CONTINUOUS EXPRESSION OF METABOTROPIC GLUTAMATE RECEPTOR 1 IS REQUIRED FOR MAINTENANCE OF TUMORIGENESIS IN GRM1 TRANSFORMED MOUSE KIDNEY EPITHELIAL CELLS

By

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A thesis submitted to the Graduate School-New Brunswick

Rutgers, The State University of New Jersey

and

The Graduate School of Biomedical Sciences

University of Medicine and Dentistry of New Jersey

In partial fulfillment of the requirements

For the degree of

Master of Science

Graduate Program in Microbiology and Molecular Genetics

Written under the direction of

Dr. Suzie Chen

And approved by

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New Brunswick, New Jersey

May 2009

ABSTRACT OF THE THESIS

Continuous Expression of Metabotropic Glutamate Receptor 1 is Required for Maintenance of Tumorigenesis in Grm1 Transformed Mouse Kidney Epithelial Cells

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Within the United States, skin cancer has become a predominant form of cancer that occurs across all age groups, racial backgrounds, and to both genders equally. Melanoma arises from malignant melanocytes that reside within the epidermis of the skin. It is the most serious form of skin cancer, causing the most mortality among all skin cancers. Currently, due to the limited treatment options available, patients have severely reduced survival rates as the disease advances toward Stage IV. Advancements in understanding of the mechanisms of melanoma development and novel targets for treatment are in great need for patients inflicted with this devastating disease.

Metabotropic glutamate receptors are normally expressed within the central nervous system (CNS) where they use glutamate, the most abundant neural transmitter, as their natural ligand. Within the CNS, these receptors have important diversified functions ranging from mediation of slow excitatory and inhibitory responses to memory formation and neuronal development. Our lab has identified the etiological role of

Metabotropic Glutamate Receptor 1 (Grm1) in melanoma development in transgene mouse models. Subsequently, our lab also detected GRM1 expression in approximately 40% of human melanomas biopsy and cell line samples. These results led us to investigate the role of GRM1 in other types of cancer.

Epithelial cancers are the abundant category of cancers to inflict humans, relating GRM1 and its possible role in solid tumors could introduce a novel target for developing cancer therapies. Since continuous expression of Grm1 was demonstrated to be required for the maintenance of the tumorigenic phenotype in Grm1-mouse melanocytes, my project is to determine whether this is true for an epithelial cell system. With the use of Grm1-transformed mouse kidney epithelial cells, an inducible tetracycline siRNA system was introduced into the cells and tested with/without the inducer, doxycycline, for the suppression of Grm1 expression levels *in vitro* and *in vivo*. The *in vivo* data demonstrated decreased tumor volumes in the doxycycline treatment groups, validating the decreased expression of Grm1 seen *in vitro,* compared to the no treatment groups. These results provide evidence that Grm1 expression is necessary to maintain the tumorigenic phenotype of Grm1 transformed cells.

Acknowledgements:

I would first like to thank my family and friends who provided me with support, encouragement, and advice. Thank you for being understanding and helping me through all the hard and trying times.

I especially want to thank Dr. Suzie Chen for allowing me to participate within her lab. I appreciate all the advice, guidance, support, and lessons that she has taught me to develop into a better scientist.

I would also like to thank all the members of the Chen laboratory for their support and sense of humor, for without them, life in the laboratory would not have been as enjoyable. I especially want to thank Jeffrey Martino for all the guidance and support he provided throughout this project.

I also want to thank Dr. Shridar Ganesan and Dr. David Axelrod for both their classroom lessons that have greatly influenced my scientific development and participating as my committee members.

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Introduction

Skin cancer is now the most common form of cancer in the United States, with more new cases being diagnosed than breast, prostate, lung and colon cancers combined (Skin Cancer Foundation, 2008). Melanoma accounts for less than 5% of all skin cancers but it causes the most fatalities, 75% of skin cancer deaths. In 2008, the American Cancer Society estimated that in the United States, about 63,000 melanomas will be diagnosed (American Cancer Society, 2008; Skin Cancer Foundation, 2008). Of these new diagnosed melanomas, about 8,500 patients will die within the year. The incidence of melanoma is the same for both males and females, 1 in 55. Cases of melanoma are found in all age groups however it is frequently found in older groups with the average age being 59 (National Cancer Institute, 2008). Among racial groups, melanoma is more common among Caucasians (2%) than for any other ethnic group (0.1% African Americans and 0.5% Hispanics) (American Cancer Society, 2008). Despite this, incidences of melanoma that do occur in non-Caucasian ethnic groups such as African Americans have an increased rate of mortality, with a overall survival rate of 77% compared to over 90% in Caucasians (Skin Cancer Foundation, 2008).

The survival rate for patients varies depending on the stage of disease at time of diagnosis. For melanomas that are detected early, that have not penetrated through the epidermis, the 5 year survival rate is 99%. If the disease is more advanced such as Stage IV when the melanoma has spread from the skin and lymph nodes to other organs, the 5 year survival rate is only 18% (American Cancer Society, 2008). Prognosis and thereby survival rates are determined by the thickness and the presence of ulceration of the primary lesion. Ren et al., states that there is growing evidence of a strong correlation

between the metastatic capacity of the lesion and its thickness (Ren et al., 2008). Overall, once a patient has developed metastasis their survival rate is greatly diminished, with 6 of every 7 skin cancer deaths being related to metastatic melanoma (Ren et al., 2008). Progression of the disease goes from a radial phase to a vertical phase with an unknown switch changing the direction of growth. Generally, the vertical growth of the primary lesion results in metastasis to the draining lymph nodes and then commonly to the lung, liver, and brain (Rodolfo et al., 2004). Currently, the treatment is surgical removal, if possible combined with adjuvent chemotherapy (depending on the diagnosed stage). However, current available therapies are not effective for the most advanced stages of melanoma thereby decreasing the survival rates of the patients (American Cancer Society, 2008).

Melanocytes are skin cells that synthesize melanin, a protective brown pigment that tans the skin and protects the deeper layers of the skin from the sun. Melanoma is a type of skin cancer that arises from melanocytes that have lost control of their growth within the epidermis or top layer of the skin (American Cancer Society, 2008). Malignant lesions can occur on several areas of the body. Typical sites include cutaneous, leptomeningeal, ocular, and mucosal areas. The majority of all cases are of cutaneous origin (Fescher et al., 2007). There are four subtypes of cutaneous melanoma: superficial spreading melanomas, lentigo maligna, acral lentiginous melanoma, and nodular melanoma (Fescher et al., 2007; Skin Cancer Foundation, 2008). Superficial spreading melanoma is the most common and accounts for 70% of all melanomas. It remains within the epidermis for an extended period of time before penetrating deeper into the skin. Superficial spreading melanoma can occur from a previously benign mole

and can be found anywhere on the body (Skin Cancer Foundation, 2008). Like superficial spreading melanoma, lentigo maligna and acral lentiginous melanomas could remain in the epidermis for a period of time before spreading further. The differences among these three subtypes are the location of the malignancy and the ethnic or age group in which it affects (Skin Cancer Foundation, 2008). Lentigo maligna appears commonly among the elderly where it occurs mostly on the face, ears, arms, and upper trunk. Acral lentiginous melanoma stands out by appearing as black or brown discoloration on the soles of the feet, palms, or under the nails. This subtype is mostly found in African Americans and Asians. The fourth subtype is nodular melanoma which is invasive when first diagnosed and appears as a bump that can be black, blue gray, white, brown, tan, red, or skin tone. (Skin Cancer Foundation, 2008). Development of any of the four cutaneous subtypes can occur through familial inheritance or through exposure of risk factors such as ultraviolet radiation exposure (American Cancer Society, 2008; Skin Cancer Foundation, 2008). Regardless of the different melanomas that patients have, there are common mutations that are observed within these malignancies.

Like most malignancies, there is not a single mutation that causes transformation from a normal cell to a malignant one. Transformation is suspected to occur after multiple mutations take place in genes affecting cell proliferation, apoptosis, survival, and invasion. For melanomas, there is evidence that indicates the existence of different subtypes of melanomas that differ in their genetic mutations and thus their clinical behavior (Rodolfo et al., 2004). In particular, genetic testing has shown that the MAP kinase pathway is constitutively activated due to various mutations in most melanomas (Davies et al., 2002; Satyamoorthy et al., 2003; Huntington et al., 2004). The Mitogen-

Activated Protein (MAP) kinase pathway is involved in the regulation of cell proliferation, survival, and invasion (Mansour et al., 1994; Curtin et al., 2005; Fecher et al., 2007). Activation of this pathway has been proposed to be an early and frequent event in melanomas (Cohen et al., 2002; Satyamoorthy et al., 2003).

The MAP kinase pathway involves a large number of components that together influence cellular proliferation, survival, and invasion. There are two common constituents that are mutated in melanomas, BRAF and RAS. RAS consists of multiple isoforms such as K-RAS and N-RAS and virtually all the mutations that appear in melanomas are with N-RAS (Rodolfo et al., 2004; Eskandarpour et al., 2005; Fecher et al., 2007). The most frequent mutation in N-RAS is in amino acid 61 and only occurs in 15 percent of cutaneous melanomas (Ball et al., 1994; Van Elsas et al., 1996). This mutation does not have any correlation with the amount of exposure to ultraviolet radiation or frequency of occurrence in a particular body site (Fecher et al., 2007). BRAF acts downstream from the RAS in the MAP kinase pathway and is frequently mutated on amino acid 600 (V600E) of the gene (Davies et al., 2002; Pollock and Meltzer, 2002; Rodolfo et al., 2004). Typically, both N-RAS and BRAF mutations are not present together in malignant melanomas. However, BRAF V600E is found in approximately 66 percent of malignant melanomas and 82 percent of benign nevi (Davies et al., 2002; Pollock et al., 2003b). The presence of this mutation appearing in benign nevi indicates that its activation is not sufficient for malignant transformation (Pollock et al., 2003b). Although mutations in the MAP kinase pathway are common in melanomas there are other mutations and disruptions that occur and impact the malignant state.

Cellular proliferation is important in the life of the cell and is essential for malignancies to maintain growth. In normal cells, proliferation is controlled in part by cyclin-dependent kinases that are regulated by cyclin-dependent kinase inhibitors such as p16INK4a (CDKN2A) and p21 (Fecher et al., 2007). CDKN2A inhibits the cell cycle by binding to the cyclin D/CDK4 complex. In familial melanomas this protein is mutated in 10 to 40 percent of all cases and does not function properly thereby allowing uncontrolled cellular proliferation. However in sporadic cases, mutations of CDKN2A are not common but affects 75% of melanomas are affected through silencing. CDKN2A is silenced in sporadic melanomas by hypermethylation (Fecher et al., 2007; Ren et al., 2008). Another critical protein that controls cellular proliferation is p53. In most cancers p53 is mutated but in melanomas, this is not the case. The frequency of mutation of p53 is less than 25% of melanomas (Fecher et al., 2007). In population studies, occurrence of mutated p53 is found to associate with freckling tendency and nevus density. Patients with normal p53 had melanomas that are associated with their inability to tan, melanomas on sun-exposed skin, and histories of other skin cancers (Rodolfo et al., 2004). In 95% of melanomas there are two commonalities, chromosome loss and gain. Melanomas acquire losses on chromosomes 1, 6, 9, and 10 and gains on chromosome 6, 7, and 8 (Rodolfo et al., 2004). Due to the aggressive nature of melanoma and the lack of effective therapies, many researchers have been studying the genetic nature of melanoma and are searching for other possible cellular components that affect tumorigenesis.

