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Metabotropic glutamate receptors (mGluRs) and cellular transformation

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Introduction

Glutamate is one of the major neurotransmitters in synapses in the central nervous system (CNS). In order to mediate the excitatory signal between neurons, glutamate activates two different types of receptors: ionotropic glutamate receptors (iGluRs) and metabotropic glutamate receptors (mGluRs). iGluRs are ligand-gated ion channels that allow cations such as calcium and potassium to pass through the plasma membrane of the cell after binding of glutamate to the receptors. iGluRs are further subdivided into three distinct types of receptors according to the response to agonists: N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-isoxazole-4-propionate (AMPA) and kainate (KA) receptors (Hollmann and Heinemann, 1994; Monaghan et al., 1989). Until the mid 1980's, the actions of glutamate in the mammalian brain were thought to be mediated exclusively by activation of iGluRs. However, in 1985, Sladeczek and colleagues discovered that glutamate had more complex roles in that it could stimulate phospholipase C (PLC) accumulation in cultured striatal neurons via a receptor that did not belong to the iGluRs (Sladeczek et al., 1985). Soon after that, a similar effect of glutamate was described in hippocampal slices (Nicoletti et al., 1986a), cultured cerebellar granule cells (Nicoletti et al., 1986b), and cultured astrocytes (Pearce et al., 1986). In 1987, Sugiyama and coworkers also reported the possibility of new types of glutamate receptors being implicated in phosphoinositide hydrolysis (Sugiyama et al., 1987). In 1991, two different research groups reported the sequence and structure of a G-protein coupled glutamate receptor which was referred to as metabotropic glutamate receptor 1 (mGluR1) (Houamed et al., 1991; Masu et al., 1991). mGluRs showed relatively large molecular masses and had no sequence homology with any other type of G-protein coupled receptor (GPCR), suggesting that these receptors were indeed a new family of GPCRs (Conn and Pin, 1997; Pin and Duvoisin, 1995). Currently, eight subtypes of mGluR have been identified. According to sequence homology and response to agonists, these mGluRs are subdivided into three groups: Group I (mGluR1 and 5), II (mGluR2 and 3), and III (mGluR4, 6, 7, and 8).

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mGluRs in non-neuronal cells

Most early research efforts on mGluRs had been confined to neuronal cells, where these receptors were described to be involved in processes such as learning and memory through long term potentiation (LTP) or depression (LTD) (Kato, 1993; Nakanishi, 1992), nociception (Pin and Bockaert, 1995), pain (Meller et al., 1993), startle reaction (Koch, 1993) and mood disorders (Fendt and Schmid, 2002). However, mGluRs, for a long period considered to be restricted to neuronal cells, are now known to be expressed in many different types of tissues in the body. This suggests that mGluRs are not just neuronal receptors implicated in neurotransmission, but also involved in maintaining the homeostasis of the body. mGluRs are expressed in various non-neuronal cell types including bone (osetoclasts) (Morimoto et al., 2006), skin (melanocytes and keratinocytes) (Frati et al., 2000; Genever et al., 1999), pancreas (pancreatic islets and β-cells) (Storto et al., 2006; Tong et al., 2002), liver (hepatocytes) (Storto et al., 2000b), heart [(myo)cardiocytes] (Gill et al., 1999), thymus (thymocytes) (Storto et al., 2000a), and embryonic stem (ES) cells (Melchiorri et al., 2007). It is of particular interest to understand the implication of mGluRs in the physiology of ES cells, since the presence of cancer stem cells in different tumor types including melanomas has been proposed recently by several investigators (Fang et al., 2005; Grichnik et al., 2006; Klein et al., 2007; Schatton et al., 2008). An expression profile of mGluRs in non-neuronal cells is summarized in Table 1.

