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REVIEW

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Involvement of metabotropic glutamate receptor 1, a G protein coupled receptor, in melanoma development

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Abstract Melanoma is the aberrant proliferation of melanocytes, the cells in the skin responsible for pigment production. In the United States the current lifetime risk of melanoma development is 1 in 57 in males and 1 in 81 in females [1]. In its early stages melanoma can be surgically removed with great success; however, advanced stages of melanoma have a high mortality rate due to the lack of responsiveness to currently available therapies. The development of animal models to be used in the studies of melanoma will provide the means for developing improved and targeted treatments for this disease. This review focuses on the recent report of a mouse melanoma model, TG-3, which has implicated the ectopic expression of the metabotropic glutamate receptor 1 (Grm1), a G protein coupled receptor (GPCR), in melanomagenesis and metastasis [2]. The involvement of other GPCRs in cellular transformation, particularly GPCRs in melanoma biology, and signaling of Grm1 are also discussed.

Keywords G protein coupled receptors · Melanoma · Transgenic models · Glutamate receptors · Transformation

Abbreviations *AC:* Adenylate cyclase · *AMPA:* α-Amino-3-hydroxy-5-methyl-isoxazole-4-propionate · *CAMK:* Calcium-calmodulin dependent protein kinase · *CHO:* Chinese hamster ovary · *CREB:* cAMP-responsive element binding protein · *DAG:* Diacylglycerol · *Dct:* Dopachrome tautomerase · *ERK:* Extracellular-regulated kinase · *ET:* Endothelin · *ETR:* Endothelin receptor · *FGF:* Fibroblast growth factor · *GAPDH:* Glyceraldehyde-3-phosphate dehydrogenase · *GPCR:* G protein coupled receptor · *GPCRK:* G protein coupled receptor kinases · *Grm1:* Metabotropic glutamate receptor 1 · *IκB:* Inhibitor of NF-κB · *IP3:* Inositol triphosphate · *MAPK:* Mitogen-activated protein kinase · *MC1R:* Melanocortin receptor 1 · *MEK:* MAPK kinase · *mGluR:* Metabotropic glutamate receptor · *MGSA/GROα:* Melanoma growth stimulatory activity/growth-regulated protein alpha · *NF-κB:* Nuclear factor κB · *NMDA: N*-Methyl-D-aspartate · *PCR:* Polymerase chain reaction · *PDGF:* Platelet-derived growth factor · *PIP2:* Phosphoinositol bisphosphate · *PKA:* Protein kinase A · *PKC:* Protein kinase C · *PLC:* Phospholipase C · *PND:* Postnatal day ·

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RT: Reverse transcriptase · *RTK:* Receptor tyrosine kinase · *SCF:* Stem cell factor · *TPA:* 12-*o*-tetradecanoyl phorbol-13-acetate · *Tyrp:* Tyrosinase-related protein

Introduction

General melanoma mouse models

Animal models for the studies of human disease are an invaluable tool for understanding the nature of the disease and devising better, more advanced treatments. Several mouse models developed for the studies of melanoma have been reported. Transgenic mouse lines constructed with known oncogenes such as SV40 T antigen, Ret (a receptor tyrosine kinase, RTK), or a mutant form of Ras, under the regulation of ubiquitous or tissue-specific promoters, develop melanocytic hyperplasia, retinal pigmented epithelial tumors, and melanoma, which in some cases can metastasize to a variety of organs [3, 4, 5]. Hepatocyte growth factor/scatter factor, a melanocyte mitogen which stimulates the RTK Met, has also been used to construct a transgenic mouse line that develops tumors in the mammary glands and among other tumor types, melanomas [6, 7]. Transgenics made with an activated mutant Ras (G12V) under the control of a melanocyte-specific promoter, bred into a $p16^{INK4A/Arf}$ deficient background, develop cutaneous and ocular melanomas; however, no metastasis is observed [8]. Many of these transgenic animals require different combinations of chemical carcinogens and extensive UV irradiation or expression of known oncogenes to develop melanomas at low rates and with long latencies. These animals may also display other primary tumors such as fibrosarcomas, papillomas, and squamous cell carcinomas [9]. Although these models are useful for the studies of melanoma, these properties make them more complex to study. Therefore the generation of a model system that develops melanoma tumors in the absence of any other tumor type, with short latency, high penetrance, and metastatic potential, is of great importance for the studies of melanoma.

TG-3 mouse model

Melanomagenesis

In our laboratory several transgenic mouse lines were made with a genomic clone (clone B). This DNA fragment was shown earlier to commit fibroblast cells to adipogenesis in culture [10, 11]. Although all five independent lines of transgenic founder mice (TG-1 to TG-5) bear insertion of five to seven copies of rearranged clone B DNA, the expected obese phenotype was never observed [12]. Instead, in one of the five transgenic lines, TG-3, the founder developed pigmented lesions at about 8 months. Mice from TG-1, TG-2, TG-4, and TG-5 lines were normal even past 2 years of age. Characterization was carried out with respect to the distribution, histology, and clinical progression of the pigmented lesions in TG-3. Lesions were detected in skin, eyes, lymph nodes, lung, inner ear, brain, and muscle. Additional studies showed that primary tumors initiate in tissues in which normal neural-crest derived melanocytes reside, and that the development of pigmented lesions in additional tissues was likely due to metastasis [13, 14]. The integration of the transgene, clone B, resulted in the deletion of approximately 70 kb of the host DNA (see below for details). The appearance of the initial pigmented lesions and tumor progression among TG-3 mice depends on the zygosity of the inserted transgene/deleted host region. Homozygosity at this region results in the onset of the tumor at 2–4 months of age; if heterozygous, the lesions are first detectable at 6–8 months. Regardless of the difference in ages at which the first pigmented lesions are detected in the animals the final melanoma phenotype remains the same.

In order to determine the origin and early timepoints in the development of melanoma in TG-3 transgenics histological analyses were performed in mice ranging in age from postnatal day (PND) 1 to 30 [14]. Tissue sections were stained with L-dopa to enhance the identification of melanocytes. In control wild-type littermates the number of melanocytes increased during the first few days after birth in all tissues normally containing melanocytes. At around PND 15 most of the melanocytes in the trunk skin migrated to and remained in the hair follicles. By PND 30 very few melanocytes could be detected in the dermis. These findings are very similar to those reported earlier for mouse melanocyte location in the skin [15]. In TG-3 transgenic mice the overall tissue distribution of melanocytes was similar to that of the control mice described above. However, the number of melanocytes in TG-3 mice was significantly greater than that of the control mice at all time points examined. These differences became more pronounced as the animals matured. At PND 1 the number of nonfollicular melanocytes in the dorsal skin of the transgenic mice was twice that of controls. By PND 15 the number of melanocytes in the transgenic mice was 11 times that of controls. Until PND 7 the morphology of the melanocytes of transgenic and nontransgenic littermates was very similar except that the melanocytes in TG-3 mice stained more darkly with both L-dopa and hematoxylin and eosin. At PND15 clusters of melanocytes were noted in TG-3. These clusters likely represented clonal expansion of "transformed melanocytes." By PND 30 large, round, heavily pigmented dopa-positive cells could be detected in the ear, eyelid, and perianal regions. These cells were indistinguishable from the large, round, and heavily pigmented cells in tumors of adult transgenic mice [13]. No abnormality was found in hair-follicular melanocytes from transgenic mice at any age.

