Identification of Important Regulators of Colon Cancer Tumorigenesis By Functional Screening

Haowei Yi

University of Nebraska Medical Center

Let us know how access to this document benefits you

http://unmc.libwizard.com/DCFeedback

Follow this and additional works at: https://digitalcommons.unmc.edu/etd

Part of the Cancer Biology Commons

Recommended Citation


https://digitalcommons.unmc.edu/etd/192
IDENTIFICATION OF IMPORTANT REGULATORS OF COLON CANCER TUMORIGENESIS BY FUNCTIONAL SCREENING

by

Haowei Yi

A DISSERTATION

Presented to the Faculty of
The University of Nebraska Graduate College
in Partial Fulfillment of Requirements
for the Degree of Doctor of Philosophy

Genetic, Cell Biology & Anatomy
Graduate Program

Under the Supervision of Professor Jenny Wang

University of Nebraska Medical Center
Omaha, Nebraska

April, 2017

Supervisory Committee:
Andrew Dudley, Ph.D.         Jennifer Black, Ph.D.
Shantaram Joshi, Ph.D.         Xu Luo, Ph.D.
ACKNOWLEDGMENTS

I would like to owe my sincere gratitude to my adviser, Dr. Jenny Wang, for her patience, constant support, encouragement, and scientific guidance throughout my studies. Her continuous support helped me to overcome stressful situations, and to complete this dissertation successfully.

I would also like to thank my committee members, Dr. Michael Brattain, Dr. Runqing Lu, Dr. Samming Wang, Dr. Jennifer Black, Dr. Xu Luo, Dr. Shantaram Joshi, Dr. Andrew Dudley for their suggestions, thoughtful ideas, and scientific evaluations throughout my study. Their insightful comments and criticisms helped me to enthusiastically focus my research.

I am thankful to my colleague Dr. Liying Geng, for her constant help and constructive advice that helped me to sort out technical difficulties and conceptualize my work. I am also thankful to my previous lab mates, Dr. Yang Zhang, Dr. Xiaolin Zhou for their support all along. I would thank Dr. Geoffrey Talmon and Dr. Adrian Black for their kind help on the histology part of my study.

I would like to thank all faculties and staffs in the Department of GCBA for their help on my study in this department.

I especially thank and appreciate my wife, Ting Jia for her love, spiritual support to me. I sincerely thank my parents, for their love, encouragement and support. I also offer my sincere thanks to all my friends, for their help on my work and on my life.

Lastly, I would like to thank my country, China. I am thankful to the China Scholarship Council for providing financial support to pursue my PhD program.
ABSTRACT

TGFβ signaling is an important regulator in colon cancer. miRNAs regulate TGFβ signaling at multiple levels. In this study, through a functional screening, we identified miR-487b-3p and miR-656-3p, which modulate TGFβ effect in colon cancer cells. Further studies revealed that GRM3 and VGLUT3 are their respective targets.

GRM3, a Metabotropic glutamate receptor in glutamatergic pathway is significantly upregulated in majority of human colonic adenocarcinomas tested and colon cancer cell lines. Knockdown of GRM3 expression or inhibition of GRM3 activation in colon cancer cells reduces cell survival and anchorage-independent growth in vitro and inhibits tumor growth in vivo. Mechanistically, GRM3 antagonizes TGFβ-mediated activation of protein kinase A and inhibition of AKT. In addition, TGFβ signaling increases GRM3 protein stability and knockdown of GRM3 enhances TGFβ-mediated tumor suppressor function. Since miR-487b-3p directly targets GRM3, overexpression of miR-487b-3p mimics the effects of GRM3 knockdown in vitro and in vivo. Expression of miR-487b-3p is decreased in colon adenocarcinomas and inversely correlates with GRM3 expression.

VGLUT3, a vesicular glutamate transporter, is also markedly upregulated in human colonic adenocarcinomas and colon cancer cell lines. Knockdown of VGLUT3 expression in colon cancer cells reduces cell survival and anchorage-independent growth in vitro and inhibits tumor growth in vivo. Mechanistically, VGLUT3 antagonizes TGFβ-mediated suppression of cell survival and clonogenicity by maintaining AKT activation. MiR-656-3p represses VGLUT3 expression and mimics the effects of VGLUT3 knockdown in vitro and in vivo.
Moreover, expression of miR-656-3p is decreased in colon cancer specimens and inversely correlates with VGLUT3 expression.