G-protein-coupled receptors (GPCRs) are seven-transmembrane α-helices that span the cellular membrane and regulate physiological functions of the cell. Generally, all GPCRs are expressed in proliferating cells and become tumorigenic either through

mutations causing continuous activation without the presence of a ligand or when exposed to excess amounts of ligands or agonists (Gutkind et al., 1991; Julius et al., 1989). The roles of G -protein coupled receptors are to regulate various functions within the cell and when the expression and/or functions of these receptors are altered due to mutations or disregulation, this impairs the cellular homeostasis resulting in cell transformation and tumor formation (Allen et al., 1991; Pollock et al., 2003). In melanoma, members of GPCR called metabotropic glutamate receptors are gaining recognition for its potential role in tumorigenesis.

Glutamate receptors are commonly expressed and function within the CNS using L-glutamate, the predominant excitatory neurotransmitter, as their ligand (Pin and Duvoisin, 1995; Dhami and Ferguson, 2006). Glutamate acts upon two classes of glutamate receptors; ionotropic and metabotropic receptors. Ionotropic glutamate receptors are multimeric cation-specific ligand gated ion channels that function by fast synaptic transmission (Nakanishi 1992; Ferraguti et al., 2006; Pellicciari et al., 1999). Ionotropic ligand-gated channels include N-methyl-D-asparate (NMDA), α-amino-3-hydroxy-5-mehtyl-4-isoxazole proprionic acid (AMPA) and kainic acid subclasses of receptors (Nakanishi 1992; Pin and Duvoisin, 1995; Pellicciari et al., 1999; Dhami and Ferguson, 2006). Metabotropic glutamate receptors (mGluRs) are GPCRs that function by slow transmission through intracellular second messengers, IP_3/DAG or cAMP depending on the cell type (Nakanishi et al., 1998). There are eight metabotropic glutamate receptors which are divided into three groups based on their amino acid sequences, intracellular coupling mechanisms, and related pharmacology (Nakanishi 1992; Nicoletti et al., 2007). Originally, mGluRs were thought to only be found in the

central nervous system however studies over the last decade have found these receptors to be present in various non-neuronal tissues as shown in Table 1 (Shin et al., 2008a; Shin et al., 2008b). There is increasing evidence that metabotropic glutamate receptors are involved in non-neuronal cellular proliferation, migration, and differentiation (Shin et al., 2008a; Shin et al., 2008b; Nicoletti et al., 2007). Within the CNS, mGluRs have multiple functions that impact neurological performance which include mediating slow excitatory and inhibitory responses, induction of long-term potentiation and long term depression, forming memory, and regulating neuronal development (Linden et al., 1991; Glaum and Miller 1992; McCormick and von Krosigk 1992; Nakanishi 1992; Bortolotto and Collingridge 1993; Aiba et al., 1994; O'Connor et al., 1995; Fiorillo and Williams 1998; Packard et al., 2001; Minura et al., 2002; Otani et al., 2002; Wu et al., 2004). Since mGluRs were first discovered in the central nervous system, there have been examples of neuronal tumors such as neuroblastomas and gliomas, which were found to have overexpression of mGluRs (Albansanz et al., 1997; Arcella et al., 2005; Aronica et al., 2003; D'Onofrio et al., 2003; Naarela et al., 1993; Shin et al., 2008b; Shinno et al., 1994). Further studies by others suggest several mGluRs could be involved in malignancy by being aberrantly expressed in cancers of various tissues (Albasanz et al., 2003; Aroncia et al., 2003; Chang et al., 2005; D'Onofrio et al., 2003; Iancovelli et al., 2006; Kalariti et al., 2007; Park et al., 2007; Shin et al., 2008b; Shinno et al., 1994; Pollock et al., 2003).

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/β-cells		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 1. Expression of metabotropic glutamate receptors in selected non-neuronal cells (Shin et al., 2008b).

Implication of mGlus in cancers

	Subclass	Cancer types	Reference
Group I	mGlu1	Melanoma	Pollock et al., 2003
	mGlu5	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	mGlu2	Glioma	D'Onofrio et al., 2003
	mGlu3	Glioma	D'Onofrio et al., 2003; Aronica et al., 2003
Group III	mGlu4	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
	mGlu6	?	
	mGlu7	?	
	mGlu8	?	

Table 2. **Implication of mGlus in cancers** (Shin et al., 2008b).

Expression of mGluRs in neuronal cells is important for normal neuronal function. Mouse models with deletion of the Group 1 mGluRs (mGlu1 or GRM1 and mGlu5 or GRM5) have been described; these mice have reduced long-term potentiation, mild ataxia, and impaired context specific associative learning (Aiba et al., 1994; Conquet et al., 1994; Lu et al., 1997). From these studies, the normal functions of mGluR were identified. It is known that GRM5 is normally expressed in normal melanocytes. Marin et al., used transgenic melanoma-prone mice and cell lines with knockouts of Grm5 but maintained Grm1 to demonstrate that Grm5 is not required for the development of melanoma but Grm1 is (Marin et al., 2005).

Namkoong et al., examined over 120 melanoma biopsies and 24 human melanoma cell lines and found approximately 40 percent of these samples expressed GRM1 at both the RNA and protein levels (Namkoong et al., 2007). Lee et al.,

investigated GRM1 expression in a tissue array consisting of quadruplicate cores made from 38 melanomas and 15 normal samples. Of these samples, 39.5 percent of the melanoma samples expressed GRM1 whereas none of the normal samples showed GRM1 expression (Lee et al., 2008). These studies suggest that GRM1 may be involved in some human melanomas.

Metabotropic glutamate receptors respond to its natural ligand, L-glutamate or agonist and activate the downstream pathways through second messengers. The receptor can be activated through either mutation or excess of ligand presence (Allen et al., 1991; Gutkind et al., 1991; Julius et al., 1989; Pollock et al., 2003). Marin et al., used mouse melanoma cell lines without N-Ras/BRAF mutation and observed that the MAP kinase cascade was activated as indicated by the phosphorylation of extracellular signal-regulated kinase (ERK) with agonist stimulated Grm1, in addition the PKCε isoform was found to be critical in mediating this activity. This further suggests that signaling mediated via Grm1 has an effect on MAP kinase activation (Marin et al., 2006). Namkoong et al., demonstrated that most human melanoma cell lines released elevated levels of glutamate and activated GRM1 possibly through an autocrine loop (Namkoong et al., 2007). Glutamate's role in cellular proliferation on human melanoma cell lines was demonstrated when a glutamate release inhibitor (Riluzole) was administered and resulted in suppression of cellular growth. This demonstrated that glutamate as a ligand affects cellular proliferation of malignant melanocytes (Namkoong et al., 2007). Understanding the mechanisms of how GRM1 works in promoting melanocyte growth is important in identifying better designs of therapies. One of the ways for studying this disease is through animal modeling.

Currently the trend to investigate cancer disease is by using animal models since the *in vitro* conditions do not accurately reflect the *in vivo* microenvironment. The best model system in use now is the mouse model. Mice are ideal for cancer investigations due to their small size, ability to breed in captivity, and 3 year lifespan (Frese et al., 2007). In addition, the genome of the mouse is entirely sequenced which enables researchers to map abnormalities to specific genes. Mice and humans are also similar both physiologically and on molecular levels, which provide the basis for why studying disease in mice can be related to human disease (Frese et al., 2007). Researchers can develop genetically modified mice that express oncogenes or dominant-negative tumor suppressor genes by making transgenic mice (Tuveson and Jacks, 2006). These transgenic mice are then used for genetic and preclinical studies to gain an understanding of diseases. Additionally, researchers can use immunocomprised mice for xenograft or allograft studies for rapid assessment of carcinogenesis. The immune response within these animals is severely decreased or null and thus allows the transplanted cells to establish themselves where studies can be carried out recreating the complex microenvironment (Kendall et al., 2006). Despite all the benefits to using the mouse model, there are some limitations. Some of the mouse models may not develop tumors spontaneously or need environmental exposure such as radiation (Hardisty 1985; van Kranen et al., 1995). Not all of the models will accurately reflect the mode or common forms of the exact human cancer. It may take extensive amounts of time to develop a mouse model that efficiently exhibits 100% penetrance and recapitulates the human disease (Frese et al., 2007).

Our laboratory has developed a mouse model that developed melanoma spontaneously with 100% penetrance. These transgenic mice were created through pronuclear injection of a 2 kb genomic fragment; clone B, which was shown to commit transfected mouse fibroblast cells to adipogenesis (Colon-Teicher et al., 1996; Chen et al., 1996). However, one out of the 5 founder mice (TG-3) developed multiple melanomas (Chen et al., 1996). Genetic mapping identified insertion of the transgene within intron 3 of the Grm1 gene and a deletion of 70 kb of the host sequence, resulted in ectopic expression of Grm1 (Pollock et al., 2003). Another transgenic line, E line, was developed with mouse Grm1 cDNA under the regulation of a melanocyte specific promoter, Dopachrome tautomerase (Pollock et al., 2003). The E line like TG-3 mice developed melanoma, showed 100% penetrance, and validated that ectopic mGlu1 expression in Grm1 melanocytes is sufficient to induce melanoma development *in vivo*. In both transgenic lines, the expression of Grm1 was only detectable in the tumors but not in normal tissues (Pollock et al., 2003).

Ohtani et al., developed another melanoma transgenic mouse model that conditionally expresses Grm1 through a tetracycline regulatory system. The addition of doxycycline to the water of the mice repressed expression of Grm1 when administered throughout the life of the mouse (Ohtani et al., 2008). Mice that were not given the doxycycline in adulthood expressed Grm1 and developed melanoma. This finding suggests that expression of Grm1 in adult mice was sufficient to induce melanoma (Ohtani et al., 2008).