As early as PND 3 the melanocytic cell layer of the choroid in TG-3 was two to three times thicker than that of nontransgenic mice. By PND 30 rounded, heavily pigmented cells with morphology indistinguishable to the previously described tumor cells in adult mice were present in both the Harderian gland and choroid of TG-3.

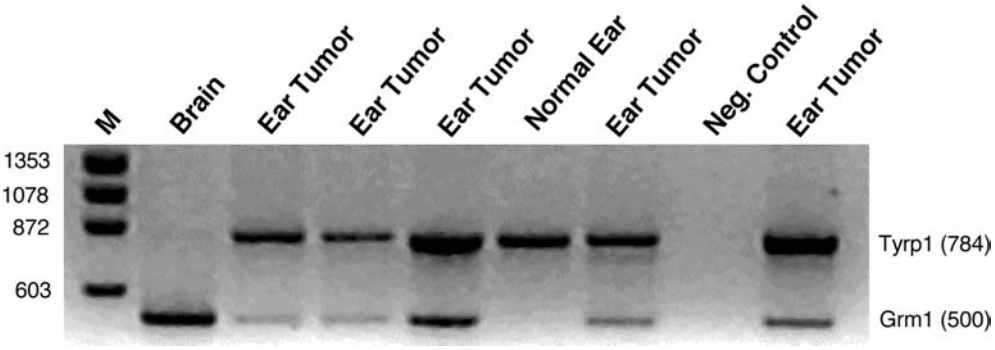

Fig. 1 Expression of Grm1 is detected only in tumor ears. RT-PCR using Grm1 specific interexon primers on ear tumor RNAs from five different TG-3 mice and normal C57BL/6 ear. These RT-PCR reactions were normalized to Tyrp-1 transcripts. Brain RNA from C57BL/6 was used as positive control for Grm1. M marker

In contrast to the thickened choroid, no abnormalities were noted in the retinal pigmented epithelium in any transgenic mouse at any age. Based on these studies we concluded that melanoma in TG-3 originated in all sites where normal neural-crest derived melanocytes are populated, and that optic-cup derived melanocytes remained normal [14].

Characterization of molecular alterations in TG-3

In TG-3 the integration of the transgene resulted in a deletion of host DNA. This 70 kb deleted host region was identified to be part of intron 3 of the gene that encodes the metabotropic glutamate receptor 1 (Grm1, formerly known as mGluR1 or Gprc1a). Grm1 is a seven-transmembrane domain G protein coupled receptor (GPCR) normally expressed in the brain and is stimulated by glutamate.

In order to assess whether the disruption of intron 3 of Grm1 had resulted in changes in Grm1 protein expression the following experiments were performed. Considering that we had shown earlier the ear to be one of the sites of primary tumor formation [13, 14], RNA and protein for molecular and biochemical analyses were isolated from control and tumor ears. Based on histopathological analyses, and western blots of various melanocyte markers (tyrosinase-related protein 1, Tyrp-1; dopachrome tautomerase, Dct; tyrosinase) these tumors were shown to contain significantly more melanocytes than the normal tissue. Under these conditions in order to fairly examine and compare Grm1 transcript levels between normal and tumor tissues reverse transcriptase polymerase chain reaction (RT-PCR) templates were normalized in tumor and control ear tissue samples to melanocyte-specific (Tyrp-1) transcripts, instead of the usual actin or GAPDH (glyceraldehyde-3-phosphate dehydrogenase). Expression of Tyrp-1 was detected in both normal and tumor ears; however, Grm1 was only detected in ear tumors (Fig. 1). These observations were confirmed by western blots using anti-Grm1 antibodies on protein extracts from normal brain (positive control) and normal or tumor ears. Expression of Grm1 protein was detected only in the ear tumor extracts, not in the control ear extracts [2].

To evaluate whether the observed phenotype was due to the expression of a mutated form of Grm1 in melanocytes Grm1 was cloned from several tumor tissue samples by RT-PCR, sequenced and compared to the sequence of the endogenous wild-type brain Grm1. No mutations in the cDNA of tumor Grm1 were found. Taken together these results showed that expression of wild-type Grm1 is detectable only in tumors, not normal tissues.

A new line of transgenics with Grm1 expression targeted to melanocytes (E line). In order to definitely demonstrate that Grm1 has a direct etiological role in melanoma development in our model we generated a new line of transgenic mice. These transgenic animals were constructed with wild-type mouse Grm1 cDNA under the melanocyte-specific Dct promoter [2]. Three transmitting founder lines (A, C, and E lines) from 53 live offspring were obtained. In one of the founder mice, E line, development of pigmented tumors on the tail was observed by 6–7 months of age, and later tumors appeared on the ears. Subsequent offspring developed tumors on the tails and ears as well. Expression of Grm1 in tumor but not normal tails was first demonstrated by RT-PCR and then confirmed by western blots of Grm1 in tail and ear tumors [2]. Histopathological analysis of ear and tail biopsy specimens showed tumors to be very similar to those of TG-3 (Fig. 2) [2, 12, 13]. The other two transgenic lines A and C did not show expression of Grm1 protein and did not develop melanoma [2]. These results confirmed that in our system the ectopic expression of Grm1 is sufficient to transform melanocytes and give rise to malignant melanoma in vivo. The notion that perhaps aberrant expression of Grm1 also occurs in human melanomas was tested. As we had done in the mouse system, the differences in melanocyte numbers between normal and tumor human tissues were normalized using Dct transcripts in RT-PCR reactions. Expression of Grm1 was detected in 7 of 19 melanoma biopsy samples examined, but not in

