clones had reached a mean size approaching 1400 mm³, whereas D3-vec cell tumors averaged only approximately one-fifth that volume (P < 0.001, ANOVA) (Fig. 3b). Only excised W2-Grm1 and D3-Grm1 tumor samples expressed Grm1, as evaluated by immunoblots (Fig. 3a and 3b). Despite that parental D3 cells are already partially transformed/tumorigenic by engineered apoptosis-defect [28], Grm1-receptor expression most obviously enhanced *in vivo* tumorigenic growth potential. Figure 3 also illustrates Grm1-iBMK epithelial cell allograft-tumor growths, and the characteristic effects of tumor angiogenesis overtly visible through skin as feeder blood vessels. As previously seen by Degenhardt et al., local invasion of iBMK allograft-tumors was noted along with frequent angiogenesis, and were classified as highly aggressive carcinomas. Representative

high magnification photomicrographs of H&E stained formalin-fixed sections of tumors at termination of a study are also shown (Fig. 3).

Sustained expression of Grm1 is required to maintain W2- or D3-Grm1 tumorigenic phenotypes *in vivo*

To determine whether sustained Grm1 expression is required to maintain the tumorigenic phenotypes, a doxycycline inducible anti-Grm1 siRNA expression system was introduced into the W2- and D3-Grm1 clones. Anti-Grm1 and anti-GFP-control siRNA constructs respectively permitted assessing the effect of successful knockdown of Grm1, and the effect of an unrelated negative control (siGFP). Several independent Grm1-siRNA clones were isolated and evaluated first *in vitro*, by immunoblotting. siGrm1 - but not siGFP - clones demonstrated consistent reduction of Grm1 protein levels in the presence of inducer; examples shown in Fig. 4a. Next we assessed by allograft whether a reduction of Grm1 levels modulated tumorigenesis *in vivo*. Upon initial appearance of palpable tumors mice were randomly segregated into two groups with equivalent tumor size (10 mm^3), and doxycycline was thereafter included in the drinking water of the treated group (0.1% w/v). Each study was terminated after a set period, or when tumor burden became excessive. Whereas there were no significant differences in allograft tumor growth observed between doxycycline treated and untreated W2- and D3-Grm1-siGFP (control siRNA) mice, there was sizeable inhibition of tumorigenesis in W2- (n=20, $P < 0.05$, t test) and D3-Grm1-siGrm1 (n=20, $P < 0.001$, t test) mice treated with doxycycline to induce siRNA knockdown of Grm1 (Fig. 4b).

GRM1 and the glutamatergic system in renal cell carcinoma

Although originally designed only to substantiate the hypothesis that components of the glutamatergic system such as metabotropic glutamate receptors can play a role in tumor cell growth, the clear results from our mouse kidney epithelial cell model system compelled us to undertake preliminary investigation of relevance to human disease in the corresponding tumor type of renal cell carcinoma (RCC). We determined to first extend the investigation with RCC cell lines.

RCC cell lines express GRM1—Total RNA was isolated from a panel of four standard RCC cell lines, UOK115, UOK117, UOK121, and UOK122, originally established from resected stage IV disease tumors [29]. Results from initial reverse transcription-polymerase chain reaction (RT-PCR) performed using gene specific primers, demonstrated GRM1 transcripts in these RCC cell lines, but not in HEK293, a human embryonic kidney cell line used as control (Supp. Fig. 2). Fittingly, HEK293 commonly is used as a recipient for exogenous metabotropic glutamate receptors to study individual mGlu function away from the synaptic environment [30–32]. Expression was also seen in the RCC cell lines at the protein level, but not in HEK293 cells (Fig. 5). Included as controls in the immunoblot were GRM1-negative p'mel* immortalized human melanocytes [33], as well as a stable GRM1+ p'mel* clone transfected with exogenous human GRM1 cDNA, and the GRM1+ human metastatic melanoma C8161+ cell line [34].

RCC cell lines release glutamate—We measured the levels of extracellular glutamate in these RCC cell lines, as varied studies, including ours with melanoma, have suggested that G protein-coupled receptors with oncogenic activity frequently display locally enhanced levels of ligand produced and released by tumor cells (autocrine) or surrounding cells (paracrine) [35, 36]. These measurements were done in parallel with MTT cell proliferation/viability assays to be sure any increases in extracellular glutamate were not due to release by cell death (Fig. 6a). Each of the RCC lines showed several-fold excess in levels of extracellular glutamate compared to control HEK293 cells (ex. under these conditions, UOK117 a maximum upwards of 300 μ M glutamate, and HEK293 never more than 1/10 of that). Even when adjusted for differences in growth rate over the course of the experiment as measured by MTT, the contrast between the RCC cells and HEK293 remained striking (Fig. 6b).

RCC cells respond to the anti-glutamatergic drug riluzole—Originally approved by the FDA as a neuroprotective agent known to inhibit glutamate release, riluzole previously was shown by our group to reduce the growth of melanoma tumor cells that release elevated levels of extracellular glutamate [14, 15]. Analyses were performed on RCC cells treated with this anti-glutamatergic drug at a working concentration guided by pilot experiments and previous studies in melanoma [14, 15]. First, MTT cell proliferation/viability assays demonstrated two RCC lines were growth-inhibited by riluzole beyond the response of control HEK293 cells to the drug. UOK115 by about 20% and UOK117 by about 50%, after treatment for 96 h at 25 μ M (Fig. 7). Next, cell cycle analyses were performed on RCC cells treated with riluzole at the same concentration, for the indicated time periods. Population distributions of HEK293 cells were not affected by riluzole at this concentration, however UOK115 and UOK117 showed consistent alterations (Fig 8a). At 72 h, RCC cell line UOK115 responded to riluzole with accumulation at G2/M. For UOK117, there was a considerable increase in cells accumulated in the sub-G1 phase of the cell cycle after 48 and 72 h, indicating cellular apoptosis. The customary marker of PARP cleavage corroborated this as shown by immunoblot (Fig 8b). In summary, two of the panel of four RCC lines responded to riluzole with either the apparent cytostatic (UOK115) or cytotoxic/apoptotic (UOK117) effect of the drug each typically seen in our lab for different human melanoma cell lines [14, 15].

Sustained expression of GRM1 is required to maintain UOK117 RCC tumorigenic phenotype in vivo—To examine whether interference in the expression of GRM1 in RCC cells would affect their tumorigenic phenotype *in vivo*, an anti-GRM1 siRNA expression system was introduced into UOK117 RCC cells, in an experimental protocol exactly paralleling that of engineered iBMK-Grm1 clones described above. An evaluation of doxycycline-induced silencing in three of the independently isolated GRM1-siRNA lines is shown by immunoblotting in Fig. 9a. From these, Clones 2 and 4 were selected for xenograft. At the termination of the *in vivo* studies, a marked inhibition of tumor progression was noted (Fig. 9b) in doxycycline treated mice (both clones n=20, P < 0.001, t test). Results thus implied sustained expression of GRM1 is at least in part necessary to maintain UOK117 tumor progression *in vivo*.