Small interfering RNA (siRNA) recently has been gaining recognition for its role in gene regulation within human diseases. SiRNA are short RNA sequences of

approximately 20 to 30 nucleotides (Castanotto et al., 2009; Jinek et al., 2009; Moazed, 2009). They are functional in both the nucleus and cytoplasm of cells and are involved in post-transcriptional gene silencing, transcriptional gene silencing, and co-transcriptional gene silencing (Castanotto et al., 2009; Moazed, 2009). Small interfering RNA are generated from double-stranded RNA precursors that are introduced into cells. Within the cell resides an endonuclease, Dicer, which processes the double-stranded RNA into shorter fragments (Chen et al., 2006b; Jinek et al., 2009; Moazed, 2009). Following the cleavage by Dicer, one strand of the double-stranded RNA duplex (guide strand) is loaded onto an Argonaute protein within the RNA-induced silencing complex (RISC). As loading is occurring, the other strand of the RNA (non-guide strand) is cleaved and ejected from the complex (Jinek et al., 2009; Moazed, 2009). The Argonaute protein will use the guide stand to bind to target RNAs with complementary sequences and slice them. Once sliced, the cleaved RNA is released and incapable to complete its function (Jinek et al., 2009). The usage of this system in cancer research allows researchers to manipulate particular oncogenes for thorough determination of their roles in tumorigenesis.

Using a siRNA system, allows further identification or validation of what genes are necessary in a given function by down regulating them through inducible systems. The inducible siRNA system is attractive because they can be modified conditionally instead of constitutive suppression (Matsukura et al., 2003). A tetracycline-inducible siRNA plasmid with a U6 promoter was shown to effectively down regulate DNA methyltransferarse I in a human colorectal cell line *in vivo* by exhibiting growth arrest in established tumors (Matsukura et al., 2003). In an *in vivo* study of metastatic melanoma,

usage of a siRNA plasmid resulted in knockdown expression of CD147, a transmembrane immunoglobulin glycoprotein, which inhibited proliferation, invasion, and metastatic activity (Chen et al., 2006). To understand the role of VEGF in melanoma, a siRNA plasmid for VEGF was evaluated *in vivo* and showed efficient downregulation that suppressed the growth of the melanoma tumor (Tao et al., 2005). Since the MAP kinase pathway is shown to be activated in melanomas, a siRNA targeting mutation in activated N-RAS was developed and tested *in vitro* with human melanoma cell lines. The siRNA suppressed N-RAS, increased apoptosis of the cells, and decreased activation of ERK within the MAP kinase pathway (Eskandarpour et al., 2005). In stable Grm1-murine melanocytic clones, transfected with a tetracycline-inducible siGrm1 system was used to determine that Grm1 is necessary for tumorigenic maintenance. *In vivo* allografts resulted in a 60% decrease in tumor volume with the siRNA targeting Grm1 (Shin et al., 2008b).

In our laboratory, Grm1 has been extensively studied in the human and mouse melanoma cell lines. Since most cancers are of epithelial origin, investigating the role of Grm1 on epithelial cells has the potential for usage in novel therapeutic developments. The purpose of this study was to determine whether expression of Grm1 is required to maintain tumor progression in the stable Grm1 epithelial clones. The epithelial cell line that was chosen for this project was baby mouse kidney epithelial cells, W2, derived from mice that are wildtype for key apoptosis regulators, Bax and Bak (Degenhardt et al., 2002). The W2 cell line was immortalized and transformed by E1A and dominant-negative p53. E1A is an oncoprotein that stimulates deregulation of the cell cycle and apoptosis and inhibits transformation when p53 is functional by inducing apoptosis

(Cuconati et al., 2002). At first, the cell line was transformed with E1A alone however; this triggered the p53 apoptosis pathway and lead to cell death. Transforming with E1A had to be accompanied with p53 dominant negative to avoid activating apoptosis (Degenhardt et al., 2002). Immortalization of the W2 cell line maintained their normal epithelial characteristics and tumorigenicity with long latency, tumor formation occurred after 3 months within mice with the injection of 10^7 cells per site (Degenhardt et al., 2006). We showed that W2 cell line did not express endogenous Grm1. The cell line was then transfected with Grm1 cDNA and several stable clones were isolated and tested for positive Grm1 expression as seen in Figure 1(Martino, J.J., unpublished data). These stable W2 Grm1 clones were then tested in allografts *in vivo* to determine the extent of their tumorigenicity. Two clones used in my project, W2g1 and W2g14 were allografted into nude mice with 10^6 cells per injection site. After a latency of approximately 8 weeks, tumors were detected, thereby suggesting these stable Grm1-W2 clones are tumorigenic. (Figure 2, Martino, J.J., unpublished data).

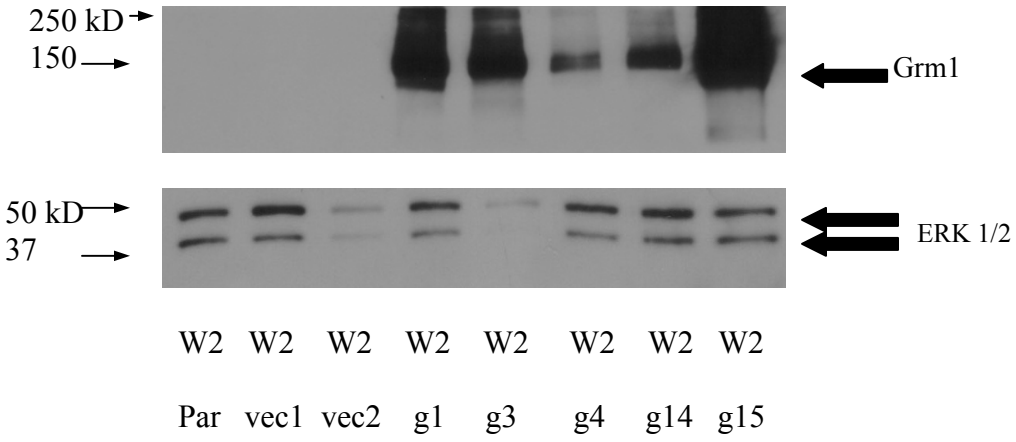


Figure 1. Western immunoblot of Grm1 expression in the parental cells of W2 Baby Mouse Kidney epithelial cells, two different vector controls clones (W2 vec1 and W2

vec2), and several stable Grm1 W2 clones. Total ERK 1/2 was used as loading controls and Mouse Grm1 Antibody. (Martino, J.J., unpublished data).

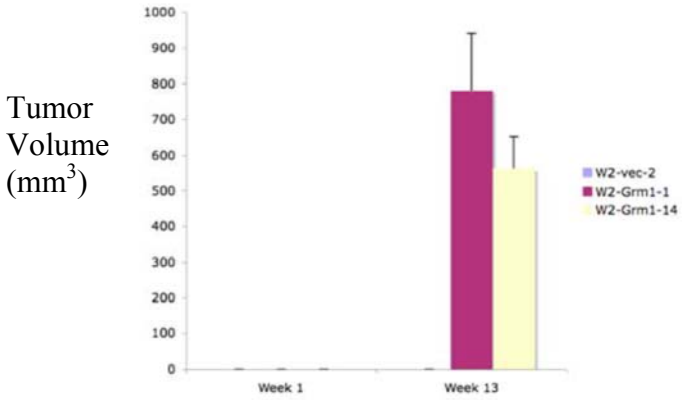


Figure 2. Development of tumors in the stable W2-Grm1 clones (W2g1 and W2g14) but not in W2-vec2 clone. (Martino, J.J., unpublished data).

My project is aimed at identifying whether expression of Grm1 is required to maintain tumorigenesis in these mouse kidney epithelial cells. The specific aims are 1) Isolate Tet^R-Grm1-W2 clones, 2) Isolate inducible siGrm1 and inducible siGFP clones of W2, and 3) allograft W2-Grm1 siGrm1 clones into nude mice. We showed that similar to the mouse melanocyte system; continued expression of Grm1 is required for the maintenance of tumorigenic phenotypes.

Materials and Methods

Cell Line Maintenance

All W2 Grm1 clones were grown in Dulbecco's Modification of Eagle's Medium (DMEM, Sigma) supplemented with 5% Fetal Bovine Serum (Sigma), Penicillin-Streptomycin (Gibco) and 1mg/ml geneticin (Gibco), in 37°C CO_2 water-jacketed incubator. Each transfection required the cells that have taken up the DNA to grow in a selective medium. The W2g1 and W2g14 clones were cotransfected with Tet^r and puromycin were grown with 2.0 and 1.5 ug/ml respectively, of puromycin. Clones of W2g1 and W2g14 transfected with either siGrm1 or siGFP were selected with 70 ug/ml and 90 ug/ml respectively, of hygromycin (Sigma). Media was changed biweekly.

Plasmid Purification

Plasmid purification for large scale production of plasmid DNA was needed for Tet^r, puromycin, siGrm1, and siGFP. The QIAfilter Plasmid Maxi Kit from Qiagen was used for preparation of plasmid samples. The procedure was conducted following the protocol provided by Qiagen.

The concentration of each plasmid DNA preparation was determined by electrophoresis on a 0.8% agarose gel comparison to the known concentration of Lambda DNA digested with HINDIII (Promega). Each preparation was confirmed by diagnostic restriction enzyme digestion. The plasmid DNA retrieved was precipitated with 25mM ammonium acetate, 25mM $MgCl_2$, and two and half times the volume of 100% ethanol.

Plasmids

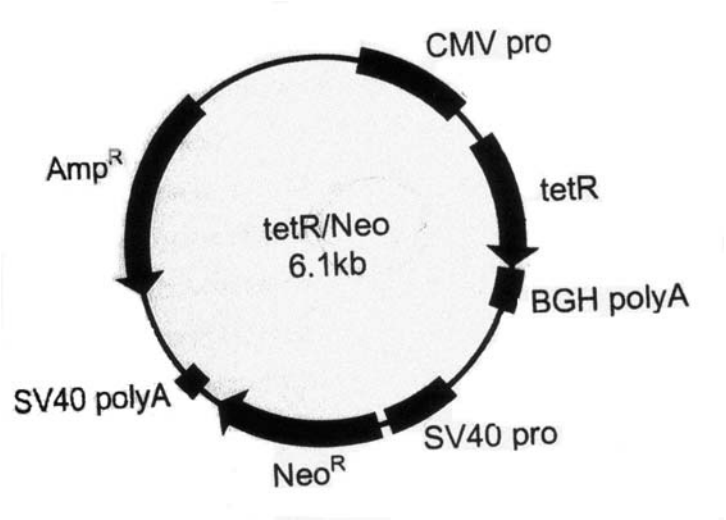


Figure 3. Tet^r DNA Plasmid Map (Matsukura et al., 2003)

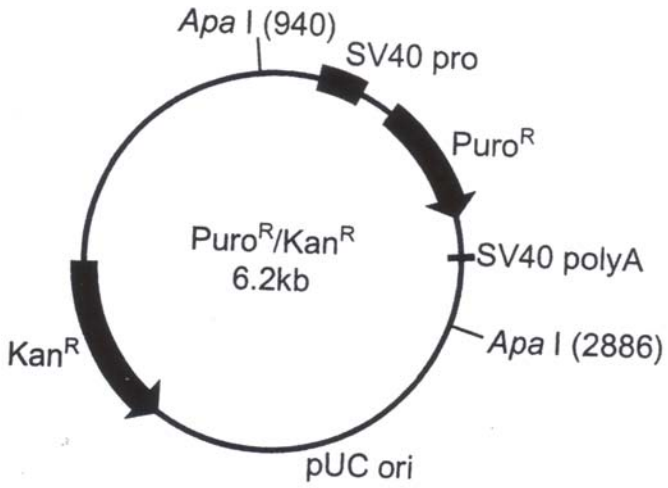


Figure 4. Puromycin DNA Plasmid Map (Matsukura et al., 2003)