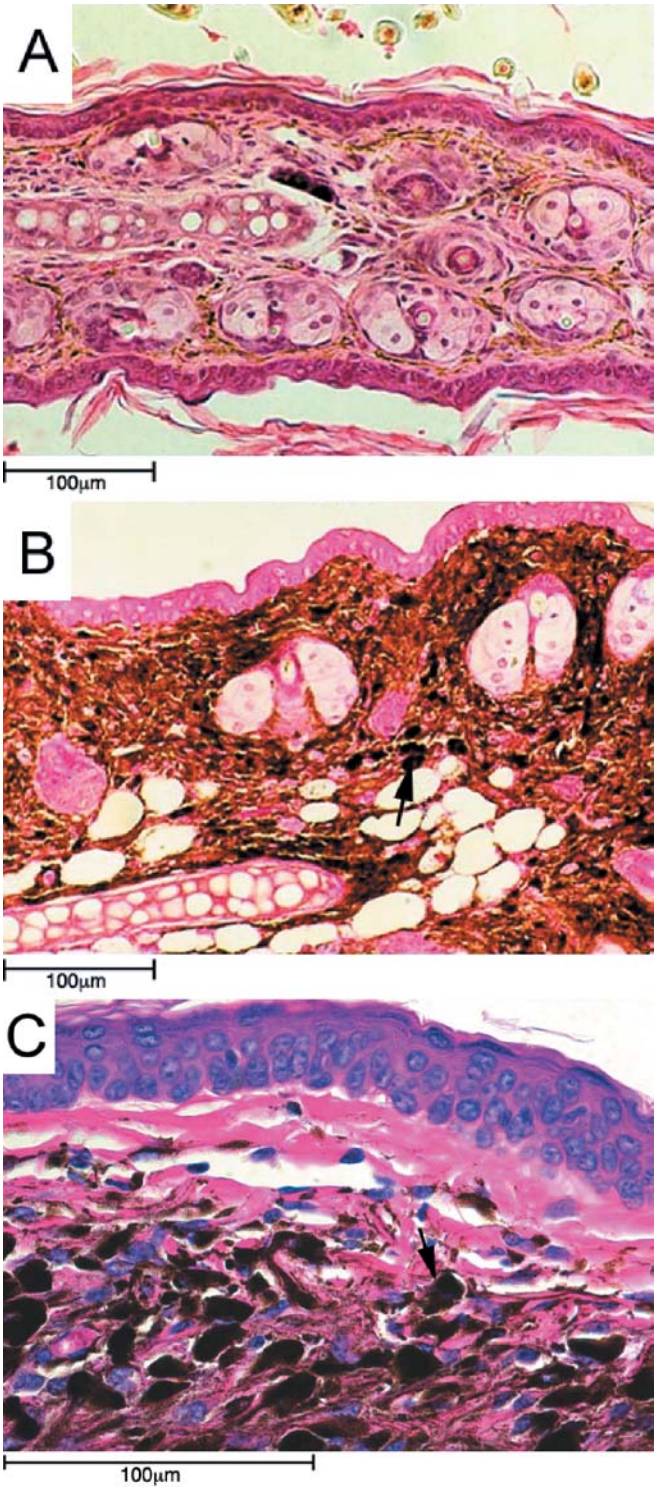

Fig. 2 Similar histopathology of ear biopsy specimens in TG-3 and E lines. **A** Normal pinnae. **B**, **C** Early lesions in the pinnae of TG-3 (**B**) and E line (**C**) transgenic mice. All animals were 1 month of age. *Arrow* Example of melanocyte staining. *Scale bar* 100 μm. Hematoxylin and eosin

normal skin samples or nevi [2]. In addition, Grm1 protein expression was detected by western blot in 12 of 18 human melanoma cell lines tested [2] and was confirmed by fluorescent immunostaining with Grm1 antibodies (unpublished results).

Amelanotic melanoma model. We had shown earlier that the pigment in TG-3 tumor cells is melanin [12]. The presence of excessive melanin in these tumor cells hampered our ability to establish cell cultures. For this reason an albino version of TG-3 was engineered. F1 and backcrosses between TG-3 and various albino strains were performed, and only albinos were selected for further studies. Spontaneous cutaneous amelanotic melanomas developed in these albino transgenic mice [16]. The onset and progression of these amelanotic melanoma tumors is very similar to that reported for TG-3. Protein extracts from tumor ears of these mice has also shown aberrant expression of the Grm1 protein as compared to normal ears (unpublished results). These tumors provided an excellent source for the derivation of melanoma cell cultures without melanin.

GPCRs as oncoproteins

GPCRs are routinely functional in fully differentiated cells, as is the case for Grm1 expression and function in neurons or MC1R (melanocortin receptor-1) in melanocytes (see below for more details). Interestingly, a growing number of GPCR family members have been implicated in the regulation of cell proliferation. The finding that GPCRs can act as proto-oncogenes is not recent. In 1986 Young and coworkers [17] demonstrated that *mas*, a GPCR, is able to transform mouse fibroblasts in the absence of any activating mutations. The ectopic expression or overexpression of other wild-type receptors, such as serotonin 1C receptor, muscarinic acetylcholine receptor, and thrombin (PAR-1) receptor in the presence of their respective ligands can transform cells [18, 19, 20]. Several neuropeptides including gastrin-releasing peptide, neuromedin, bombesin, and galanin, which act through their cognate GPCRs, have been shown to play a role in cell proliferation and transformation [21, 22]. In small-cell lung cancer gastrin-releasing peptide and neuromedin are secreted by the cells, leading to the activation of growth-stimulatory autocrine loops [23, 24]. Treatment of small-cell lung cancer cells with antagonists or neutralizing antibodies to bombesin receptors, results in a partial reduction in cell growth [23, 25, 26]. Furthermore, another neuropeptide, neurotensin, has also been shown to promote the growth of prostate cancer cells [27]. Taken together, results from these studies suggest that many of these receptors mediate their effects on cell proliferation by an increase in the number of receptors per cell or an increase in ligand production leading to amplified or sustained growth-promoting signaling in these cells without the need for any activating mutations [22].

Naturally occurring mutations in GPCRs were later described in human tumors. For example, mutations in the thyroid-stimulating hormone receptors were found in about 30% of human thyroid adenomas [21]. Activating mutations have also been detected in the G proteins of several tumor types, including thyroid adenomas and carcinomas, ovarian sex cord tumors, ovarian small-cell carcinomas, and colorectal cancers [28, 29]. Moreover, expression of a dominant negative $G\alpha_{i2}$ protein slowed the growth of melanoma cells in vitro and in vivo. Injection of nude mice with melanoma cells transfected with this mutant G protein resulted in a delayed appearance of smaller tumors and a longer survival period than in controls [30]. Some virally encoded GPCRs can also lead to cellular transformation; for example, Kaposi's sarcoma-associated herpesvirus is the causal agent for Kaposi's sarcoma. Transformation of fibroblasts by Kaposi's sarcoma-associated herpesvirus is mediated by the ectopic expression of vGPCR, a virally encoded GPCR with an activating mutation [31]. In vivo expression of vGPCR in a cell-specific manner induced Kaposi-like lesions in endothelial cells of transgenic mice that greatly resembled human Kaposi's sarcomas, whereas transgenic mice with an inactive mutant of the vGPCR were unaffected [32]. Adding to the growing evidence implicating GPCRs in cellular transformation, we provided unequivocal evidence from in vivo studies that a GPCR, Grm1, normally expressed in the central nervous system, gives rise to melanoma when expressed ectopically in melanocytes [2]. Therefore the idea that activating mutations, ectopic expression, or overexpression of a GPCR, or components in its signaling network may lead to transformation is an exciting concept that demands further investigation.