Implication of mGluRs in cancers

The previous findings elucidated by many researchers imply that the physiological functions of mGluRs are more complex than expected. Recent studies unequivocally support the notion that mGluRs are not only involved in the maintenance and normal regulation of homeostasis of the body, but also participate in the progression of a variety of neoplasms or malignancies in humans (Table 2). Since mGluRs are highly expressed in the CNS, first speculations of these receptors in human malignancies were in neuronal tumors such as neuroblastoma (Naarala et al., 1993), medulloblastoma (Iacovelli et al., 2006), and glioma (Albasanz et al., 1997;Arcella et al., 2005;Aronica et al., 2003;D'Onofrio et al., 2003;Shinno et al., 1994). Interestingly, among these neuronal tumors, glioma cells releasing an excess of glutamate, which is the natural ligand of mGluRs, showed more aggressive growth than parental glioma cells (Takano et al., 2001). In addition, glutamate antagonists inhibited the growth of human tumor cells including neuroblastoma, rhabdomyosarcoma, brain astrocytoma, thyroid carcinoma, lung carcinoma, colon adenocarcinoma, and breast carcinoma, whereas no effect was observed in either normal human fibroblast or bone marrow stromal cells (Rzeski et al., 2001). The notion that mGluRs may play a critical role in neoplasms of non-neuronal tissues was first noted by our laboratory in 2003, where a transgenic mouse line with mGluR1 expression targeted to melanocytes showed predisposition to melanoma (Pollock et al., 2003).

Since then several other types of malignancies involving mGluRs were also reported. For example, Chang and colleagues demonstrated mGluR4 expression in more than 40 % of colorectal adenocarcinomas, malignant melanomas, laryngeal squamous cell carcinomas, and breast carcinomas tested. In addition, colorectal carcinoma patients displaying mGluR4 overexpression had a lower survival rate than those without mGluR4 expression. However, expression levels of mGluR4 were not correlated with the progression of colorectal cancers (Chang et al., 2005). Expression of mGluR5 was also detected in MG-63 osteosarcoma cells, where dexamethasone upregulated the expression of mGluR5, suggesting a functional glutamatergic system in bone pathophysiology (Kalariti et al., 2007). Park and coworkers also demonstrated that mGluR5 is overexpressed in oral squamous cell carcinoma. Moreover, patients with elevated levels of mGluR5 expression showed a decreased survival rate relative to those who were mGluR5 negative (Park et al., 2007). Although mGluRs 1 and 5 belong to Group 1, their downstream transduction signaling pathways are likely to be different.

Furthermore, the involvement of mGluRs 1 and 5 in the progression of human cancers may differ by type of neoplasm.

Transgenic mouse models and mGluR1 in melanocyte transformation

As transgenic techniques have advanced, genetically modified animal models have become valuable and powerful tools in understanding the progression of many different types of human disease. Unlike spontaneous melanoma formation in humans, rodents rarely develop melanoma. In the past 20 years, several transgenic murine melanoma models have been developed. Bradle and coworkers have reported that expression of a transgene (SV40 early region) under the control of the melanocyte-specific tyrosinase promoter demonstrated ocular melanoma with metastatic competence with a broad range of latency from 4 to 11 weeks. Several progenies also showed cutaneous melanoma with metastasis, however, the source of the metastasis was not clear (Bradl et al., 1991). Chin and colleagues have developed several transgenic mouse lines to investigate function of $p16^{INK4A}$ in melanomagenesis. Mice that were deficient for $p16^{INK4A}$ did not develop melanoma. However, when activating mutant of H-RAS (G12V) under the control of tyrosinase promoter was introduced into mice with INK4A-deficient background, they developed melanoma with the average latency of 5.5 months without evidence of metastasis (Chin et al., 1997). Powell and coworkers also have reported transgenic mouse lines that developed melanoma. These mice were established by the introduction of mutated Ha-RAS (TPras), topical applications of 7, 12-dimethylbenz- [a] - anthracene (DMBA), 12-O-tetradecanoylphorbol-13-acetate (TPA), and UV radiation, which resulted in cutaneous melanoma with low incidence. In most cases, the resulting tumors were a mixed population of papillomas and carcinomas, in addition to melanoma (Broome Powell et al., 1999). Taken together, most murine models required carcinogenic stimuli, UV exposure, or a combination of several oncogenes, to promote melanoma development with low penetrance, long latencies, and limited metastatic potential.