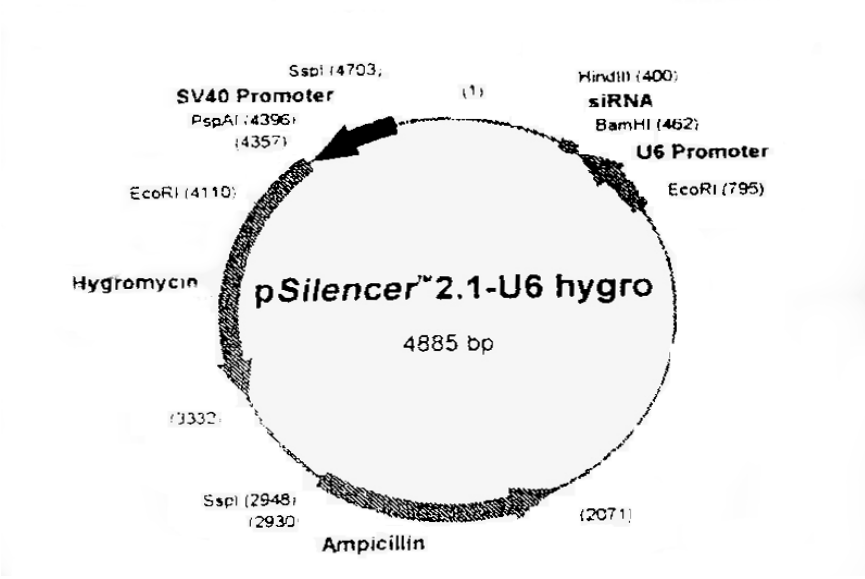


Figure 5. Inducible SiGrm1 and SiGFP DNA Plasmid Map (GenScript)

pRNA-H1 Forward	5′TAATACGACTCACTATAGGGAGAGAGAGAGAATTACCCTCACTAAAGATAT TTGCATGTCGCTATGTGTTCTGGGAAATCACCATAAACGTGAAACTCTATCAT TGATAGAGTTCTTATAAGTTCTGTATGAGACCACTCGGATCC**GATGTACATCA TTATTGCC**TTCAAGAGA**GGCAATAATGATGTACATC**TTTTTTGGAAAAGCTT AAGTTTAAACCGCTGATCAGCCTCGACTGTGCCTTCTA
pRNA Reverse	5′TAGAAGGCACAGTCGAGGCTGATCAGCGGTTTAAACTTAAGCTTTTCCAAA AAA**GATGTACATCATTATTGCC**TCTCTTGAA**GGCAATAATGATGTACATC**G GATCCGAGTGGTCTCATACAGAACTTATAAGAACTCTATCAATGAATAGAGTT TCACGTTTATGGTGATTTCCCAGAACACATAGCGACATGCAAATATCTTTAGT GAGGGTAATTCTCTCTCTCTCCCTATAGTGAGTCGTATTA

Table 3. Inducible SiGrm1 Insertion Sequence (In Bold). (GenScript)

pRNA-H1 Forward	5′TAATACGACTCACTATAGGGAGAGAGAGAGAATTACCCTCACTAAAGATAT TTGCATGTCGCTATGTGTTCTGGGAAATCACCATAAACGTGAAACTCTATCAT TGATAGAGTTCTTATAAGTTCTGTATGAGACCACTCGGATCC**GGTTATGTACA GGAACGCA**TTCAAGAGA**TGCGTTCCTGTACATAAC**CTTTTTGGAAAAGCTT AAGTTTAAACCGCTGATCAGCCTCGACTGTGCCTTCTA
pRNA Reverse	5′TAGAAGGCACAGTCGAGGCTGATCAGCGGTTTAAACTTAAGCTTTTCCAAA AAG**GTTATGTACAGGAACGCAT**CTCTTGAA**TGCGTTCCTGTACATAACC**GG ATCCGAGTGGTCTCATACAGAACTTATAAGAACTCTATCAATGATAGAGTTTC ACGTTTATGGTGATTTCCCAGAACACATAGCGACATGCAAATATCTTTAGTGA GGGTAATTCTCTCTCTCTCCCTATAGTGAGTCGTATTA

Table 4. Inducible SiGfp Insertion Sequence in Bold. (Ambion/GenScript)

Agarose Gel Electrophoresis

DNA and PCR samples were run on 0.8% agarose gels made in 0.04M Tris-acetate and 0.001M EDTA (TAE) buffer with ethidium bromide for visualization under UV light. A standard DNA marker, Lambda DNA digested with HINDIII (Promega) or PhiX174 DNA digested with HAEIII (Promega) was included for each gel. Samples were prepared with water and DNA dye (0.04% Xylene cyanol, 1.14% Bromophenol Blue, 2.5% Ficoll). The DNA and PCR samples were electophoresed in TAE buffer. Visualization was performed with a UV Transilluminator and Kodak 1D 3.0 LE Alias program with the Kodak Digital Science Electrophoresis Documentation and Analysis System 12.0.

RNA samples were run on a 1% agarose (Invitrogen) gel made with 0.09 M Tris-Borate and 0.002M EDTA (TBE) buffer made with RNAse free water. The RNA samples were mixed with RNAse free water and heated to 65°C for 15 minutes with the RNA ladder 0.5-10 kb, (Invitrogen) was cooled on ice for 5 minutes and then RNA dye

(0.04% Xylene cyanol, 1.14% Bromophenol Blue, 2.5% Ficoll) was added. RNA samples were electrophoresed and placed in TBE with 0.5% ethidium bromide for 20 minutes and visualized with a UV Transilluminator and Kodak 1D 3.0 LE Alias program with the Kodak Digital Science Electrophoresis Documentation and Analysis System 12.0.

Plasmid DNA Transfection

Cells were plated on a 60mm plate with 7 x 10^5 cells a day before the transfection. The plasmid DNA was transfected following Lipofectamine 2000 protocol (Invitrogen) and plated. At 24 hours after transfection, each plate was trypsinized into 3 plates in Dulbecco's Modification of Eagle's Medium (DMEM) supplemented with 5% Fetal Bovine Serum (Sigma), and Penicillin-Streptomycin (Gibco). The following day (24 hours later), the plates were placed under appropriate drug selection for clonal isolation with either hygromycin or puromycin.

Isolation of SiGrm1/SiGFP Clones

Each transfected plate was inspected under a microscope for clones that were well established and distant from other cells. Each clone was isolated with a clone ring (Bellco) and placed in a 24 well plate. The clones were allowed to grow and expand in a 24 well plate until 50% confluent. The cells were then typsinized and transferred to a 60 mm plate for further studies.

RNA Extraction

Cell plates were grown to near confluency for RNA extraction. The medium was aspirated; one ml of TRI Reagent LS from Molecular Research Center was added to the plate, and scraped with a cell scraper (Sarstedt). The RNA extraction and precipitation

was completed following the TRI Reagent protocol from Molecular Research Center. The RNA samples were stored in -80°C freezer for further studies.

Reverse-Transcriptase PCR (RT-PCR)

The RNA samples were treated with DNase (Promega) prior to RT-PCR. The samples were then mixed with 0.002 mM Oligo dT (Promega), 10 mM dNTP, and sterile water. The mixture was heated at 65°C for 5 minutes, chilled quickly on ice and then centrifuged briefly to collect all the liquid. To each sample,250 mM Tris-HCl (pH 8.3), 375 mM KCl, 15 mM $MgCl_2$, First Strand Buffer (Invitrogen) and 0.1M DTT (Invitrogen) was added and incubated at 43°C for 2 minutes. SuperScript Reverse Transcriptase, 200 units, (Invitrogen) was added and incubated at 42°C for 50 minutes for full reaction activity then inactivated at 70°C for 15 minutes. The cDNA that was made will be used for PCR to verify our Tet^r plasmid was incorporated into our selected clones. The antibody for Tet^r was not good therefore it was not possible to perform Western immunoblots.

Each PCR sample contained the following: 2uL cDNA, 25 ul PCR mix (Qiagen), 21 ul sterile water, 1ul pCMV 366F 10uM, and 1 ul Tet^r 10uM. A negative control was used with the same components as the test sample but 1ul DNased RNA sample was used in place of cDNA and 22ul of sterile water. GAPDH was used as a positive control to assure that the cDNA made was good.

Tet^r R primer	5'-TTAAGACCCACTTTCACATTTAAGTTGT-3'
CMV 366 F primer	5'-CGCTATTACCATGGTGATGCGG-3'
GAPDH R primer	5'-CTGCTTCACCACCTTCTTGAT-3'
GAPDH F primer	5'-GTGATGGGTGTGAACCACGAG-3'

Table 5. Primer sequences used for RT-PCR.

In Vitro Doxycycline Treatment

Cells were plated with 1 x 10^5 cells and placed in appropriate antibiotic selection with DMEM, 5% Fetal Bovine Serum, and Penicillin-Streptomycin, a day before doxycycline treatment. Each clone was tested with 1.5 ug/ml of doxycycline for 6-7 days with protein extractions on Day 4 and Day 6 or 7 dependent on confluency. A parallel set of plates were used as a negative control that were not given doxycycline.

Protein Extraction

Clones transfected with siGFP and siGrm1 were grown with or without doxycycline treatment in duplicates and protein was extracted. The plates from the in vitro doxycycline treatment were aspirated of the medium and washed once with cold Phosphate Buffered Saline (PBS). PBS was aspirated and extraction buffer consisting of phosphatase inhibitors 1 and II (Sigma), Complete protease inhibitor cocktail (Roche), 50mM Tris-HCL, 150mM NaCl, 1mM EDTA, 1% NP40, 5% Glycerol, and 1mM Dithiothreitol, was added to the plate. The plate was scraped with a cell scraper, transferred to a 1.5 ml tube, and incubated on ice for 20 minutes. The samples were then spun at 14,000 rpm for 20 minutes at 4°C. The supernatant obtained was then transferred to a clean 1.5 ml tube and placed at -80°C for future analysis.

Protein Concentration

Protein concentration for each protein lysate was determined by Lowry method using a commercially available kit (Bio-Rad, DC protein assay) with standards (0.1-1.6 mg/ml).