RCC tumor tissues express GRM1—Although both the available anti-human GRM1 antibodies, and the inherent heterogeneity and variability of tissues obtained during surgical procedures can be problematic, the results from cultured RCC cells compelled us, for completeness, to perform initial experiments to assess GRM1 expression in tumor tissue. A small collection of six pairs of anonymized tumor and adjacent “normal” biopsy-tissue sections from patients with RCC, was obtained through the Tissue Retrieval Service at the Cancer Institute of New Jersey and analyzed for GRM1 protein by immunohistochemistry (IHC). Initial results, both visual by photomicroscopy, and unbiased quantitative assessment of IHC staining of digitized slides using an Aperio ScanScopeGL system with ImageScope software, indicated that GRM1 is expressed in at least a subset of these tissues. It is readily apparent in a representation (Fig. 10a) of the automated quantification, that in these samples, there was on average a greater number of GRM1+ cells in those tissues that had been designated tumor, than those termed adjacent normal. Interestingly, in some slides the IHC revealed visually provocative patterning of GRM1+ stained cells displaying classic crab-like invasion into an area of unstained cells, or in others, characteristic tumor-like cellular disorganization (Fig. 10b).

Discussion

Previously our laboratory reported a line of transgenic mice (TG-3), which displays spontaneous melanoma with 100% penetrance due to an insertional mutagenesis causing a constitutive mis-expression of *Grm1* in its melanocytes [11, 13]. The significance of *Grm1* (mGluR1) receptors in the progression of human melanoma was subsequently demonstrated [11, 15]. It was established that in TG-3 tumor-derived cells, like in the CNS, *Grm1* signaling coupled through a G-protein to phospholipase C (PLC) and its second messengers inositol-1,4,5-triphosphate (IP₃) and diacylglycerol (DAG) [26] - part of a signaling apparatus available to many mammalian cell types. Although melanoma is one of the most deadly cancers, carcinomas are by far the most common. We thus set out to assess whether *Grm1* is able to transform epithelial cells.

We hypothesized that activity of an otherwise normal glutamate receptor in an ectopic cellular milieu can activate signaling pathways which dysregulate cell growth and ultimately lead to tumor formation in epithelial cells. Indeed, functional expression of *Grm1* in normal W2 baby mouse kidney epithelial cells enabled a tumorigenic potential as evidenced in allograft assays, whereas empty-vector transfected W2-vec cell inoculations remained tumor free *in vivo*. Although D3 cells already have been partially transformed by engineered apoptosis-defect and are tumorigenic [24], it is readily apparent from our results that expression of functional *Grm1*-receptor significantly enhanced and accelerated the tumorigenic potential of D3 cells relative to multiple empty-vector control lines *in vivo*. Inducible silencing RNA against *Grm1* was used to demonstrate that sustained expression of *Grm1* is required to maintain W2- or D3-*Grm1* tumorigenic phenotypes. It additionally was shown that in both W2- and D3-*Grm1* epithelial cells, the exogenous receptor specifically activated endogenous MAPK and AKT signaling pathways when stimulated by *Grm1*-agonist.

It is an attractive concept that signaling from a single cell-surface metabotropic glutamate receptor can activate at least two of the hallmark activated signaling pathways thought to play essential roles in growth, survival, invasion, and even angiogenesis and in human tumors, MAPK and AKT [37–41].

It may be argued that the human tumor type corresponding to that of our experimental mouse model system is kidney cancer, a neoplasm newly affecting over 65,000 persons and responsible for more than 13,000 deaths in the United States this year [42]. The incidence of this neoplasm has been steadily increasing worldwide for decades [43–45]. By far the most common type, renal cell carcinoma or RCC is thought to originate in the lining of the proximal convoluted tubule. Interestingly, renal cell carcinoma is one of the coexistent or subsequent primary neoplasms that have been reported in statistically significant higher incidence in patients diagnosed with melanoma [46, 47]. Our initial studies demonstrated GRM1 expression in tested subsets of RCC cell lines and tumors, and indicated a susceptibility of RCC cells to drugs which modulate the glutamatergic system. Further investigation into the necessity for sustained expression of GRM1 in human RCC cells implied that, at least in a subset of cases, it may be one of the requirements for maintenance of the tumorigenic phenotype *in vivo*. These findings suggest a possible role for glutamate signaling apparatus in RCC cell growth and maintenance.

Much early effort researching the glutamatergic system was restricted to the CNS. Metabotropic glutamate receptors (mGlu), originally thought to be confined to neural cells, may have more diverse physiological functions as they have been found to be expressed in a range of non-neuronal tissues in recent years. After Grm1/mGluR1 first was linked to melanomagenesis by Chen et al., various mGlu subsequently have been implicated in human melanoma and an increasing subset of human malignancies [17, 18, 48]. The metabotropic glutamate receptors are highly druggable GPCRs [49]. For example appropriate pharmacologic modulation of mGlu2/3 and mGlu4, respectively, inhibited the growth of human glioma cells in culture [50] and medulloblastomas in xenografts [51].

It may thus be suggested that modulation of the glutamatergic system can offer therapeutic benefits in non-neuronal neoplasms as well. Significant investigation over the past few years has gone into design and testing of therapeutics to modulate the glutamatergic system to address a diversity of neurological and neuropsychiatric disorders [52–54], and it may be possible to leverage these studies in the quest for anticancer therapeutics. Riluzole, the anti-glutamatergic drug used in this study, is the only US FDA approved treatment for the neurodegenerative disease amyotrophic lateral sclerosis (ALS) [55, 56]. Recent work by Chen and Goydos groups indicated that riluzole reduced melanoma-cell glutamate release, resulted in cell cycle arrest and subsequent apoptosis *in vitro*, and impeded xenografted melanoma tumor growth *in vivo* [15, 57, 58]. These preclinical results led to an exploratory Phase 0 clinical trial of oral riluzole in patients with advanced melanoma. Pre- and post-treatment tumor biopsies and PET scans were performed to monitor cellular response and tumor metabolic activity. Assessment of proliferation and apoptotic markers revealed that four of eleven patients who completed the protocol had pronounced responses to riluzole administration as shown by increased apoptosis and tumor shrinkage [16]. The completion

of a more significant Phase II clinical trial of the drug for anti-melanoma therapy is expected presently.