GPCRs and their signaling in melanoma

Normal human melanocytes in culture require a combination of at least two chemical reagents or growth factors that act synergistically to promote cell proliferation. These growth-stimulating agents include the phorbol ester 12-*o*-tetradecanoyl phorbol-13-acetate (TPA) and cholera toxin. Potent melanocyte growth factors include fibroblast growth factor (FGF), stem cell factor (SCF), and endothelins (ET) [33]. Interestingly, the requirement for growth factors by normal human melanocytes is lost when they are cocultured with keratinocytes. These cells provide basic FGF and ET to melanocytes, suggesting that keratinocytes also feed these types of synergistic growth factors in vivo to melanocytes [34]. In the case of mouse melanocytes in culture, complementation of serum with TPA in the media is enough to promote proliferation [33]. One of the characteristics of transformed melanocytes is the loss of their requirement for additional TPA or growth factors in the culture media [35]. The gain of autonomous cell growth by transformed melanocytes may be mediated by anomalous extracellular signals exerted by autocrine loops, and/or alterations in intracellular signaling pathways. Ultimately, the sustained activation of certain signaling pathways may lead to uncontrolled cell proliferation. Selected GPCRs that may mediate extracellular signals that control different aspects of melanoma tumor biology, including transformation, proliferation, migration, invasion, and angiogenesis are discussed below (see Table 1).

Grm1

Our studies showed that alterations in the expression of Grm1 are sufficient to promote mouse melanocyte trans-

Table 1 Role of GPCRs in melanoma and potential therapies

Selected GPCRs in melanoma	Signaling molecule(s)	Role(s) in melanoma biology	Potential therapies against several molecular targets
Grm1 (glutamate receptor)	Receptor	Ectopic expression is sufficient for melanoma development in vivo [2]	Specific antagonists, siRNA, and antisense oligonucleotides against Grm1 may be tested
MC1R (melanocortin receptor)	Receptor	Possible correlation of variants of the receptor with increased melanoma risk [36], and melanoma susceptible phenotypes such as red hair and poor tanning ability [37]	Possibility of therapy has not yet been investigated
ETRB (endothelin receptor)	Receptors and ligands	Required for the proliferation of normal melanocytes, as well as the proliferation, adhesion, migration and invasion of melanoma cells [38, 39, 40]	Specific antagonists affect melanoma tumor growth [40, 41]
Frizzled receptor	Ligand (Wnts)	Involved in neural-crest derived melanocyte differentiation [42], and the invasiveness of melanoma cells [43]	Antibodies against the receptor decrease melanoma cell invasiveness [43]
CXCR2 (chemokine receptor)	Ligand (MGSA/ GROα)	Ability of the ligand to transform melanocytes [44, 45]; possible role in the metastatic phenotype of melanoma [46]	Antibodies against CXCR2 limit tumor development in nude mice [47]; NF-κB has been identified as a molecular target of CXCR2 activation and may be a candidate for therapy [68]; mutant MGSA/GROα peptide may serve as a partial antagonist to the receptor [49]
PAR-1 (thrombin receptor)	Receptor	Involved in the metastatic potential and angiogenesis of melanoma [50]	Thrombin inhibitors show encouraging results in clinical trials [51, 52]

formation in vivo [2]. Grm5, but not Grm1, is normally expressed in melanocytes (see below for more details) [53]. Grm1 and Grm5 knock-out mouse lines have been described. These mice showed reduced long-term potentiation, impaired context specific associative learning, and mild ataxia, but no melanocyte-related abnormalities have been reported [54, 55, 56].

MC1R

MC1R is a melanocortin receptor, a member of the GPCR family of receptors. MC1R couples to adenylate cyclase (AC) upon activation by its ligand α-melanocyte stimulating hormone. Activation of MC1R ultimately leads to the synthesis of eumelanin (black-brown pigment). The human MC1R gene is highly polymorphic, and some of the variability in this gene is in part responsible for the phenotypes of red hair, and poor tanning ability [57, 58]. Abdel-Malek's group [59] have studied the consequences of MC1R polymorphisms on cell viability. They reported that UV irradiation was highly toxic to human melanocyte cultures that harbored loss of function mutations in the MC1R gene. In contrast, cells with functional MC1R can better survive UV irradiation. It is well known that UV irradiation can induce cells to undergo apoptosis, and melanocytes that escape cell death could initiate tumor development by alteration of genes in the apoptotic pathway [60, 61]. Studies on MC1R variants as possible indicators for skin cancer susceptibility are therefore of great relevance in the studies of melanoma.

Endothelin receptors

Endothelin receptors are also members of the GPCR family. These receptors have been shown to be of critical importance in many tumor types. Antagonists to endothelin receptor A (ETR) A are in phase I clinical trials for patients with refractory adenocarcinomas, including prostate, colorectal, and lung cancer [62]. Many of these tumor tissues have increased expression of ETRA. Moreover, its preferred ligand, ET-1, serves as a mitogen for normal melanocytes and colorectal, ovarian, and prostate tumor cells [33, 38]. In humans mutations in ETRB, result in Hirschsprung's disease, which is characterized by failed development of melanocyte precursors. Further evidence that ETRs and another of their ligands, ET-3, are involved in melanocyte differentiation came from studies by Lahav and colleagues [39]. In their studies the presence of ET-3 in cultures established from quail neural crest cells resulted in an increase in melanocyte–early-marker positive cells (premelanocytes). Treatment of the neural crest cells with ET-3 for 16 days resulted in 90% of them labeled as premelanocytes as compared to 5% in control cultures. These results suggested critical roles for ET signaling in melanocyte differentiation and proliferation [39]. The same group also demonstrated a reduced viability of human melanoma cell lines upon treatment with antagonist to ETRB, but not to ETRA [41]. In order to validate in vivo these in vitro results, human melanoma cells were injected into nude mice and tumors were allowed to form. Approximately 2 weeks later mice were treated with ETRB antagonist, either intratumorally or intraperitoneally; slower tumor progression or tumor regression was observed. Even though many normal cell types express ETRB, when receptor-expressing 293 kidney cells were treated with ETRB antagonist, no toxic response was detected [41]. These results suggest different roles for the same receptor in different cell types. The potential for therapeutic application of ETRB antagonists in the treatment of melanoma is therefore promising and requires further evaluation.