SDS-PAGE and Western Immnoblot

Ten percent SDS-Polyacrylimide Gel Electrophoresis (SDS-PAGE) gels were used for all Western immunoblots with 4 ug of protein from each test sample. Following electrophoresis, the gels were transferred to a nitrocellulose membrane in 48mM Tris base, 39mM Glycine, 0.037% SDS and 20% Methanol. The nitrocellulose membrane was removed, stained with Pouseau Red for band visualization. The membrane was blocked with 15mL of 5% milk in 25mM Tris Buffered Saline with 0.1% Tween 20 for one hour at 4°C. The membranes were placed in a plastic bag with primary antibody (Anti-mouse Grm1 at 1:3000 dilution from BD Biosciences and p42/44 for ERK at 1:1500 dilution from Cell Signaling Technologies) with 3mLs of 5% milk in 25mM Tris Buffered Saline with 0.01% Tween 20 overnight incubation at 4°C. The following day the membranes were washed with 25mM Tris Buffered Saline with 0.01% Tween 20 for a total of 10 minutes, 5 minutes each, and then given the secondary antibody from GE Healthcare (ECL™ Anti-mouse IgG: Horseradish Peroxidase-linked whole Antibody (from donkey at 1:3000 dilution) and ECL™ Anti-rabbit IgG: Horseradish Peroxidase-linked whole Antibody (from sheep at 1:3000 dilution)) in 10mLs of 5% milk in 25mM Tris Buffered Saline with 0.01% Tween 20 for 1 hour at room temperature on a shaker. The membranes were washed again on a shaker with 25mM Tris Buffered Saline with 0.01% Tween 20 for four 15 minute intervals.

Chemoluminescent ECL Plus (GE Healthcare) was added to each membrane and incubated for 3 minutes at room temperature. After incubation, the membranes were exposed to HyBlot CL Autoradiography film in the dark for various time points and then developed with Kodak X-OMAT 1000A Processor.

Animals

The Institutional Review Board for the Animal Care and Facilities Committee of Rutgers University approved all the animal studies. The siGrm1 clones were prepared in PBS at 10^7 cells per ml with 10^6 cells injected into two dorsal sites of 4 to 5 week old male nude mice from Taconic (Hudson, NY). When the tumor volumes were around $10mm^3$, the animals were divided into two groups with 10 mice in each group. One group received doxycycline (0.1% w/v) in the drinking water while the other one only received plain water. Doxycycline water was changed biweekly. Tumor measurements were taken on a biweekly basis with a vernier caliper and animals were euthanized once tumor burden reached 465.5 mm^3.

Results

Transfection of Tet^r and Puromycin into Stable W2 Grm1 Clones

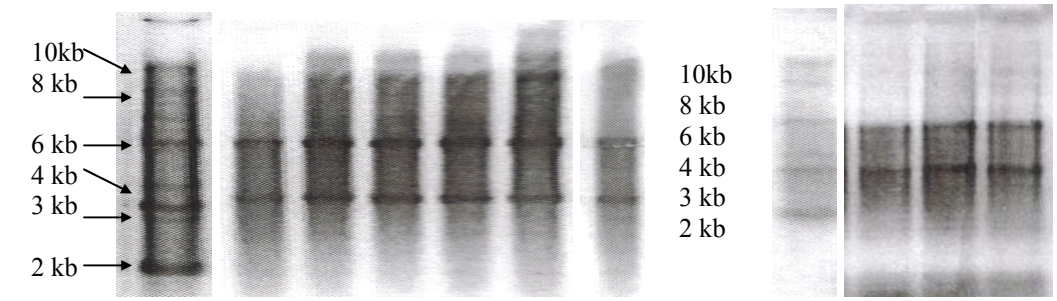
Two stable Grm1 W2 clones, W2g1 and W2g14, were selected for cotransfection of DNA plasmids Tet^r and puromycin. Following transfection, the cells were placed under puromycin selection (1.5 ug/ml W2g14 and 2.0 ug/ml W2g1). Puromycin-resistant clones were then tested by RT-PCR for Tet^r expression. The RNA were assessed first for their integrity in agarose gels (Figure 6A and 6B). All the clones were shown to have two bands indicative of the two ribosomal RNAs, 18S and 28S, the most abundant RNA present in the cells.

Once the integrity of RNA in the sample was determined, the samples were treated with DNase to remove any possible DNA contamination that may interfere with the PCR. The cDNAs were made by reverse transcriptase. The cDNA samples were then prepared for PCR. Upon completion of PCR cycling, each sample was analyzed on an agarose gel with HAE III digest PhiX174 DNA Marker to identify the presence of the Tet^r plasmid with an expected size of approximately 1.0 kb in length. There were three conditions set in the PCR: 1) test samples, cDNA were mixed with Tet^r R and pCMV 366F primers, which flank the Tet^r cDNA to determine which sample expressed Tet^r, 2) negative control, DNased RNA samples mixed with the same set of primers as in (1) to establish whether there is any DNA contamination, and 3) positive control, cDNA was mixed with GAPDH primers to establish that the integrity of the cDNA. A positive control exhibiting Tet^r expression, C8161-Tet^r-3, was included in each sample set.

Each W2g1 clone, except for clone 1, showed expression of Tet^r including the positive control C8161-Tetr clone 3 (Figure 7A). The negative control test group

indicated that all the clones had no DNA contamination because there were no visible bands found including the positive control (Figure 7B). The positive control group all showed bands corresponding to GAPDH which verified that the cDNA made was good (Figure 7C). The success rate for W2g14 was not as high as in W2g1. Figure 8A shows that only W2g14 clone 3 and 6 expressed Tet^r but all the clones were found to be expressing GAPDH (Figure 8C) indicating that cDNA made was good and that the other clones had not taken up the Tet^r plasmid. All the clones were found to have no DNA contamination since there was a lack of visible bands for the negative control test group (Figure 8B).

A) W2g1 clones



B) W2g14 clones

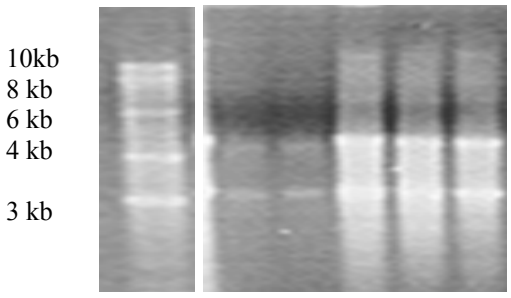


Figure 6. Integrity and presence of RNA extracted from W2g1 and W2g14 clones was assessed on a 1% Agarose gel. The bands lengths of the RNA Marker are the same in each gel. A) RNA from W2g1 clones. B) RNA from W2g14 clones.

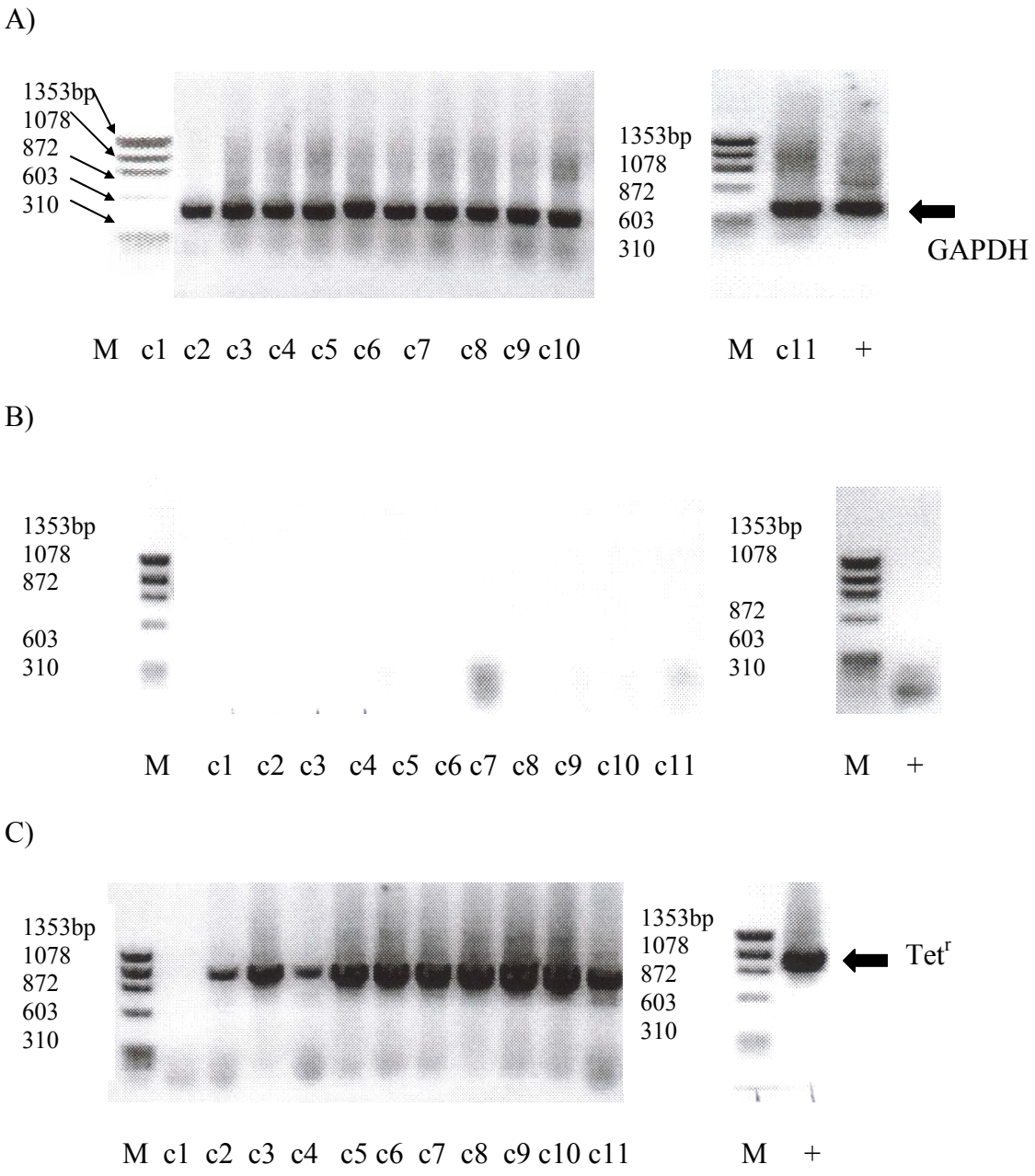


Figure 7. Presence of Tet^r plasmids in cDNA made for putative W2g1-Tet^r clones. W2g1 PCR products were run on a 0.8% gel with a positive Tet^r control, C8161 Tet^r 3 (+), to determine the clones expressing the Tet^r plasmid. HAEIII digested PhiX174 DNA was used as the marker (M) in all the gels. The bands lengths of the marker (M) are the same in each gel. A) PCR was performed with GAPDH primers to assess synthesized

cDNA. B) Same PCR conditions as in (A) except DNase-RNA samples were used as templates. C) cDNA made from several isolated samples were tested for the presence of the Tet^r plasmid.