In addition to having been observed in a subset of aggressive melanoma cell lines as compared to normal melanocytes and where so far linked with GRM1 expression [14, 15], amplified release of extracellular glutamate has been noted in malignant gliomas [59], in cell lines of varied tumor-types in bone metastases [60, 61], and more recently in triple-negative breast cancer cells [62], again implying correlation of the glutamatergic system with malignant potential. Current interest in the distinctive metabolic changes in cancer cells and their relationships with signaling networks and proliferation includes investigations of glutamate both as a cellular messenger and as a metabolic support, particularly as a product of glutaminase in glutaminolysis [63]. Given our own published and unpublished observations, and examples such as in bone metastasis [61] and breast cancer [62], it is thus somewhat encouraging for prospective therapeutic benefit that the glutamate transport system in cancer cells can be modulated pharmacologically.

Taken together, the results presented in this report establish that Grm1 indeed can be oncogenic in epithelial cells and can activate multiple characteristic cancer-signaling pathways - and that components of the glutamatergic system are active in renal cell carcinoma. Although beyond the scope of the current work, our preliminary observations offer a rationale for both comprehensive survey of expression of GRM1 and other metabotropic glutamate receptors, which are highly druggable GPCRs, and systematic examination of other elements of glutamate signaling, as possible novel targets for renal cell carcinoma therapy.

Materials and Methods

Cell lines, culture conditions, and DNA transfection

DMEM and RPMI were supplemented with 100 U/ml – 100 µg/ml Penicillin-Streptomycin (Invitrogen, Carlsbad, CA, USA). Incubators were maintained at 37°C and 5% CO₂. For signaling studies and glutamate assays, exogenous glutamate was minimized by use of glutamate and glutamine-free medium supplemented with GlutaMax at 2 mM, (Invitrogen, Carlsbad, CA, USA), with or without dialyzed Fetal Bovine Serum (FBS) [15, 26]. The derivation of W2 and D3 baby mouse kidney epithelial cell lines has been described [28]. W2 and D3 were cultured in DMEM supplemented with 5% FBS. All DNA transfections were performed with Lipofectamine 2000 according to the manufacturer (Invitrogen, Carlsbad, CA, USA). Coding sequence for the full-length form of the receptor was subcloned from mouse-brain Grm1 cDNA [14, 64] into mammalian expression vector pCI-neo (Promega, Madison, WI, USA). Stable Grm1- or empty vector- transfected clones were selected in 1 mg/ml (W2) or 2 mg/ml (D3) Geneticin (Invitrogen, Carlsbad, CA, USA). siGrm1 or siGFP sequence [14] was cloned into the inducible siRNA expression vector pRNATin-H1.2/Hygro (GenScript, Piscataway, NJ, USA) and co-transfected with TetR plasmid [65]. Stable siRNA/TetR-transfected clones were selected in Hygromycin-B (Invitrogen, Carlsbad, CA, USA) at a concentration determined for each line (200–240 µg/ml). Renal cell carcinoma cell lines UOK115, UOK117, UOK121, and UOK122 [29], were

routinely grown in DMEM with 10% FBS. siGRM1- [66] UOK117 clones were selected in 300 µg/ml Geneticin and 25–40 µg/ml Hygromycin-B.

Inositol-1,4,5-triphosphate measurement

IP₃ measurement was a modification of Thandi et al., [67] performed as described previously [26]. Briefly, sequential changes of medium starved cells of stimulatory factors, glutamate, and other cell-surface receptor ligands to minimize signaling, and also replaced the intracellular pool of inositol with radiolabeled precursor to render IP₃ production measurable.

Antibodies and Western immunoblotting

Anti- total-ERK1/2, phospho-ERK1/2, total-AKT, phospho-AKT, were obtained from Cell Signaling Technology, Danvers, MA, USA; anti- Grm1/mGluR1 from BD Biosciences, San Jose, CA, USA; anti- GRM1/mGluR1 from Upstate/Millipore, Billerica, MA, USA; anti- α -tubulin from Sigma, St. Louis, MO, USA. Cells and tissue extracts were prepared in NP40-containing buffer with protease and phosphatase inhibitors as described previously [68]; protein concentrations were determined with Detergent Compatible Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA). Proteins were resolved by Tris-Glycine polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane by wet tank electroblotting. After overnight incubation with primary antibodies, blots were visualized with Amersham ECL-HRP Linked Secondary Antibodies and ECL Plus Western Blotting Detection Reagents (GE Healthcare Life Sciences, Piscataway, NJ, USA). Densitometry by OptiQuant software (PerkinElmer, Shelton, CT).

Immunohistochemistry (IHC)

The Tissue Analytical Services at the Cancer Institute of New Jersey performed standard IHC for GRM1, and unbiased quantitative assessment of IHC staining was completed using a digital Aperio ScanScopeGL system and ImageScope software (v 10.1.3.2028) (Aperio Technologies Inc., Vista, CA) according to the manufacture's protocol with modifications as described [69].

Cell proliferation/viability (MTT) assay

Cells were typically seeded at $1 - 2 \times 10^3$ cells per well in a 96-well culture plate. At designated time-points, 0.1 volumes of 5mg/ml Thiazolyl Blue Tetrazolium Bromide (Sigma, St. Louis, MO, USA) in 1X PBS were added to growth medium, incubated for 4 hours at 37°C, then solubilized overnight at 37°C with an equal volume of 10% SDS/0.1 M HCl. A 96-well plate reader (Infinite 200 Tecan USA, Durham, NC, USA) was used to measure absorbance at 550 nm with a reference wavelength of 750 nm.

In vivo tumor growth assay

Animal protocols were approved by the Institutional Review Board for the Use and Care of Animals and the Animal Care and Facilities Committee of Rutgers University. Expanded from independent clones, 10^6 cells were resuspended in 0.1 ml sterile PBS and injected subcutaneously into the dorsal flanks of nude mice (Taconic, Hudson, NY, USA) to

establish allografts or xenografts. Tumors were measured with a vernier caliper twice-weekly, and tumor volume was calculated as $V = d^2 \times D/2$ [70]. Studies were terminated as soon as tumor volumes reached maximally permissible size.

Glutamate release assay

Glutamate concentration in culture medium “conditioned” by tumor cells was measured by enzyme-based colorimetric assay in a microplate reader at 450nm as per the manufacturer (Glutamate Assay Kit, Biovision, Mountain View, CA, USA). Cells were generally seeded at $1 - 2 \times 10^3$ per well and cultured in 200 μ l glutamate/glutamine-free medium as described above, with 10% dialyzed FBS. At measurement half the volume was removed from each well for glutamate assay, and cell-viability immediately assessed by MTT assay.