Our laboratory has developed a transgenic mouse model (TG-3) that is predisposed to spontaneous melanoma development. Onset of melanoma in TG-3 occurs with 100% penetrance, short latency and high metastatic potential. Offspring of TG-3 developed externally visible melanocytic lesions in the perianal region, pinnae of the ear, eyes, snout, legs, tail, and skin within 2–4 weeks of birth. With the onset of melanocytic lesions, these mice showed metastasis first to the lymph nodes, and progress to other organs includes lung, muscle, bone, brain, liver, spleen, and skull. There were no other types of cancer seen in these mice with the progression of melanoma (Chen et al., 1996; Zhu et al., 2000; Zhu et al., 1998). Spontaneous development of melanoma and tumor progression in TG-3 is similar to that reported in humans, in several aspects. For example, most human melanomas also develop in a stepwise fashion, beginning with the transformation of normal melanocytes, and progress to benign nevi, atypical nevi, primary melanoma in situ, invasive primary melanoma, and metastatic melanoma (Clark, 1991). In TG-3, it was shown that insertional mutagenesis by a transgene resulted in the appearance of melanoma (Chen et al., 1989; Chen et al., 1996). Extensive molecular studies revealed that the transgene had integrated into intron 3 of metabotropic glutamate receptor 1 (Grm1) resulting in ectopic expression of mGluR1 in melanocytes and leading to tumor development (Pollock et al., 2003). In order to distinguish whether the aberrant expression of mGluR1 in tumors was a cause or a consequence of melanoma, a new transgenic line (E line) was constructed by targeting expression of Grm1 cDNA to melanocytes under the regulation of a melanocyte-specific promoter, dopachrome tautomerase (DCT). E line developed melanoma with similar onset and progression as observed for TG-3. Taken together, results from these studies demonstrated that aberrant expression of mGluR1 in melanocytes is sufficient to induce melanoma development *in vivo* (Pollock et al., 2003). Furthermore, we verified that mGluR5 does not contribute to melanomagenesis in our system, since progenies from TG-3 crossed to produce mice with a Grm5 homozygous null background developed

melanoma in a manner indistinguishable from TG-3 (Marin et al., 2005). These results again showed that ectopically expressed mGluR1 is involved in moribundity in the absence of functional mGluR5.

Oncogenic potential of mGluR1 in melanocyte transformation: In vitro and allograft studies

In previous studies by others, transfection of Grm1 cDNA into heterologous systems such as human embryonic kidney 293 (HEK293) cells (Abe et al., 2003; Mundell et al., 2001; Pula et al., 2004), routinely had been used only as a tool to study receptor function. We, however, assessed the transforming ability of a full-length mGluR1 cDNA that had been previously cloned from a mouse brain cDNA library (Zhu et al., 1999), by transfection into normal melanocytes.

Expression levels of mGluR1 varied in these melanocytic clones suggesting mGluR1 expression is not toxic and does not impair cellular homeostasis. The functionality of mGluR1 in these stable mGluR1-melanocytic clones was assessed by the measurement of several features including the accumulation of the second messenger, inositol-1, 4, 5-triphosphate (IP3), and activation of ERK using a mGluR1 agonist, L-quisqualate. One distinct feature of these mGluR1 clones is that they do not require 12-O-tetradecanoylphorbol-13-acetate (TPA) supplement for proliferation. Although several growth factors are capable of compensating for the need of TPA, normal melanocytes require the presence of TPA for their optimal growth, otherwise they enter growth arrest phase (Bennett et al., 1987; Wellbrock et al., 2004). Thus, independence from TPA supplementation for these mGluR1-melanocytic clones represented an initial step towards a transformed phenotype.