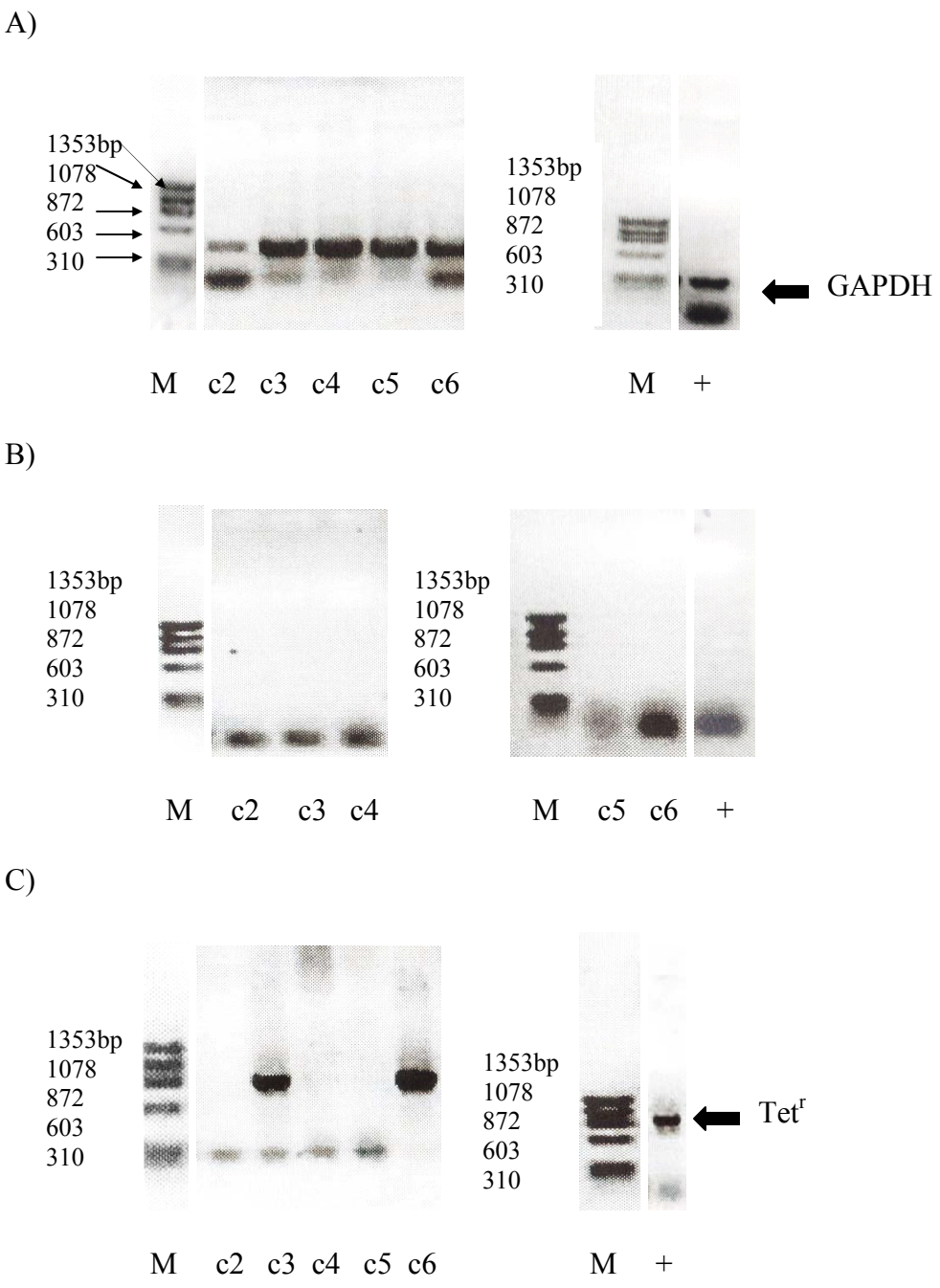


Figure 8. Presence of Tet^r plasmids in cDNA made for putative W2g14-Tet^r clones. W2g14 PCR products were run on a 0.8% gel with a positive Tet^r control, C8161-Tet^r-3 (+), to determine the clones expressing the Tet^r plasmid. HAEIII digested PhiX174 DNA was used as the marker (M) in all the gels. The bands lengths of the marker (M) are the same in each gel. A) PCR was performed with GAPDH primers to assess synthesizing

cDNA. B) Same PCR conditions as in (A) except DNase-RNA samples were used as templates. C) cDNA made from several isolated samples were tested for the presence of the Tet^r plasmid.

Transfection of Inducible SiGrm1 and SiGFP Constituents into W2g1- and W2g14-Tet^r Clones

Once the positive Tet^r clones were established, two clones each were picked from W2g1 (c7 and c11) and W2g14 (c3 and c6) to be transfected with siGrm1 and siGFP separately. Each clone was transfected with either siGrm1 or siGFP, the negative control. Following transfection, the selection placed upon the cells was 70 ug/ml (W2g1-Tet^r-c7-siGrm1-A) and 90 ug/ml (W2g14-Tet^r-siGrm1-C/ W2g14-Tet^r-c3-siGrm1-F) of hygromycin. Approximately 70 clones were isolated with hygromycin resistance and expanded for further analysis.

Under the inducible siGrm1 and siGFP system, the cells have to be administered the inducer, doxycycline, to activate the siRNA and therefore affect the expression levels of respective genes. SiGFP clones were included as negative controls. Dozens of clones were isolated and tested in the presence or absence of the inducer, doxycycline (an analog of tetracycline) for possible suppression of Grm1 expression. Protein was extracted at two time points, Day 4 and Day 6 or 7. Initial siGrm1 clones showed decreased Grm1 expression at Day 6 and therefore subsequent experiments were performed with doxycycline treatment for 6 days. Once all the protein samples were collected, western immunoblots were run to determine the Grm1 expression levels for siGrm1 and siGFP stable W2g1 and W2g14 clones with total ERK used as loading controls. Candidate clones were tested at least two times to verify the results. The siRNA nomenclature of each W2g1 or W2g14 Tet^r clone is designated by either siGrm1 or siGFP and a letter starting with A.

Several siGFP clones were also isolated and tested by western immunoblot, we showed that there was no change in the Grm1 expression levels at any time point and in both doxycycline treatment and no treatment groups of four clones (Figure 9).

For W2g1-Tet^{r}-c7 clones tested, only one clone (W2g1-Tet^{r}-c7-siGrm1-A) was found to have decreased levels of Grm1 expression (Figure 10A). While for W2g14-Tet^{r}-clones, we identified two clones, W2g14-Tet^{r}-c3-siGrm1-C and W2g14-Tet^{r}-c3-siGrm1-F, showed much reduced Grm1 expression (Figure 10B and 10C).

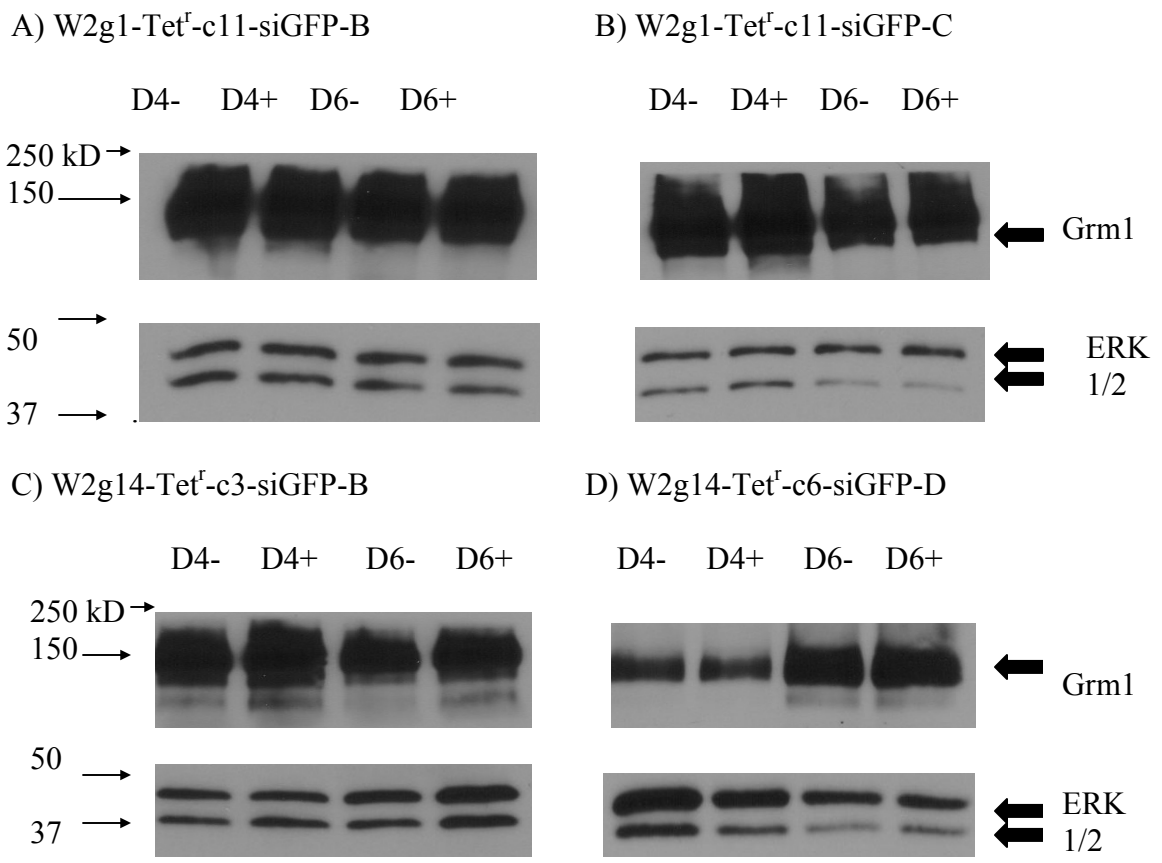
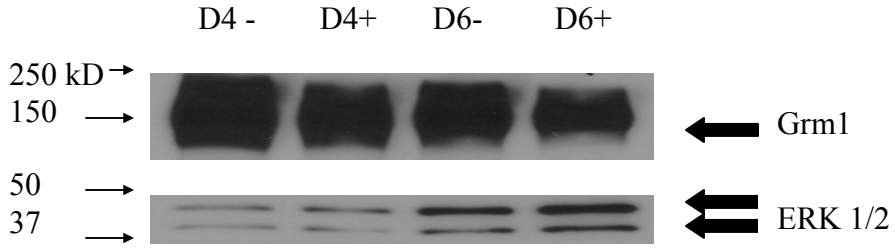
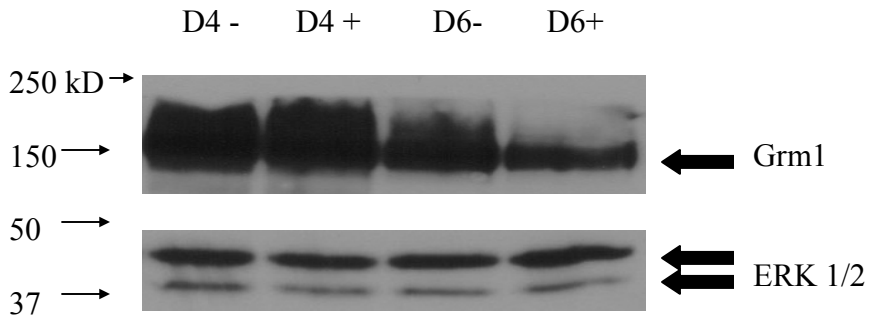


Figure 9. Western immunoblots of W2g1 and W2g14 siGFP clones. The immunoblots of Grm1 (150 kD) and total Erk (42kD) expression levels were completed for Day 4 and Day 6 with and without doxycycline treatment. A and B) W2g1-Tet^r-c11-siGFP clones indicates no change in Grm1 expression on Day 4 or 6 of doxycycline treatment. C and D) After 6 days of doxycycline treatment W2g14-Tet^r-siGFP clones B and D exhibited no change in Grm1 expression compared to the no treatment groups.