Cell cycle analysis

Cells were plated at $5 \times 10^5 - 1 \times 10^6$ per 100-mm dish and treated with 25 μ M riluzole, vehicle (DMSO), or left un-treated, for 24–72hrs as specified. Adherent and floating cells were pooled, pelleted, washed twice with ice-cold 1X PBS, fixed by drop-wise addition of ice-cold 70% ethanol while mixing, and stored at -20°C . Fixed cells were washed twice with and resuspended in 1X PBS, treated with RNase A solution (Sigma, St. Louis, MO, USA) at 100 μ g/ml and stained with propidium iodide (Sigma, St. Louis, MO, USA) at 10 μ g/ml for 30 min. Cell cycle analysis was performed on a Coulter Cytomics FC500 Flow Cytometer (Beckman Coulter, Fullerton, CA, USA) at the Analytical Cytometry Core Facility, Rutgers University.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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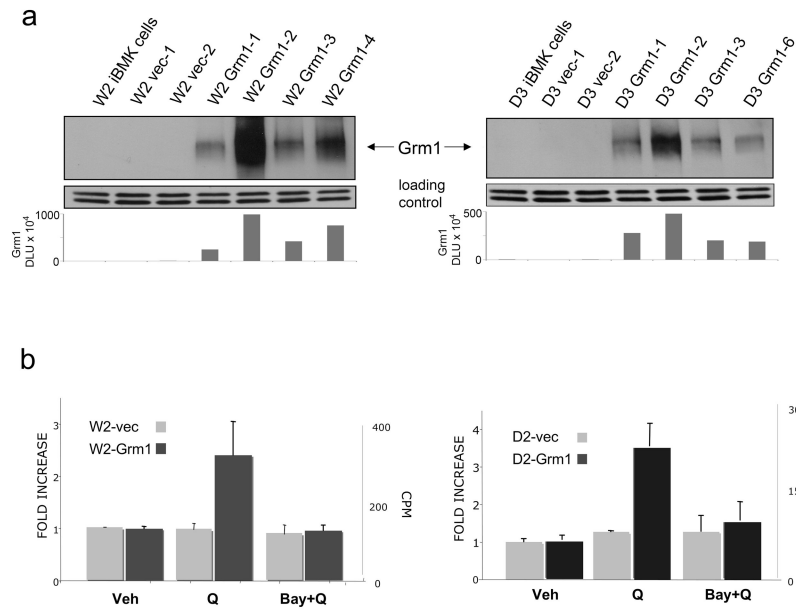
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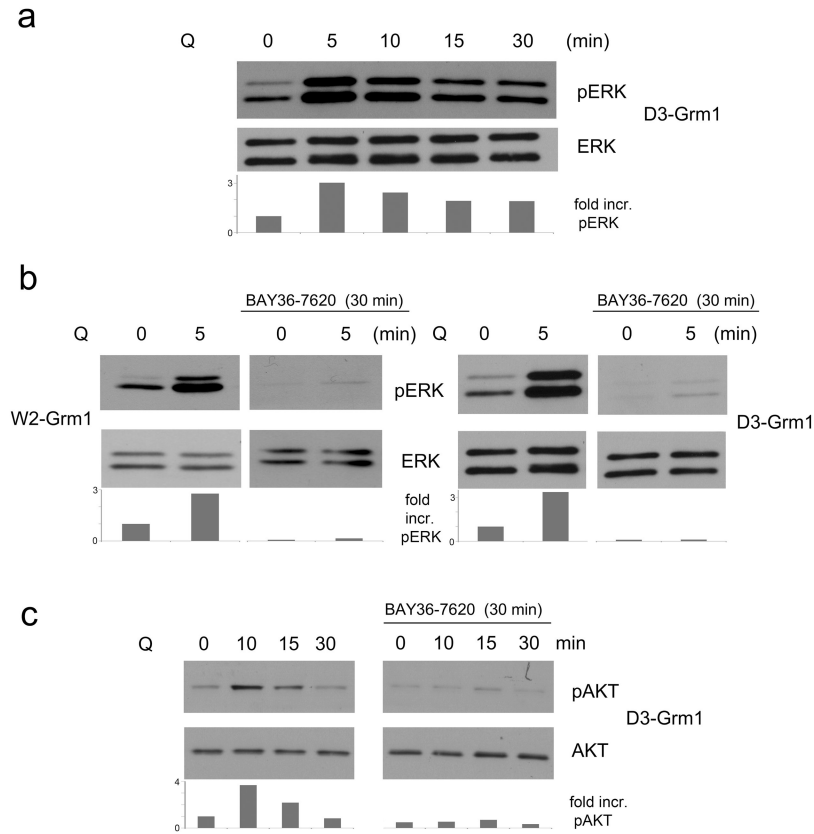
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**Fig. 1.**

A. Expression of exogenous Grm1/mGluR1 receptor in stable W2- and D3 iBMK epithelial cell clones. iBMK epithelial cells were transfected with the full-length form of the receptor in pCI-neo or empty-vector pCI-neo alone (vec). Independent stable G418^r clones exhibited varying levels of Grm1 expression detectable at approximately 150 kD by immunoblotting. No expression was observed in parental iBMK or vec transfected cells. Total ERK at 42/44 kD was used as a loading control. Densitometry presented graphically.

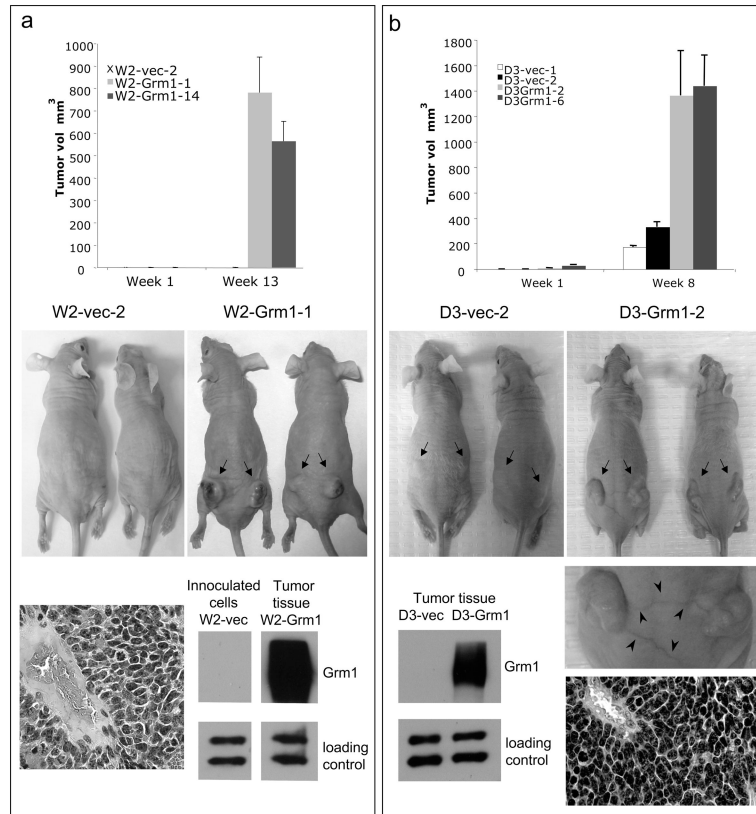
B. Exogenous Grm1/mGluR1 receptor is functional in W2- and D3-Grm1 cells as assessed by accumulation of second messenger IP₃ that is specifically induced by Grm1-agonist. Stimulation by the pharmacological agonist, L-quisqualic acid (Q), led to a significant increase in IP₃ over vehicle-only (DMSO) treated W2- and D3-Grm1 cells; the increase was abrogated by preincubation with the Grm1-receptor specific non-competitive antagonist BAY 36-7620 (BAY). No significant responses were noted for empty-vector transfected iBMK cells.