More striking features of these mGluR1 clones were observed when allografted into both immunodeficient nude (Figure 1A) and immunocompetent syngeneic mice (Figure 1B). Allografts of mGluR1 clones formed robust tumors with a very short latency of 5 days, suggesting exogenously introduced mGluR1 led normal melanocytes to fully transformed ones. The malignancy of these cells was verified by the formation of new blood vessels (angiogenesis) (Figure 2A) and their invasion to muscle and intestine (Figure 2B). These observations are consistent with the earlier result where tumor-bearing TG-3 mice showed metastases to the muscle at stage 3 (14 %) and stage 4 (55 %) (Zhu et al., 1998). Clinically, the intestinal metastases are very rare in other cancers; however, previous reports by others demonstrated that tumors from transformed human melanocytes induced with SV40ER, hTERT, and oncogenic RAS (G12V) showed metastasis to the intestine (12 %) (Gupta et al., 2005). The malignancy of mGluR1 clones is also demonstrated by the formation of tumors in syngeneic C57/BL6 mice suggesting that the intact immune systems in these mice did not impair the malignancy of mGluR1-induced tumorigenesis.

Although it is not clear how mGluR1 mediates melanocyte transformation and what cellular targets are activated by mGluR1, we speculate several possible candidates conferring advantages to melanoma cells. It has been reported by others that transfection of oncogenic E1a or myc into normal melanocytes, B10.BR, resulted in tumor formation in immunodeficient nude mice but not in immunocompetent syngeneic mice. In contrast, oncogenic H-RAS-B10.BR transformants resulted in tumor formation in both nude and syngeneic mice (Dotto et al., 1989). These results suggest that mGluR1-induced tumorigenesis is likely to be different from that of E1a or myc since mGluR1 led to tumor formation in both nude and syngeneic mice. Possible implication of effectors common with oncogenic RAS being activated by stimulation of mGluR1 in mGluR1-mediated transformation, is under investigation in our laboratory. Taken together, our results unequivocally demonstrate that mGluR1 has a crucial

function in melanocyte transformation and confers advantages to promote melanocyte growth leading to tumor formation.

Oncogenic potential of mGluR1 in transformation of epithelial cells: carcinoma

Having demonstrated the etiologic role of mGluR1 in melanomagenesis (Marin et al., 2005; Namkoong et al., 2007; Pollock et al., 2003; Shin et al., In Press) and having referenced the continuing studies which observe natural expression of various components of the glutamatergic system in a number of non-neuronal tissues and in a subset of cancers (Kalariti et al., 2005), we hypothesize that the ectopic expression of mGluRs may be involved in the pathogenesis of tumors other than melanoma.

Melanocytes and neurons may share developmental origins from the embryonic neural crest, but the intracellular signaling pathways normally activated by these receptors are widely distributed among diverse cell types. Exogenous mGluRs can connect to endogenous signal transduction apparatus. In heterologous experimental systems involving ectopic expression to study synaptic function, mGluRs including mGluR1 have been shown to exhibit promiscuous coupling to other pathways (Sharon et al., 1997). mGluR activation can be indirectly coupled to glutamate release, forming a autocrine-like loop (Hoogduijn et al., 2006; Shin et al., In Press). Not only can the concentration of free glutamate outside of the tightly regulated microenvironment of the synapse be orders of magnitude higher (Meldrum, 2000), but many GPCRs including the glutamate receptors display constitutive activity even without the necessity for endogenous agonists, in both native and heterologous systems (Milligan, 2003). It is thus proposed that activity of an otherwise normal glutamate receptor in an ectopic cellular environment actuates signaling pathways which dysregulate cell growth and ultimately leads to tumor formation. As most human cancers are of epithelial origin (carcinomas), we now wish to consider whether mGluR1 can transform epithelial cells in order to develop a model system for further investigation.

The laboratory mouse having advanced as the central experimental device to model human disease, a useful system designed to facilitate the genetic dissection of molecular mechanisms regulating tumorigenesis was developed in the laboratory of Dr. E. White (Degenhardt and White, 2006). Primary mouse epithelial cells underwent a defined immortalization allowing the retention of normal epithelial characteristics, including a lack of innate tumorigenicity. The resulting system facilitates in vivo screening for genetic or epigenetic events that enable tumor growth.