A) W2g1-Tet^r-c7-siGrm1-A



B) W2g14-Tet^r-c3-siGrm1-C



C) W2g14-Tet^r-c3-siGrm1-F

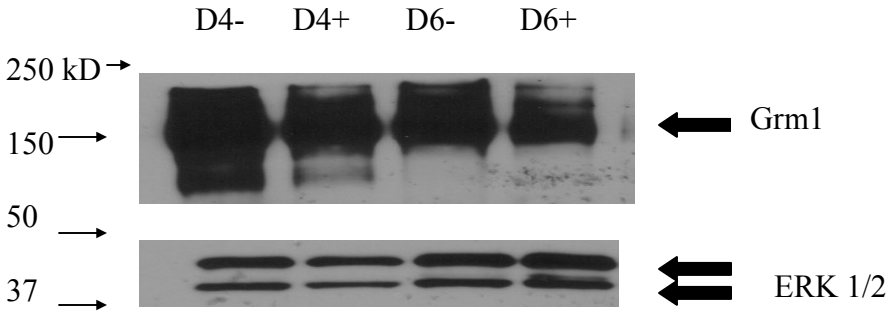


Figure 10. Western immunoblots of W2g1 and W2g14 SiGrm1 Positive Clones. The immunoblots of Grm1 (150 kD) and total Erk (42kD) expression levels were completed for Day 4 and Day 6 with and without doxycycline treatment. A) Of all the W2g1-Tet^r-c7 and c11-siGrm1 transfected clones only one, W2g1-Tet^r-c7-siGrm1 A exhibited decreased levels of Grm1 at Day 6 with doxycycline. B and C) W2g14-Tet^r-c3-siGrm1 clones C and F show decreased Grm1 expression levels at Day 6 with doxycycline compared with no treatment.

Allografts of the Inducible SiGrm1 W2 Clones

These experiments are still ongoing except for W2g14-Tet^r-siGrm1-F. The W2g1-Tet^r-c7-siGrm1-A, W2g14-Tet^r-c3-siGrm1-C, and W2g14-Tet^r-c3-siGrm1-F clones were allografted into immunoincompetent athymic nude mice with 10^6 cells per injection site. The animals were randomly placed into two groups (doxycycline treatment and no treatment) upon detection of tumor volume of approximately 10 mm³. Following measurement of the initial tumor, doxycycline was administered in the drinking water for the treatment group.

Injection of clone, W2g1-Tet^r-c7-siGrm1-A into the nude mice formed detectable tumors after five weeks. The doxycycline treatment group had an initial tumor volume of 31.20 mm^3 (standard deviation of 5.39 mm^3) compared to the no treatment group's tumor volume of 37.74 mm^3 (standard deviation of 35.63 mm^3). As time progressed, the volume difference between the groups began to increase. Over a period of a month with doxycycline treatment, there was a noticeable difference between the two groups. The no treatment group comparatively grew at a relatively faster rate indicated by a slightly larger tumor volume at the last measured time point. Since this experiment is ongoing, the last measured tumor volume for the no treatment group was 269 mm^3 (standard deviation of 294.94 mm^3) and 86.05 mm^3 (standard deviation of 51.23 mm^3) for the doxycycline treatment group (Figure 12A).

Tumor formation from the injections of W2g14-Tet^r-c3-siGrm1-C and W2g14-Tet^r-c3-siGrm1-F took approximately 9 weeks before the appearance of measurable tumors. This is 4 weeks longer than what was seen with the W2g1-Tet^r-c7-siGrm1-A clone. Each clone had different time points for the appearances of tumors. Also, within

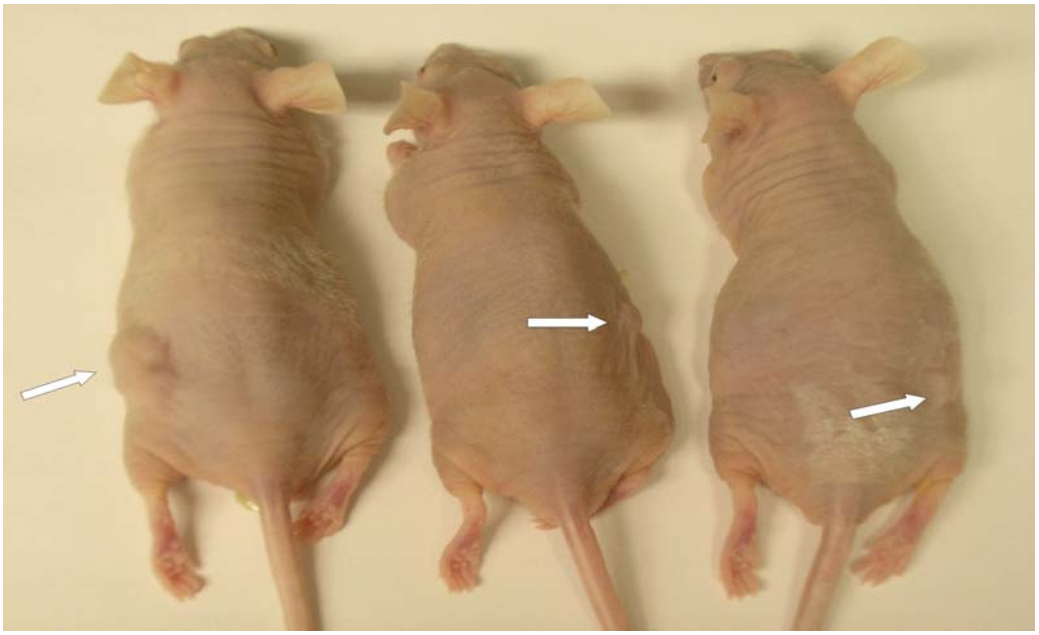
each mouse test group, there was a variation in the tumor volumes and in tumor development. The standard error for each group was calculated to indicate the fluctuations in the sampling measurements. Each mouse was injected into two sites; however, not every mouse developed tumors in both sites (Figure 11). The overall trend observed was the same for all the clones, the tumor volume in the doxycycline treatment group was much less than in the no treatment group.

Similarly to W2g1-Tet^r-c7-siGrm1-A, W2g14-Tet^r-c3-siGrm1-C was found to have initial tumor volumes that were not much different between the treatment groups. The doxycycline treatment group was found to have an initial tumor volume of 26.7 mm^3 (standard deviation of 8.5 mm^3) whereas the no treatment group's initial volume was 61.1 mm^3 (standard deviation of 77 mm^3). Despite this initial observation, the tumor volume trend changed and later showed that the tumor volume in the doxycycline treatment group (47.3 mm^3(standard deviation of 46.18 mm^3)) was much less than in the no treatment group (219 mm^3 (standard deviation of 327.84 mm^3)) (Figure 12B).

The W2g14-Tet^r-c3-siGrm1-F clone exhibited a large difference in tumor volumes within each treatment group in a 4 week time period. Initially, the no treatment group of W2g14-Tet^r-c3-siGrm1-F had a tumor volume of 57.95 mm^3 (standard deviation of 40.90 mm^3) whereas the doxycycline treatment group's tumor volume was 49.18 mm^3 (standard deviation of 50.81mm^3). The no treatment group tumors continued to grow whereas the doxycycline treatment group tumors were growing but at a much slower rate. The tumor burden in the no treatment group had reached the maximum allowed size and therefore the experiment was terminated at 91 days post injection (Figure 11). At termination, the tumor volume for the no treatment group was 1361.76 mm^3 (standard

deviation of 1903.5 mm^3) and 545.47 mm^3 (standard deviation of 940.31 mm^3) for the doxycycline treatment group (Figure 12C)

A)



B)