**Fig. 2.**

A. MAPK/ERK is activated by Grm1 agonist in both W2- and D3-Grm1 cells, as assessed by immunoblotting, which demonstrated enhanced levels of phosphorylated ERK (pERK). All functional stable W2 and D3-Grm1 lines responded similarly. Maximal activation was seen within five minutes of induction by the agonist Q (10 μ M), as seen in this representative immunoblot (D3-Grm1). Fold change in pERK relative to control, by densitometry.

B. MAPK/ERK activation is specific to Grm1. Pretreatment for 30 min with the Grm1-specific antagonist BAY (10 μ M) inhibited phospho-ERK 1/2 signal increase by induction with Q; examples of both W2-Grm1 and D3-Grm1 responses are shown here at the 5 min time-point. Fold change in pERK relative to control, by densitometry.

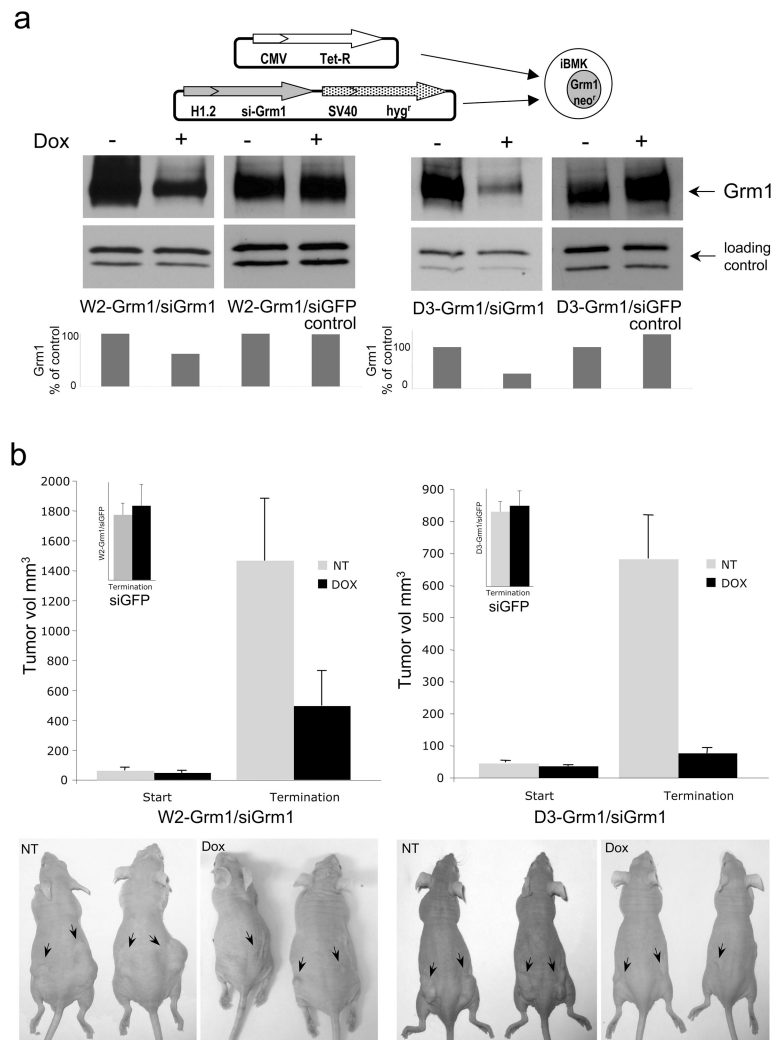
C. AKT is specifically activated by Grm1 agonist in both W2- and D3-Grm1 cells as assessed by immunoblotting. Again W2-Grm1 (not shown) and D3-Grm1 responses exactly corresponded. In this representative immunoblot (D3-Grm1), maximal activation was seen within ten minutes of induction by the agonist Q (10 μ M). Pretreatment with the Grm1-specific antagonist BAY (10 μ M) for 30 min inhibited phospho-AKT signal induction by Q. Fold change in pAKT relative to control, by densitometry.

**Fig. 3.**

A. Grm1-receptor ectopically expressed in W2 cells induces tumorigenicity in independent clones (W2-Grm1). Average tumor size upon termination at 13 weeks after inoculation with 10^6 cells ($n=20$). No measurable tumors were ever observed from empty-vector transfected cells (W2-vec) at this endpoint. Representative nude-mouse/tumor photographs, and photomicrograph of typical H&E stained tumor section with blood vessel. Immunoblotting demonstrated Grm1 expression in tissue excised from Grm1-iBMK tumors.

B. Grm1-receptor ectopically expressed in D3 cells increases tumorigenicity in independent clones (D3-Grm1). At the endpoint, 8 weeks after inoculation with 10^6 cells ($n=20$), Grm1-receptor expressing clones reached a mean tumor size over 1000 mm^3 , whereas D3-vec cell tumors only averaged approximately one-fifth that volume ($P < 0.001$, ANOVA).

Representative nude-mouse/tumor photographs include closer view of tumor angiogenesis visible through the skin as overt blood vessels, in addition to photomicrograph of H&E stained section. Immunoblotting demonstrated Grm1 expression only in tissue from Grm1-iBMK seeded tumors.

**Fig. 4.**

A. In cell culture tests, anti-Grm1 siRNA, but not anti-GFP control siRNA, constructs introduced into iBMK-Grm1 cells permitted doxycycline-inducible knockdown of Grm1 at the protein level as assessed by immunoblots. Grm1 expression as percentage of control, by densitometry.

B. *In vivo* inhibition of tumor development by anti-Grm1 siRNA. Mice bearing allografted tumors developing from W2- and D3-Grm1-siGrm1 cells from Fig. 4a were treated with doxycycline (0.1% w/v in drinking water) to induce siRNA expression to knockdown levels of Grm1. Results indicated sustained expression of Grm1 is necessary to maintain W2- and D3-Grm1 tumor progression (respectively $P < 0.05$ and $P < 0.001$, t test, $n=20$). No significant differences in tumor growth were observed between doxycycline treated and untreated W2- and D3-Grm1-siGFP (control siRNA) mice.