In preliminary work undertaken in our laboratory, these immortalized baby mouse kidney (BMK) epithelial cells were transfected and selected for stable integration of a Grm1 expression vector, and analyzed for mGluR1 protein. Clonal mGluR1-expressing lines have been tested for functional signaling and specificity of the ectopic glutamate receptor by both the accumulation of second messenger and activation of MAPK following challenge with mGluR1-agonist/antagonist. Initial observations indicate some clones grow with a reduced doubling time, relative to vector and parental controls. These cells will further be examined in vitro for changes in growth properties, and in vivo as an ultimate measure of the potential for tumorigenicity by allograft into immunodeficient or syngeneic mice.

Conclusions/Discussions

L-glutamate has not only evolved early multiple functions in nutrition, energy, metabolism, and structure, but also is a phylogenetically conserved cell signaling molecule (Young and Ajami, 2000). In fact the extracellular ligand-binding domain of the metabotropic receptor has

been shown to be related to bacterial periplasmic binding proteins (O'Hara et al., 1993). Signaling roles of glutamate have long been studied in animals, but more recently have been expanded to the plant kingdom (Forde and Lea, 2007), and even prokaryotes. Although in higher mammals acknowledgement of the glutamatergic system usually has been restricted to normal functions in the CNS and associated neuropathologies, it is likely that dysregulated function of this system can lead to diverse disease types in humans, including cancer. One fundamental goal of developing better mouse models of human cancers is to facilitate oncology drug development. Such small animal models are considered to produce initial data of the highest quality in target identification and validation, in evaluation of efficacy, toxicity, and delivery of a therapeutic. It is noteworthy that as members of the superfamily of GPCRs, which by experience are considered highly "druggable" the metabotropic glutamate receptors may merit a higher priority for validation as therapeutic targets (Martino and Chen, 2005).

Several years ago it was reported that mGluR2/3 antagonists can reduce the proliferation of cultured human glioma cells (D'Onofrio et al., 2003). Activation of mGluR4 receptors was shown to inhibit the growth of medulloblastomas (Iacovelli et al., 2006). In a recent publication, our laboratory demonstrated that exogenous Grm1 cDNA introduced into murine melanocytes resulted in malignant tumor formation in both syngeneic and immunodeficient mice (Shin et al., In Press). In addition, we verified suppression of in vitro human melanoma cell proliferation and in vivo allograft tumor growth by both mGluR1-specific antagonists and Riluzole, an FDA approved drug which inhibits glutamate release (Namkoong et al., 2007). Continued studies of the glutamatergic system in cellular transformation are a necessity, and pharmacological modulation of metabotropic glutamate receptors holds promise for future therapies.