Frizzled

Wnt/Frizzled is another interesting example of GPCR signaling in melanocytes and melanoma. Wnt is a family of secreted peptides that activate the Frizzled receptors, members of the family of GPCRs. Wnt/Frizzled signaling has been extensively studied in embryogenesis and development of vertebrates and invertebrates. In mammalian cells the induced pathways in many instances lead to the stabilization of β-catenin, ultimately resulting in altered gene expression [63]. In mouse embryos deficient for Wnt1 and Wnt3a a decrease in neural-crest derived melanocytes was detected [64]. Wnt1 itself has been shown to affect the expansion of neural-crest derived melanocytes in an ET-3 dependent manner [42]. Moreover, mutations that lead to the stabilization of β-catenin have been documented in melanoma cell lines and may be implicated in tumor progression [65]. The nuclear/cytoplasmic localization of β-catenin, rather than at the cellular membrane, is another indicator of possible activation of Wnt/Frizzled pathways in melanoma tumors [66]. Upregulation in the expression of Wnt5a in carcinomas of the lung, breast, prostate, and melanoma has also been documented [67]. Weeraratna et al. [43] reported that melanoma cells with both low Wnt5a expression and low in vitro invasion show increased protein kinase C (PKC) phosphorylation and increased invasiveness in vitro upon transfection with exogenous Wnt5a. Furthermore, treatment of the cells with an antibody to Frizzled-5 inhibited the binding of Wnt5a to the receptor, resulting in decreased PKC phosphorylation and decreased invasiveness. Taken together these results suggest a possible role for Wnt/Frizzled signaling in human melanomas.

CXCR2

Another GPCR involved in melanoma biology is CXCR2. CXCR2 may be stimulated by several chemokines, including MGSA/GROα (melanoma growth stimulatory activity/growth-regulated protein). Transfection of the chemokine MGSA/GROα into mouse melanocytes resulted in transformation of these cells in vitro, as evi-

dent by their ability to form colonies in soft-agar and tumors in nude mice [44, 45]. Exogenous addition or continuous expression of this chemokine in mouse melanocytes resulted in stimulation of the receptor CXCR2 and activation of the downstream transcription factor, nuclear factor κB (NF-κB). When the mouse melanocyte clones expressing MGSA/GROα were transfected with the inhibitor of NF-κB (IκBα), their ability to form colonies on soft-agar was severely impaired. These results suggest that transformation of melanocytes by MGSA/GROα (likely mediated by the activation of CXCR2), leads to the activation of NF-κB, ultimately resulting in the transcription of genes that control cell proliferation [69]. Moreover, human melanoma cells have been shown to secrete high levels of MGSA/GROα and have constitutively active NF-κB [70, 71]. Constitutive expression of MGSA/GROα appears to be at least in part regulated by an NF-κB element in the 5′ regulatory region of the gene [72]. Taken together these data suggest a major role in melanocyte transformation for the activation of a GPCR, CXCR2.

PAR-1

The thrombin receptor, PAR-1, has been identified as a transforming gene in NIH3T3 fibroblasts [20]. PAR-1 is a protease-activated GPCR, which is involved in blood coagulation [73]. PAR-1 overexpression has been detected in colon, breast, and pancreatic tumor cell lines, among others. Elevated PAR-1 expression is detected in metastatic melanoma cells, as compared to nonmetastatic cells [74]. This overexpression is correlated with the loss of expression of the transcription factor AP-2. In fact, it has been shown that the promoter region of PAR-1 contains binding sites for AP-2 and Sp1. Binding of AP-2 results in negative regulation of gene expression, whereas binding of Sp1 leads to the stimulation of PAR-1 transcription. These two transcription factors compete for their binding to overlapping consensus sequences on the PAR-1 promoter region, and the ratio of the two determines the level of PAR-1 transcription [74]. Accordingly, in metastatic melanoma cells a decrease in AP-2 expression allows for increased PAR-1 expression. Stimulation of the thrombin receptor is then proposed to mediate the alteration in expression of genes necessary for tumor cell invasion and angiogenesis [50]. Therapies that target thrombin pathways are being considered for the treatment of certain tumors, including melanoma [51].

There are other GPCRs whose expression has been reported in melanoma cells but require further investigation, for example, the SLC-1 and $S1P_2$ receptors [75, 76]. The vast amount of information provided by the completion of the sequencing of the human genome suggests that there are hundreds of orphan GPCRs (receptors with unknown ligand). The possibility that some of these receptors have a major role in tumor biology, and their implications on drug development must be further considered.

Grm1 functions in the brain

Physiology and pathophysiology

Functional studies of Grm1 have been conducted in the normal neuronal system and in animal models of various human diseases. Results obtained from these earlier studies can assist us in our understanding and study of Grm1 in melanoma.

In the mammalian central nervous system glutamate is considered to be the major neurotransmitter. Glutamate excites neurons through the activation of its receptors in the synapse. Glutamate receptors belong either to the ionotropic or metabotropic families of receptors. Ionotropic glutamate receptors include ligand-gated cation channels: *N*-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-isoxazole-4-propionate (AMPA), and kainate receptors, whereas metabotropic glutamate receptors (mGluRs) are part of family 3 of GPCRs, which also contains the calcium sensing receptor [77]. Eight mGluRs have been identified to date, and they are divided into three groups based on sequence homology, agonist selectivity, and effector coupling. Grm5 together with Grm1 comprise group I mGluRs. Expression of Grm1 is normally detected in the central nervous system, most abundantly in hippocampal neurons and cerebellar Purkinje cells. Grm1 is also expressed in the olfactory bulb, amygdala, thalamus, and basal ganglia. The receptor is found in the cell bodies and dendrites of neurons. Grm1 has been suggested to be involved only in excitatory responses induced by strong presynaptic stimulation. In general, agonists of group I mGluRs cause depolarization and neuron excitation, in part by modulating voltage-dependent and voltage-independent ion channels. Grm1 modulates synaptic transmission not only postsynaptically but also by mediating the modulation of neurotransmitter release from presynaptic terminals [78]. Grm1 is also implicated in the synaptic plasticity of neurons in long-term potentiation and long-term depression; both of these events can last for hours in vitro and up to weeks in vivo. These phenomena are taken as models for the studies of learning and memory formation [79]. In addition, overstimulation of glutamate receptors potentiates neuronal death as observed after brain ischemia (glucose and oxygen deprivation), traumatic brain injury, or anoxia. This type of toxicity has also been implicated in epilepsy, Parkinson's, Huntington's or Alzheimer's disease [78, 79, 80]. For these reasons Grm1 has been a major focus of studies on the processes of learning and memory formation as well as glutamatergic-induced neuronal cell death (neurotoxicity). Since group I mGluRs are potentially implicated in so many diseases, it is not surprising that agents which act specifically on these receptors are considered potential targets for therapies for neuronal cell damage after stroke, neurodegenerative disorders, epilepsy, pain, and even hypertension [80, 81].