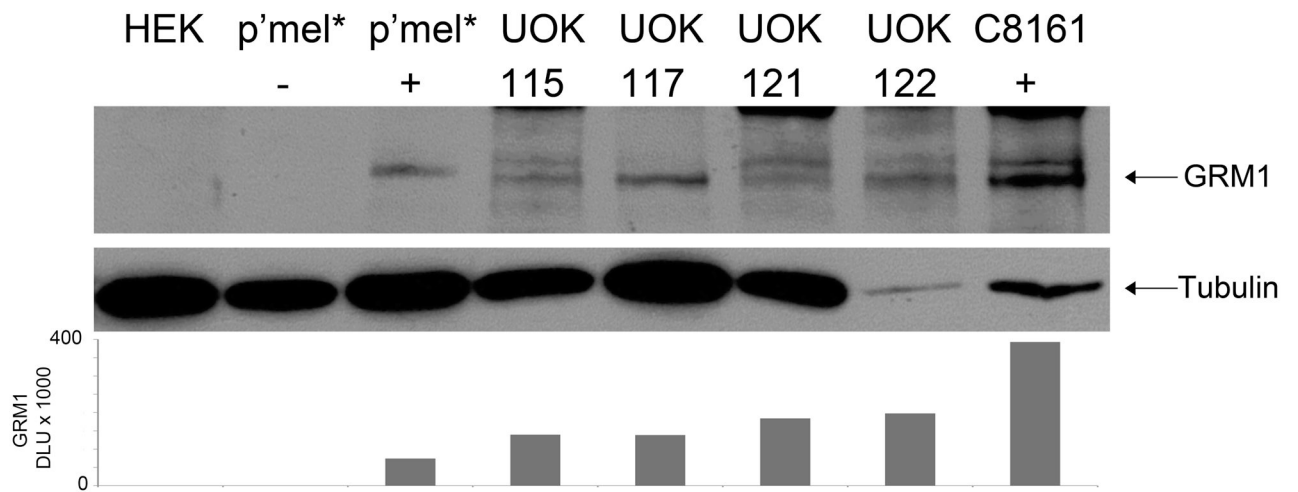
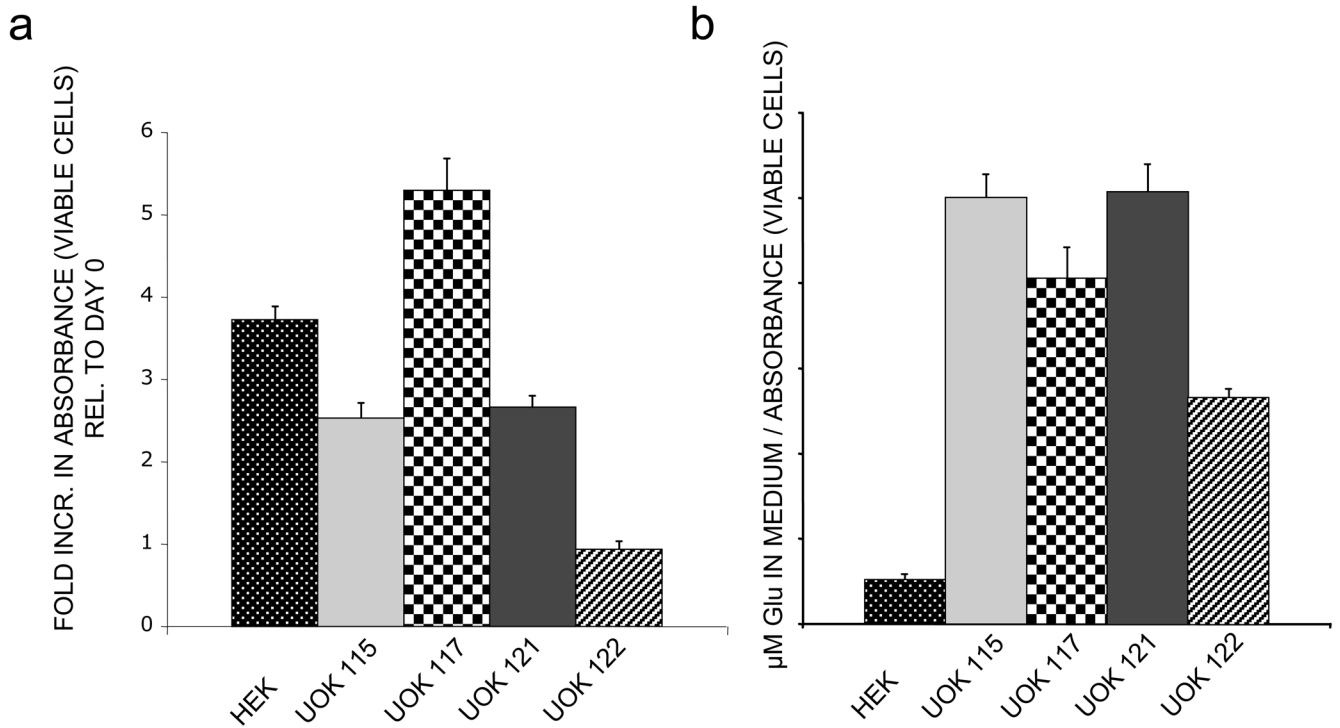


Fig. 5.

Immunoblot for GRM1 in protein extracts from a panel of representative RCC cell lines, UOK115, UOK117, UOK121, and UOK122. Negative controls are immortalized human melanocytes (p'mel*⁻) and HEK293 (HEK) cells. Positive controls are GRM1 expressing p'mel*⁻-GRM⁺ (p'mel*⁺) cells and C8161 melanoma tumor cells (C8161⁺). M is protein MW standard. Loading control is tubulin. Densitometry is presented graphically.

**Fig. 6.**

Renal cell carcinoma cell lines release glutamate. At the time of measurement, 96 h, half the volume of medium was removed for glutamate assay, and cell-viability immediately assessed by MTT. Glutamate and MTT assays were performed in triplicate and quadruplicate, respectively. A. Cell growth. Plot of increase in MTT absorbance relative to Day 0 (set at 1 relative unit).

B. Glutamate release. $\mu\text{M Glu} / \text{absorbance}$. RCC lines achieved a several-fold increase in extracellular glutamate over HEK293 cells even when adjusted for differences in growth rate over the course of the experiment as measured by MTT.