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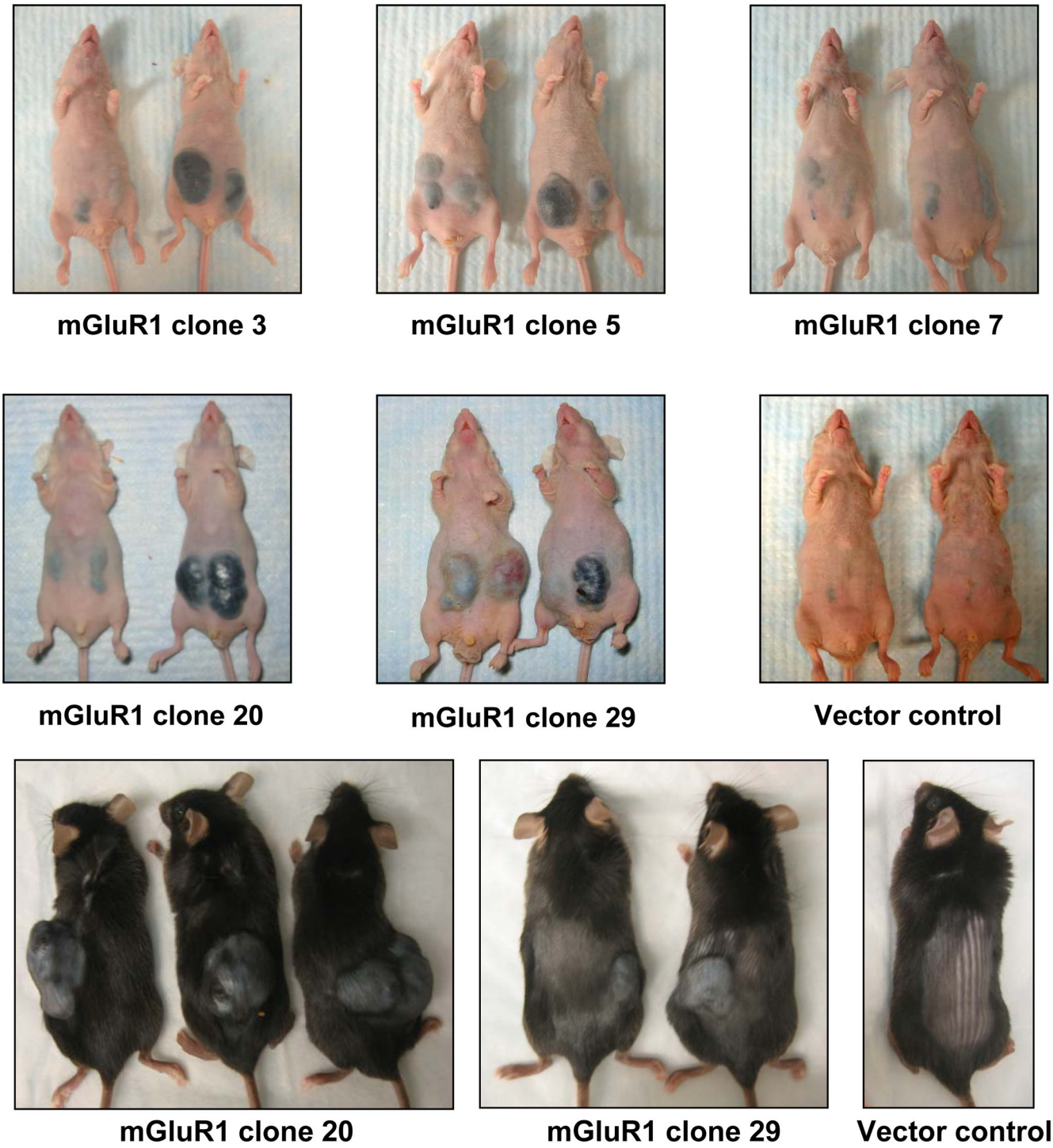
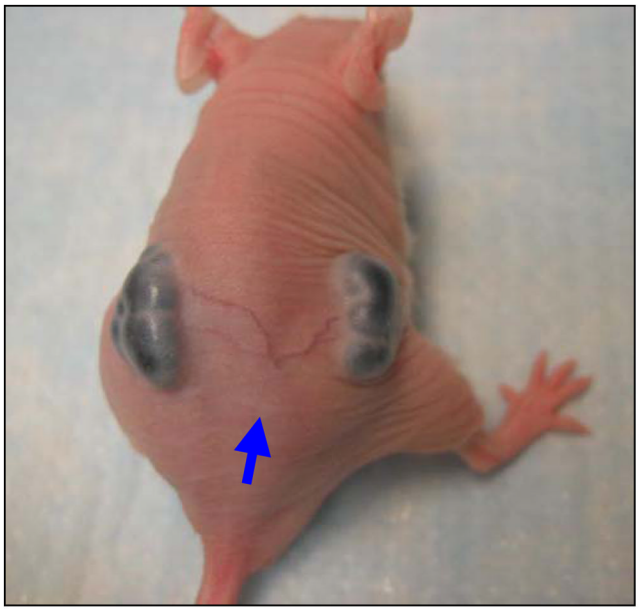