Glutamate receptor antagonists

Antagonists of Grm1 have been shown to have neuroprotective activities against neurotoxicity mediated by overstimulation of the receptor in different disease models, including ischemia [82, 83]. In terms of seizures, although still somewhat unclear, it is generally considered that the activation of group I mGluRs enhances neuronal excitability; thus agonists to these receptors have convulsant activity, whereas antagonists have anticonvulsant activity [80, 84].

Based on several studies it has been suggested that antagonists to ionotropic glutamate receptors may be a previously unrecognized tool for cancer therapy [85]. Antagonists to NMDA and AMPA have been shown to exert concentration dependent antiproliferative effects on human tumor cell lines including astrocytomas, neuroblastomas, thyroid, breast, and lung carcinomas, and colon adenocarcinomas. Human skin fibroblasts and bone marrow stromal cells are not affected by these compounds [86]. Another team reported that gliomas with high glutamate release show growth advantage in vivo, and that treatment of implanted gliomas with an NMDA antagonist limits the growth of the tumor significantly [87]. With respect to mGluRs one group reported that injection of black mice with monosodium glutamate produces depigmentation in the injected region. This effect was linked to the expression of Grm5 in normal melanocytes. The possible role of Grm5 in normal melanocyte proliferation was suggested [53]. In our model system the potential for using antagonists as a possible therapeutic treatment for melanoma is being investigated in Grm1-positive tumor bearing mice. We are particularly interested in recently developed antagonists which have been shown to be highly specific for Grm1 and have no effect on neuronal cell viability [88].

Grm1 signaling

Endogenous signaling of Grm1

Grm1 possesses the characteristic seven-transmembrane domain structure of GPCRs. Upon stimulation of the receptor by its ligand a G protein couples the activated receptor to its effector leading to intracellular signaling. G proteins are heterotrimeric proteins which consist of α and β/γ subunits. These subunits are associated with one another only when bound to GDP (inactive state). Activation of the receptor by its ligand leads to the exchange of GDP for GTP, allowing the dissociation of the G_{α}-GTP subunit from the $G_{\beta/\gamma}$ dimer, both of which can act as independent signaling molecules [89]. Group I mGluRs are positively coupled to phosphoinositide hydrolysis through preferential coupling to $G\alpha_{q/11}$, and in exogenously transfected systems Grm1 may also activate AC through coupling with $G\alpha_{s}$ [90]. In contrast, group II mGluRs (which includes Grm2 and Grm3) and group III (including Grm4, Grm6, Grm7, and Grm8), are negatively coupled to forskolin-induced AC [78, 79, 90].

The coding region of Grm1 has multiple splice sites which give rise to receptors with different C terminal domains. Grm1a is the full/length receptor, and several shorter splice variants have been reported [90]. As is the case for many other GPCRs, Grm1 can be phosphorylated on sites of its carboxy terminal domain. The longer splice variant Grm1a has been shown to have constitutive activity by activation of the G protein in the absence of ligand in heterologous model systems [91]. In the case of the endogenously expressed Grm1 in mouse cerebellar granule cells constitutive activity of the receptor has been shown to be controlled by interactions with Homer proteins, which contain PDZ domains that allow their interaction with the receptors C terminal domain [92]. This regulation of mGluRs by Homer proteins can also affect the receptor location, clustering, and cell surface expression [93, 94].

Grm1, PKC, and MAPK activation

Activation of the mitogen-activated protein kinase (MAPK) cascade has been shown to be an early event in melanoma progression, observed in radial growth but not in atypical nevi [95]. Upon activation Grm1 may lead to the activation of the MAPK cascade [96, 97]. In the extracellular-regulated kinase (ERK) 1/2 signaling pathway, Raf proteins are MAPK kinase (MEK) kinases which phosphorylate and activate MEK1/2 which in turn activates ERK1/2 (MAPK) [98].

The cascade of events triggered by Grm1 stimulation, which leads to the activation of the MAPK signaling proteins, has been described for both the endogenous receptors in neurons and the transfected receptors in heterologous systems. It has been shown that Grm1 preferentially couples to the heterotrimeric $G\alpha_{q/11}$ and may also couple to $G\alpha_{s}$ proteins. Coupling of Grm1 to $G\alpha_{q/11}$ leads to the activation of phospholipase C (PLC). Upon activation of PLC, inositol triphosphate (IP3) and diacylglycerol (DAG) are produced from the hydrolysis of phosphoinositol bisphosphate (PIP2). This results in the activation of PKC and release of calcium from intracellular stores in the endoplasmic reticulum (ER). Consequences of these events are many: calcium release leads to the activation of calcium responsive kinases, including calcium-calmodulin dependent protein kinases (CAMKs), and Pyk2. CAMKII can activate the MAPK cascade resulting in the activation of transcription factors (TF) such as cAMP-responsive element binding protein (CREB) and Elk-1 (Fig. 3) [99, 100, 101]. Pyk2, on the other hand, is a nonreceptor tyrosine kinase which has been shown to mediate the activation of MAPK upon its activation by GPCRs [102].

In many cases the pathways of GPCRs and RTKs that lead to ERK activation are convergent. It has been postulated that GPCR stimulation may lead to tyrosine phosphorylation of RTKs, a phenomenon which is termed