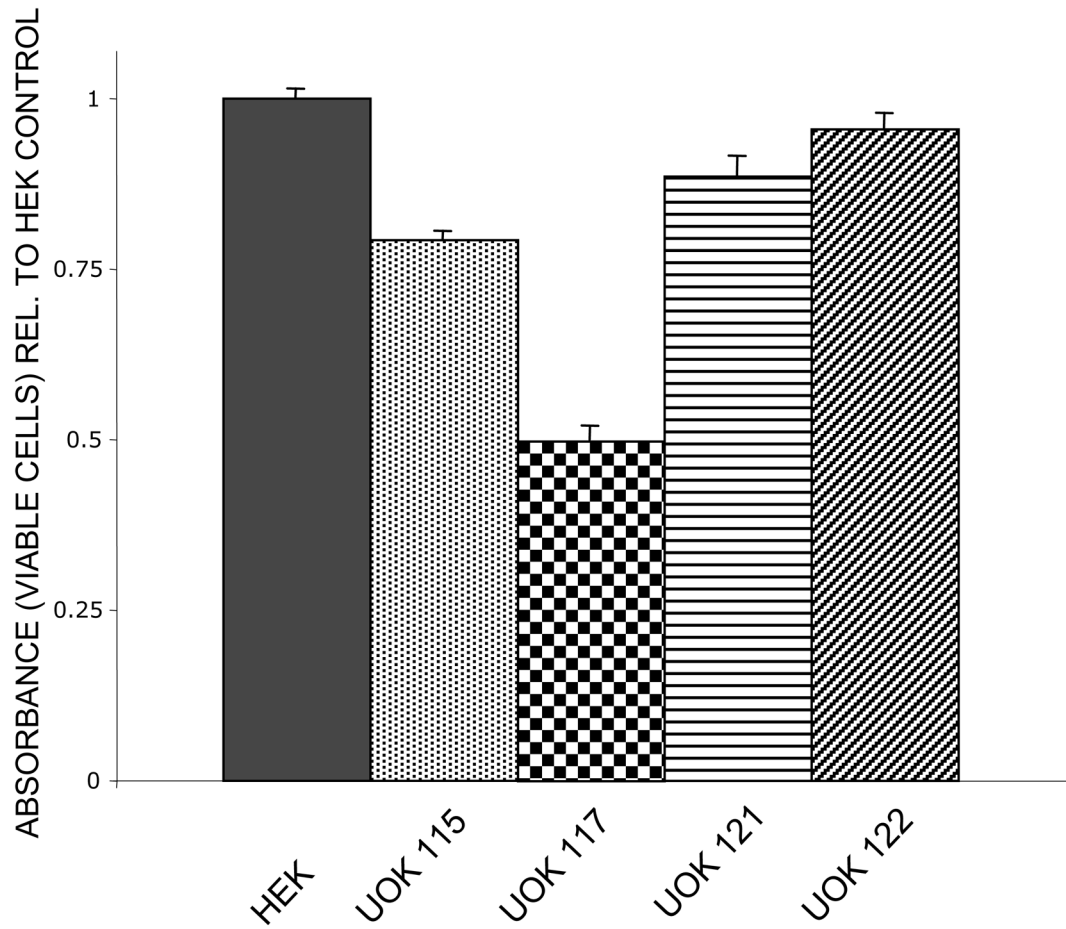


Fig. 7. Renal cell carcinoma cells respond to the anti-glutamatergic drug riluzole
MTT cell proliferation-viability assays were performed after treatment of RCC cells with riluzole for 96 h at 25 μ M. Two of the four RCC lines exhibited reduced cell proliferation in the presence of riluzole: UOK115 by about 20%, and UOK117 by about 50% in comparison to the control HEK293 cells (set at 1 relative unit).

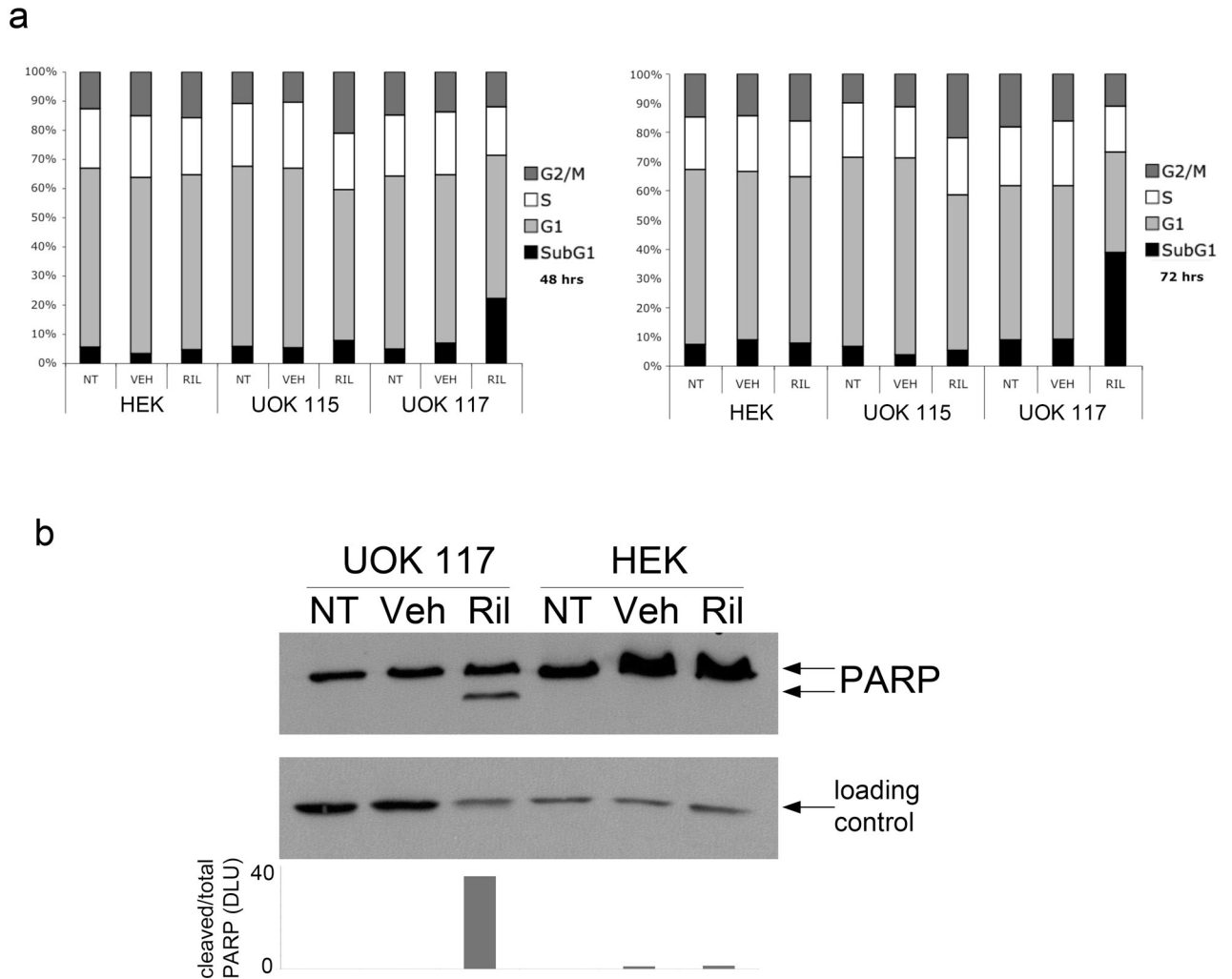
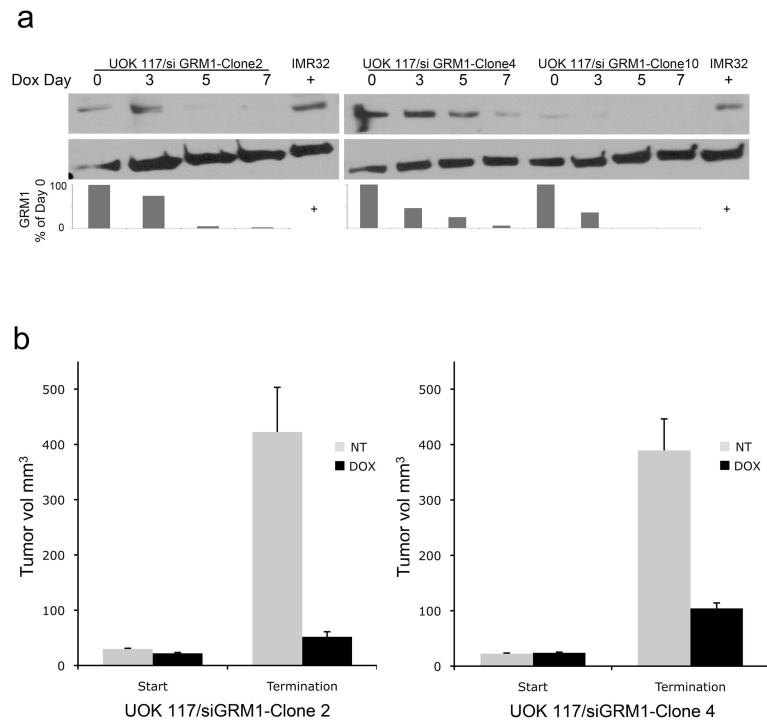