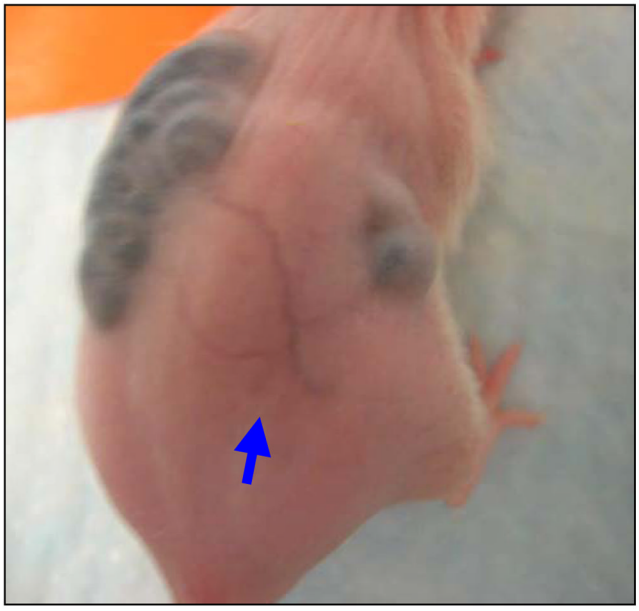
Figure 1.

Spontaneously immortalized melanocytes, melan-a, were transfected with empty vector or Grm1 cDNA. Stable clones expressing mGluR1 were selected with neomycin. Western immunoblots showed various levels of mGluR1 expression among these clones at ~150kD. Allografts of these mGluR1 stable clones into both immunodeficient nude **(A)** and immunocompetent syngeneic **(B)** mice showed aggressive tumor formation. 10^6 cells of each

mGluR1 clone and vector control were inoculated subcutaneously. Visible lesions of mGluR1 stable clones in nude mice were detected approximately after 3–5 days of inoculation and the size of tumors reached 600 mm^3 after 15 days. In contrast, vector control clones formed no tumor even after 4 weeks of inoculation. All mGluR1 allografted mice were sacrificed after 4 weeks due to the tumor burden.