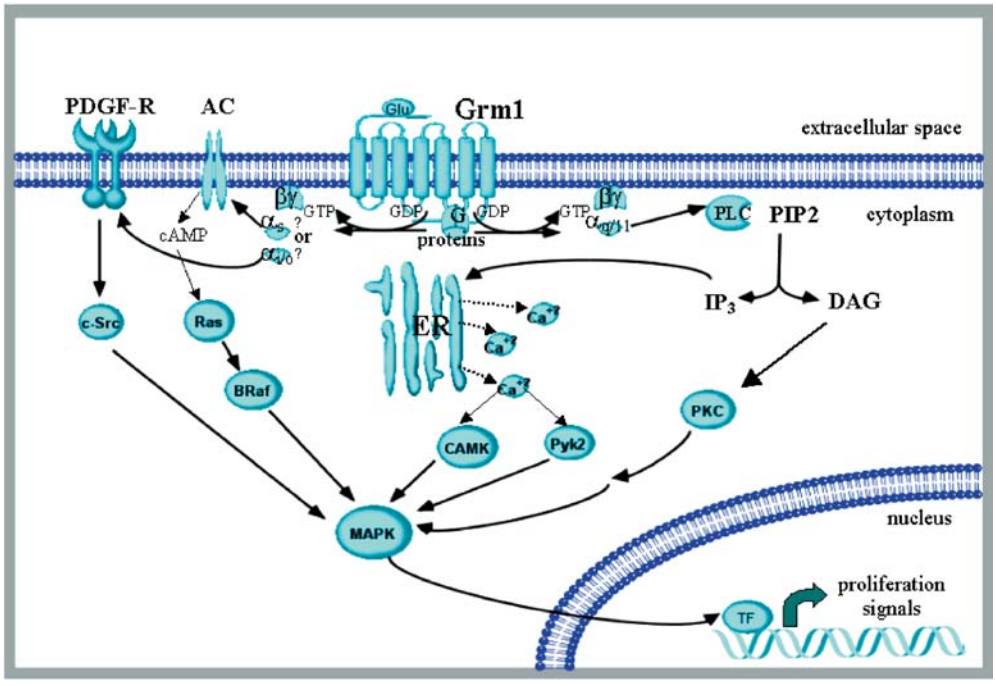

Fig. 3 Schematic view of simplified Grm1-stimulated signaling. Upon stimulation by glutamate (*Glu*), Grm1 can couple to multiple signaling pathways through different G proteins, including PLC activation via $G\alpha_{q/11}$. This in turn leads to the hydrolysis of PIP2 to IP3 and DAG. DAG activates PKC, leading to the activation of the MAPK cascade. On the other hand, IP3 promotes the release of calcium from the ER. Calcium-dependent kinases such as CAMKs and Pyk2 can then be activated leading to the activation of MAPK. If Grm1 is coupled to $G\alpha_s$, its activation leads to the accumulation of cAMP, mediated by AC. This in turn, leads to the activation of the Ras-B-Raf-MAPK signaling cascade. In addition, Grm1 stimulation can transactivate PDGF receptor, a RTK, potentially by coupling to $G\alpha_{i/o}$. The transactivation of PDGF-R leads to the activation of c-Src and MAPK. Finally, upon activation MAPKs can transmit the stimulatory signals to the nucleus where TF that regulate the expression of genes that promote cell proliferation are activated (see text for details)

transactivation [103, 104, 105]. Transactivation of RTKs can be achieved by various G proteins that couple to GPCRs depending on the cellular system [106]. In fact, Grm1 activation of ERK is attenuated by inhibitors to the platelet-derived growth factor (PDGF) receptor, which is a RTK. It has been suggested that one of the mechanisms of Grm1 transactivation of the PDGF receptor, which leads to ERK phosphorylation, may be the activation of the Src family of tyrosine kinases. These results suggest a cascade in which Grm1 activation leads to the transactivation of the PDGF receptor, which then stimulates Src, leading to the activation of MAPKs (Fig. 3) [96]. Whether any of the discussed pathways are activated in melanoma by Grm1 stimulation remains to be studied.

The signaling exerted by PKC has been extensively studied. In neuronal cells PKC activation is required for the depolarization of nerve terminals and increases glutamate release [79]. In addition, in striatal neurons PKC has been shown to play a key role in the Grm1-mediated activation of ERK in ischemic long-term potentiation [107]. In Chinese hamster ovary (CHO) cells transfected with Grm1 glutamate is able to activate ERK2, and inhibitors to PKC could abolish this activation. Glutamate-induced Grm1 activation of ERK2 was also shown to be dependent on MEK1/2 [97]. In contrast, in studies by Thandi et al. [96] using CHO–Grm1 transfected cells as well, ERK activation by Grm1 was shown to be independent of both PKC and phosphatidylinositol 3′ kinase. Activation of ERK in this case was shown to be sensitive to Src-specific inhibitors and also to pertussis toxin, implying that ERK activation is mediated through Grm1 coupling to $G\alpha_{i/o}$. Taken together these data imply that in neuronal cells the endogenously expressed Grm1 receptor can activate PKC leading to ERK activation; however, in heterologous systems with Grm1 expression the involvement of PKC in the activation of ERK remains unclear.

PKC itself is a molecule of great interest in the study of melanoma, as PKC inhibitors have been shown to decrease the angiogenic and metastatic potential of melanoma cell lines [108], and in phase I clinical trials using PKC inhibitors a partial response of a melanoma patient has been reported [109].

In transfected cells, when Grm1 is coupled to $G\alpha_s$, AC is activated leading to the accumulation of the second messenger cAMP [110]. In melanoma cells activation of MC1R, another GPCR, leads to the accumulation of cAMP and the activation of the Ras-B-Raf-MEK1/2-ERK1/2 signaling cascade, in a protein kinase A (PKA) independent manner (Fig. 3) [111]. Recently, activating mutations of B-RAF in many human malignant melanomas have been reported [112, 113]. Somatic missense mutations within the kinase domain of B-RAF have been detected in 66% of malignant melanomas. A single substitution (V599E) accounted for 80% of the mutations.

Interestingly, mutated B-RAF proteins were shown to have higher basal kinase activity than the wild-type B-RAF and were also able to transform NIH3T3 cells [112]. The high frequency of the V599E substitution in primary melanomas and melanoma metastases was confirmed in an independent study, but surprisingly this mutation was also found in 82% of nevi [113]. Whether activation of Grm1 mediates its transforming capabilities in melanocytes through B-Raf activation in our system is a subject we are currently investigating.

Grm1 desensitization

PKC activation has also been described to desensitize Grm1 by phosphorylating its C-terminal domain at threonine 695, which is within its $G\alpha_{q/11}$ coupling site. Desensitization of Grm1 by PKC phosphorylation selectively inhibits the IP3–Ca^{+2} pathway but not the $G\alpha_s$ mediated cAMP accumulation pathway [114]. The differential effects of PKC desensitization may be of great importance in the modulation of neuronal signaling by Grm1. In fact it has been proposed that group I mGluRs may have a dual role in the agonist induced facilitation or inhibition of glutamate release. The pathway leading to facilitation of glutamate release may be desensitized after the initial treatment with agonist, while the inhibition of glutamate release may not. This dual control of glutamate recycling is PKC dependent and is probably a mechanism to prevent neurotoxic accumulation of glutamate in the synapse, implying a critical role for receptor desensitization in physiological conditions [115].