Fig. 8. Riluzole can modulate RCC cell growth

A. Two out of four RCC lines examined displayed apparent cytostatic (UOK115) or cytotoxic/apoptotic (UOK117) effects in the presence of riluzole, as previously observed in melanoma cells [15]. For example, after treatment with riluzole (25 μ M) for 48 and 72 h, cell cycle analyses showed an accumulation of UOK115 in G2/M (cytostasis), and a considerable increase in UOK117 cells accumulated in the sub-G1 phase of the cell cycle, indicating cellular apoptosis.

B. Apoptosis in UOK117 was corroborated by PARP cleavage as shown by immunoblot. HEK293 cells were not affected by riluzole at this concentration. Ratio of cleaved to total PARP by densitometry.

**Fig. 9.**

A. Anti-GRM1 siRNA constructs introduced into UOK117 RCC cells permitted doxycycline-inducible knockdown of endogenous GRM1 at the protein level as assessed by immunoblots. GRM1 + control is IMR32 cell extract. Levels of GRM1 expression in three independent siGRM1-UOK117 clones as percentage of control Day 0, by densitometry.

B. *In vivo* reduction of tumor progression by anti-GRM1 siRNA. Mice bearing xenografted tumors developing from two independent clones of siGRM1-UOK117 RCC cells chosen from Fig. 9a were treated with doxycycline to induce siRNA knockdown of endogenous GRM1 levels. Results implied sustained expression of GRM1 is in part necessary to maintain UOK117 tumor progression (both clones $P < 0.001$, t test, $n=20$).

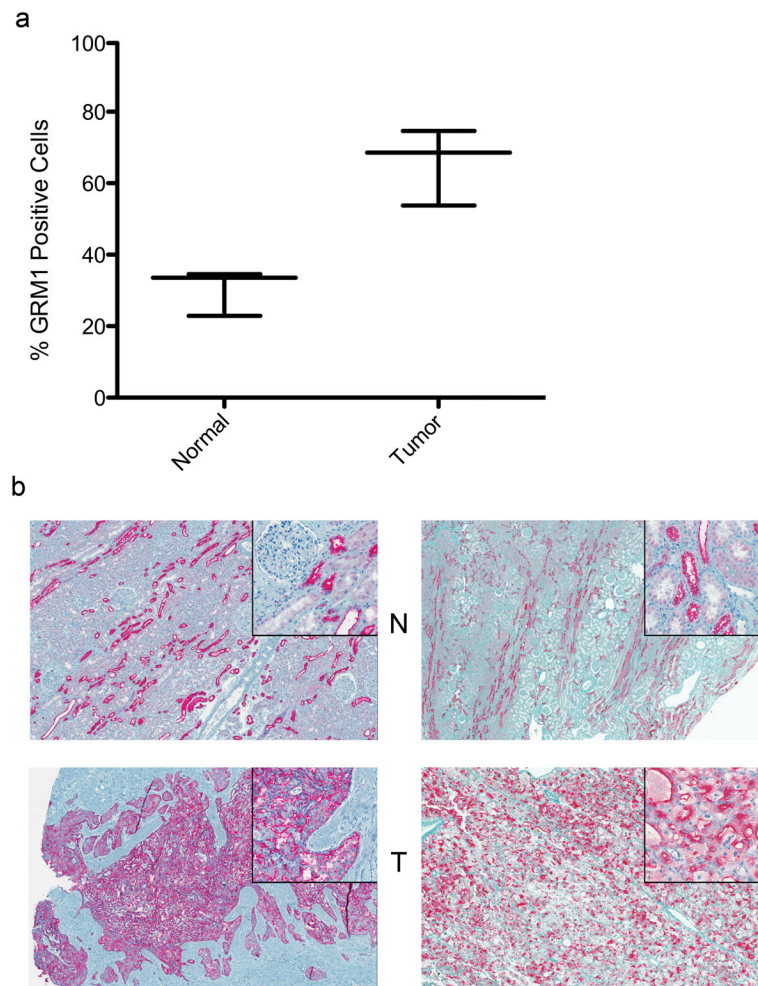


Fig. 10. Immunohistochemistry (IHC) for GRM1 in RCC-biopsies in paired sections of tumor (T) and adjacent normal (N) tissue. A. GRM1 positive cells (%) scored in random areas of each digitized slide in an unbiased quantitative assessment using Aperio ScanScopeGL and ImageScope software. B. Photomicrographs of IHC, representative paired tissue sections. Image scan at 5X; 20X inset.