mGluR1 clone 20



mGluR1 clone 29

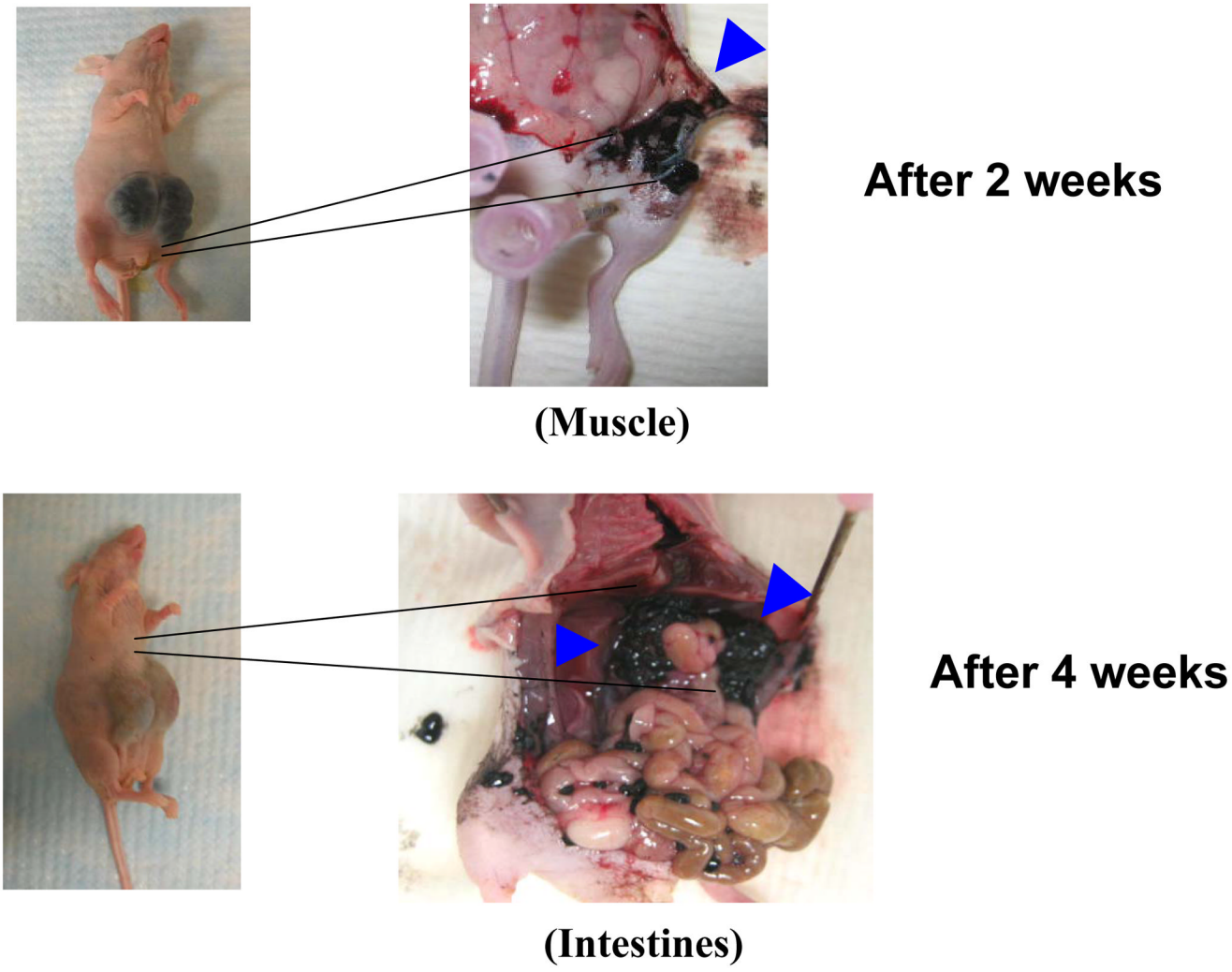


Figure 2.

mGluR1 stable clones showed malignant phenotype in nude mice. **(A)** Formation of new blood vessels (angiogenesis). mGluR1 stable clones showed strong angiogenic activities. An example of angiogenesis from mGluR1 clone 20 and 29 tumors is shown. **(B)** Invasion of MASS clones was detected in muscle (top picture) and intestine (bottom picture) after 2 weeks and 4 weeks, respectively.

Table 1

Expression of metabotropic glutamate receptors in selected non-neuronal cells

Cell type	Group I		Group II		Group III				Reference
	mGlu1	mGlu5	mGlu2	mGlu3	mGlu4	mGlu6	mGlu7	mGlu8	
Melanocytes		O							Frati et al. 2000
Keratinocytes	O		O	O					Genever et al. 1999
Osteoclasts								O	Morimoto et al., 2006
Pancreatic islets/βcells		O						O	Storto et al., 2006; Tong et al., 2002
Hepatocytes		O							Storto et al., 2000b
Myocytes	O	O	O	O					Gill et al., 1999
Thymocytes	O	O	O	O					Storto et al., 2000a
Embryonic stem cells		O			O				Melchiorri et al. 2007

Table 2

Implication of metabotropic glutamate receptors in cancers

	Subclass	Cancer types	Reference
Group I	mGluR1	Melanoma	Pollock et al., 2003
	mGluR5	Glioma	Aronica et al., 2003; Albasanz et al., 1997; Shinno et al., 1994
		Osteosarcoma	Kalariti et al., 2007
		Oral squamous cell carcinoma	Park et al., 2007
Group II	mGluR2	Glioma	D'Onofrio et al., 2003
	mGluR3	Glioma	D'Onofrio et al., 2003; Aronica et al., 2003
Group III	mGluR4	Colorectal adenocarcinoma	Chang et al., 2005
		Malignant melanoma	Chang et al., 2005
		Squamous cell carcinoma	Chang et al., 2005
		Breast carcinoma	Chang et al., 2005
		Medulloblastoma	Iacovelli et al., 2006
	mGluR6	?	
	mGluR7	?	
	mGluR8	?	