G protein coupled receptor kinases (GRKs) are a family of proteins comprised of six members, some of which have been shown to be involved in Grm1 phosphorylation leading to receptor desensitization [116, 117]. Recently Iacovelli and coworkers [118] showed that desensitization of Grm1 in their system is mediated by GRK4 phosphorylation of the receptor. This phosphorylation event leads to the recruitment of β-arrestin to the plasma membrane. β-Arrestin itself may serve as a signaling protein for the activation of c-Src and MAPK [119]. In cerebellar Purkinje cells antisense oligonucleotides to GRK4 inhibit Grm1 internalization but not receptor induced MAPK activation. Conversely, β-arrestin dominant negative mutants reduce Grm1-induced MAPK activation but not receptor internalization [118]. Phosphorylation of Grm1 is not only involved in receptor desensitization, but can also limit which partner G protein is able to couple to the receptor and mediate its intracellular signaling [114]. Thus receptor phosphorylation plays a critical role in the balance between activation and inhibition of agonist-induced signaling.

Possible transcription factors mediating cell proliferation

GPCRs can activate a variety of different transcription factors and downstream target genes, of which only two are discussed here as an illustration of how extracellular signals mediated through GPCRs may ultimately lead to an increase in cell proliferation.

CREB and related proteins

GPCR activation can lead to transcriptional activity of the downstream nuclear target CREB. For example, activation of muscarinic acetylcholine receptors, as well as group I mGluRs, can lead to the activation of CREB, and these activations are in part dependent on PKA and PKC activity [120, 121]. Specifically, Grm5 itself has been shown to be linked to the activation of CREB in primary cultures of striatal neurons [122]. CREB was initially identified as a target of the cAMP-signaling pathway through its activation by PKA. In addition to PKA, activation of CAMKs or PKC has also been linked to the phosphorylation of the transcription factor CREB at residues critical for its activation (Ser133) [100]. Expression of CREB and its related family member AP-1 is correlated with the malignant progression of melanoma cells [123]. Importantly, earlier studies demonstrated that CREB and related proteins act as survival factors allowing human melanoma cells to escape apoptosis [124, 125]. Cyclin D_1 is one of the possible transcriptional targets of activated CREB [126, 127]. The increased synthesis or reduced degradation of cyclins has been postulated to be in part responsible for the dysregulated growth of melanoma cells [128, 129]. Persistent levels of cyclins lead to the inactivation of the retinoblastoma tumor-suppressor pathway and E2F-mediated upregulation of cell cycle progression genes [129]. Amplification of the cyclin D_1 gene in some cases of human melanoma has also been reported [130]. Taken together CREB and its associated proteins are of great importance in the development and progression phenotypes of malignant melanoma and may mediate some of the extracellular signals that lead to the aberrant proliferation of melanocytes.

NF-κB and family of proteins

The NF-κB/Rel family of proteins consists of several related members. In unstimulated cells NF-κB is sequestered in the cytoplasm by IκB. Upon stimulation of the cells upstream kinases can phosphorylate the IκB kinase complex, which phosphorylates IκBs and targets them for proteasomal degradation. Free NF-κB is then translocated to the nucleus where it binds to specific target DNA sequences and activates transcription [131]. In neuronal cells glutamate specifically activates NF-κB through the activation of the NMDA receptor, another member of the glutamate family of receptors [132]. More

specifically, Richmond's group [69, 133] showed that NF-κB is constitutively activated in human melanoma cell lines. In mouse melanocyte cell lines NF-κB can be activated by incubating the cells with MGSA/GROα, a chemokine which activates CXCR2, a receptor of the GPCR family. NF-κB is deregulated in many human cancers, including Hodgkin's lymphoma, head and neck squamous cell carcinoma, non-small-cell lung cancer, colorectal cancer, thyroid cancer, pancreatic carcinoma, leukemia, multiple myeloma, prostate cancer, breast cancer, and melanoma [134]. Genes regulated by NF-κB include regulators of apoptosis, and cell proliferation [135], thus making NF-κB a key regulator in the proliferation of many human cancers and an attractive transcription factor to study in melanoma.

Future studies

Transformation and signaling studies

We are currently interested in interfering with the transforming abilities of Grm1 in melanoma cell lines by impairing its expression with antisense oligonucleotides, dominant-negative Grm1, and siRNA. These types of studies will allow us to determine whether melanomas that show Grm1 require the expression of the receptor for their uncontrolled proliferation and survival in vitro. The possible application of these techniques, which negatively affect Grm1 expression, or the use of antagonists, which inhibit the receptors function, are also being assessed in melanoma tumors in vivo. In terms of signaling we have started to examine the possible transduction pathways based on previously reported signaling by Grm1 in neuronal and heterologous systems. These studies will provide evidence as to what signaling mechanisms are used by the receptor to ultimately stimulate cell proliferation.

The human/mouse melanoma model

Normally melanocytes are present in the epidermis of the human skin. The progression of melanoma in humans has been divided into three classes. Class I is represented by nevi whose growth is restricted to the epidermis; class II is that of intermediate lesions with microinvasion into the dermis, which includes in situ and radial growth phase melanomas. Finally, class III melanomas are the vertical growth phase tumors with potential for metastasis [136]. In contrast, melanocytes in the mouse are located mostly in hair follicles. An exception to this is the epidermal location of murine melanocytes in the ear, tail, perianal region, and footpads [137, 138]. The differences in the distribution of melanocytes in the skin of humans and mice hamper our efforts to use mouse models to study human melanoma as a disease that progresses by microinvasion of cells from the epidermis into the dermis. Given these differences it is of importance to consider the relevance of mouse models of melanoma in terms of their application to the human system. Interestingly, some of the most prominent sites of tumor development in our transgenic animals, TG-3 and E line, are the ears, perianal region, and tail, where melanocytes reside in the epidermis, although in our animals the tumor melanocytes did not breach the overlying epidermis [12, 13]. As time progresses, other sites such as the eyes, snout, legs, and other parts of the skin where melanocytes are located in the dermis also develop primary tumors [2, 13].

A transgenic mouse model with melanocytes present in the epidermis has been described. These mice were constructed with the cDNA of SCF (also known as steel factor). SCF is the ligand for the c-Kit RTK, which controls the migration, development, and survival of melanocytes. In order to maintain SCF is in its membrane bound state rather than in the soluble form, the cDNA of SCF used to engineer these transgenic mice was modified to prevent cleavage. Using a keratinocyte-specific promoter to regulate SCF expression, Kunisada and coworkers [139] were able to construct a transgenic mouse line with melanocytes in the epiderm which resembles the human phenotype, yet no melanoma development was observed.

Crossing TG-3 with a mouse such as the one just described is one of our current goals and could open the door to the study of a spontaneous melanoma-developing mouse model with melanocytes in the epidermis. A true mouse model of the human disease.

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