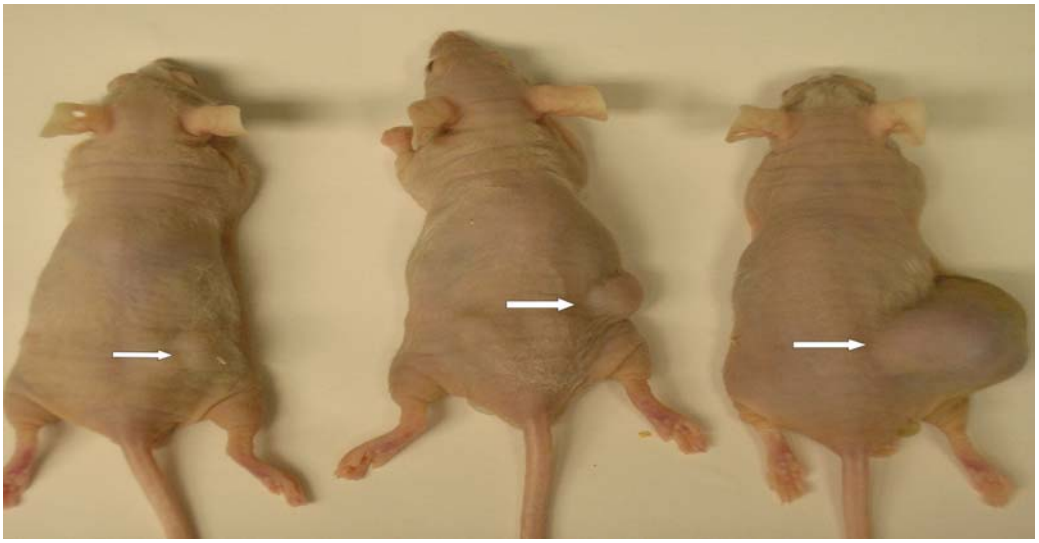
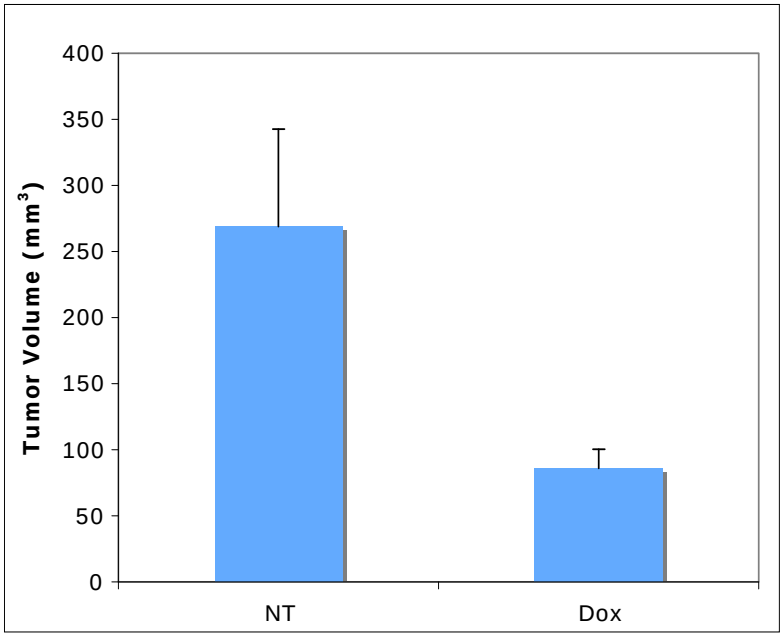
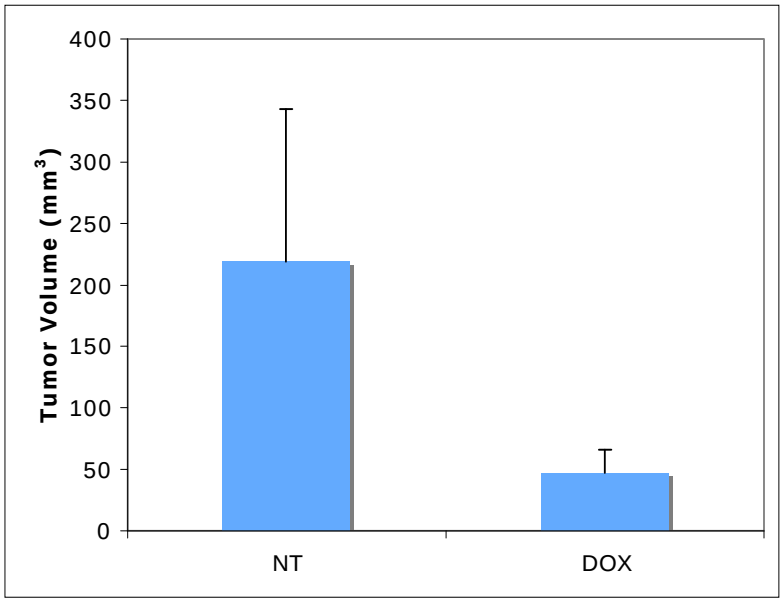


Figure 11. Tumor formation of W2g14-Tet^{r}-c3-siGrm1-F clones allografted into immunocomprised nude mice. A) The picture identifies the small tumors formed in the doxycycline treatment group over one month of dosage provided in the drinking water. B) These mice were given plain drinking water without the addition of doxycycline. The tumors are indicated by the white arrows.

A)


400
350
300
250
200
150
100
50
0
Tumor Volume (mm3)
NT
Dox

B)


400
350
300
250
200
150
100
50
0
Tumor Volume (mm3)
NT
DOX

C)

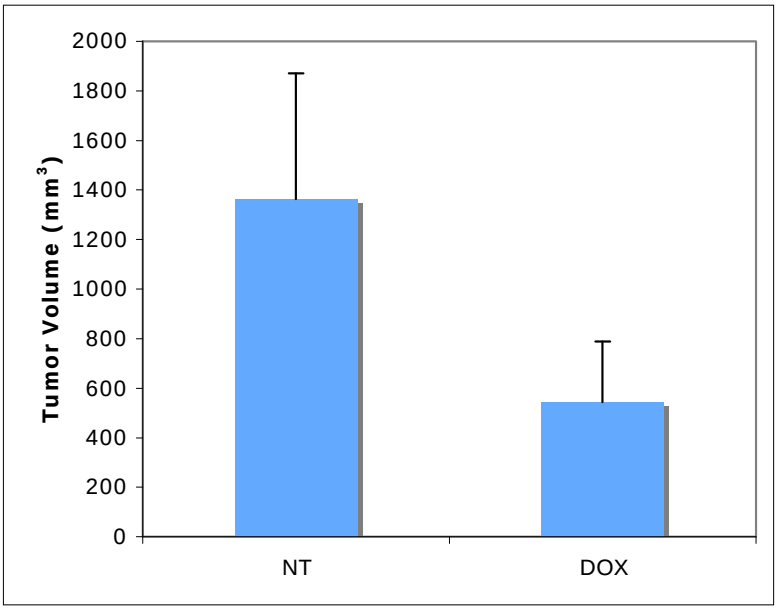


Figure 12. Comparison of the doxycycline treatment versus no treatment of all the siGrm1 clones. The average tumor volumes and standard errors of every animal in each treatment group was calculated and compared to the no treatment group. The standard error for each group was calculated to indicate the fluctuations in the sampling measurements. A) W2g1-Tet^{r}-c7-siGrm1-A after 49 days. B) W2g14-Tet^{r}-c3-siGrm1-C after 49 days.. C) W2g14-Tet^{r}-c3-siGrm1-F after 28 days.

Discussion

In recent years, skin cancer has become one of the most common forms of cancer within the United States. Melanoma in particular is the most serious form of skin cancer that is responsible for the majority of mortalities in skin cancer patients (American Cancer Society, 2008; Skin Cancer Foundation, 2008). Currently the options for melanoma patients are surgical removal and chemotherapy. This regime is proving unsuccessful for most patients beyond Stage I and provides little hope for survival for those with advanced disease (American Cancer Society, 2008; Skin Cancer Foundation, 2008). Gaining further insight into the mechanisms of melanoma tumorigenesis and identifying new drug targets will have a large impact on the growing population who are at risk for melanoma.

Metabotropic glutamate receptors are a class of G-protein-coupled seven transmembrane domain receptors commonly expressed in the central nervous system that use glutamate as their natural ligand (Pin and Duvoisin, 1995; Dhami and Ferguson, 2006). There is evidence however that the metabotropic glutamate receptors are found to be expressed in various other tissues of the body and may have a functional role in carcinogenesis (Albansanz et al., 1997; Arcella et al., 2005; Aronica et al., 2003; D'Onofrio et al., 2003; Naarela et al., 1993; Shin et al., 2008b; Shinno et al., 1994; Chang et al., 2005; Iancovelli et al., 2006; Kalariti et al., 2007; Park et al., 2007; Pollock et al., 2003). As an example, 40% of melanoma biopsies and human melanoma cell lines were found to have expression of metabotropic glutamate receptor 1, GRM1, and none in the normal human melanocytes or benign nevi (Namkoong et al., 2007). Research on melanoma cells has shown elevated levels of glutamate being released by a majority of

human melanoma cells thereby creating an autocrine loop feedback system (Namkoong et al., 2007). Inhibition of glutamate release suppresses human melanoma cell growth and promotes apoptosis *in vitro* and *in vivo*. Taken together, these results suggest the autocrine system may enable the melanoma cells to maintain active proliferation through the constitutively activated GRM1.

Grm1 was demonstrated as a complete oncogene in mouse melanocytes (Shin et al., 2008b). To further establish the oncogenic role of Grm1 in murine melanocytes, a tetracycline inducible siRNA system was used to assess if Grm1 expression is needed to maintain the tumorigenic phenotype (Shin et al., 2008b). Using the inducible siGrm1 system, the presence of doxycycline, the inducer, resulted in substantial decrease of tumor volumes (about 60%) of Grm1-melanocytes within mice compared to the no treatment group (Shin et al., 2008b). The inducible siRNA system proved to be a reliable and effective system to modify expression levels.

Since cancers of epithelial origin are the most abundant cancer types in humans, we were interested to know if GRM1 is also an oncogene in epithelial cell systems. Normally, GRM1 expression has been documented in the CNS as well as myoctes and thymocytes (Table 1) but not in epithelial cells (Gill et al., 1999; Storto et al., 2000a; Storto et al., 2000b; Shin et al., 2008a). In order to study the role of Grm1 in epithelial cells, a mouse kidney epithelial cell line (W2) that normally does not have Grm1 expression was used to receive exogenous Grm1. Several stable W2-Grm1 clones were isolated and shown to produce tumors in nude mice. We then set up a set of experiments to assess if continuous expression of Grm1 is required to maintain the tumorigenic phenotype in the epithelial cells.

A tetracycline inducible siRNA system was used. SiGrm1 or siGFP was transfected into W2-Grm1 clones. Several clones were isolated and the ability of siGrm1 but not siGFP to suppress Grm1 expression was examined using Western immunoblots. We identified three clones to have decreased expression of Grm1 in the presence of the inducer, doxycycline *in vitro*. These clones were then selected for *in vivo* assessment using allografts in immunodeficient nude mice due to the lack of additional reliable *in vitro* assays. The W2-Grm1 clones have particular characteristics, such as density dependence, that prevent further investigative *in vitro* assays. A cellular proliferation assay was initiated multiple times unsuccessfully. The cell density required for the duration of the assay was too low for the W2-Grm1 cells to survive. The *in vivo* allograft was the next appropriate step for this project and cell line. Comparison of the no treatment and doxycycline treatment western immunoblots of W2-Grm1-siGrm1 clones revealed that the siRNA system did not fully decrease the expression of Grm1. Complete suppression of Grm1 within the W2-Grm1 transformed cell line may not be possible. The integrations of this gene appear to be a large number due to appearance of the large protein bands on the western immunoblot. In order for the siRNA system to knockout Grm1 expression as oppose to knockdown Grm1 expression, would require increasing the amounts of siRNA integrations into the cell genome to match that of Grm1, which occurs randomly upon transfection and can not be controlled.

A total of three W2-siGrm1 clones were selected for the *in vivo* experiment. A siGFP clone is proposed to begin the *in vivo* experiment in the near future to ensure that any result within the doxycycline treatment group is not due to the doxycycline alone but from the siGrm1. Currently only one W2-siGrm1 clone, W2g14-Tet^{r}-c3-siGrm1-F,

allograft experiment is complete. The result for this one clone showed that similarly as the melanocyte system, Grm1 expression is required to maintain tumorigenic phenotype *in vivo*.

Determining the importance of Grm1 expression in epithelial cells is only the first step towards identification of GRM1 expression in human epithelial tumors. Moving forward with this data would logically involve relating it to human renal carcinoma. Using a well-known human embryonic kidney cells (HEK), we did not detect GRM1 expression. Currently we are examining if human renal carcinomas cell lines and tissue samples express GRM1, if so, then we can use the same experimental design described in this thesis to test if GRM1 is an oncogene. In addition, the inducible siGrm1 system can be used to determine if GRM1 expression is needed for maintenance of tumorigenic phenotype. Identifying GRM1 expression in human renal carcinoma would provide another target for future drug therapies. If we do not detect GRM1 in human renal carcinoma, we can extend our study to other epithelial cancers.

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