This is particularly interesting and important from a therapeutic standpoint because numerous glutamatergic signaling inhibitor, many of which have been found unsuitable for treatment of neuropsychiatric disorders for reasons such as inability to readily penetrate blood brain barriers. Since GRM3 and VGLUT3 are upregulated in colon cancer, but rarely expressed in normal peripheral tissues, targeting GRM3 and VGLUT3 with such agents would not likely cause adverse neurological or peripheral side effects, making them attractive and specific molecular targets for colon cancer treatment.
# TABLE OF CONTENTS

ACKNOWLEDGMENTS .......................................................................................................................... i

ABSTRACT ........................................................................................................................................ ii

TABLE OF CONTENTS ...................................................................................................................... iv

LIST OF TABLE .................................................................................................................................. viii

LIST OF FIGURES ............................................................................................................................. ix

LIST OF ABBREVIATIONS .................................................................................................................. xi

CHAPTER 1: INTRODUCTION .............................................................................................................1

  Colorectal cancer .............................................................................................................................. 1

  TGFβ and cancer .............................................................................................................................. 1

  MiRNAs ........................................................................................................................................... 6

  miRNAs and cancer ......................................................................................................................... 9

  MiRNAs and TGFβ .......................................................................................................................... 10

  Glutamate and its signaling ............................................................................................................. 11

CHAPTER 2: MATERIAL AND METHODS .........................................................................................18

  Cell lines and reagents .................................................................................................................... 18

  Western blot analysis, RT-PCR and Q-PCR assays ....................................................................... 18

  Apoptosis assays ............................................................................................................................ 19

  Soft agarose assays ......................................................................................................................... 19
Plasmids construction and lentiviral infection ............................................................. 19

Luciferase Assays .............................................................................................................. 20

In vivo xenograft model .................................................................................................... 20

IHC staining of GRM3/VGLUT3 in xenograft tumors and human patient samples .......... 21

TUNEL assay and Ki67 staining ....................................................................................... 21

In Situ hybridization .......................................................................................................... 22

Transwell assays .............................................................................................................. 22

Statistical analysis ............................................................................................................. 22

CHAPTER 3: MIRNA LIBRARY SCREENING ................................................................. 25

CHAPTER 4: THE MIR-487B-3P/GRM3/TGFβ SIGNALING AXIS IS AN IMPORTANT REGULATOR OF COLON CANCER TUMORIGENESIS .......................................................... 28

INTRODUCTION ............................................................................................................... 28

RESULTS ......................................................................................................................... 30

Expression of GRM3 is markedly increased in colon cancer specimens ......................... 30

GRM3 is critical for tumor growth in vivo ......................................................................... 33

A GRM3 antagonist mimics GRM3 knockdown in vitro and in vivo ................................. 39

GRM3 antagonizes TGFβ to regulate cell survival through the PKA/AKT signaling axis ... 42

MiR-487b-3p regulates GRM3 expression in colon cancer cells ....................................... 46

MiR-487b-3p regulates PKA/AKT activation and mimics GRM3 knockdown effect in colon

......
Regulation of glutamate homeostasis in cancers.................................................................94

Glutamate activates multiple pathways through multiple receptors.....................................96

Regulation of GRM3.............................................................................................................96

Other insights of VGLUT3 and GRM3................................................................................97

Pharmacology of glutamate pathway inhibitors in cancer treatment .....................................98

Literature Cited .....................................................................................................................99
LIST OF TABLE

Table 2.1 Antibodies ................................................................................................................23

Table 2.2 Primers .......................................................................................................................24

Table 3.1 List of increased and decreased miRNAs from screening .......................................27
LIST OF FIGURES

Figure 1.1. Schematic diagram of TGF\(\beta\) signaling pathway. ......................................................... 2

Figure 1.2 Biogenesis of miRNAs. ................................................................. 8

Figure 1.3. iGluR structure and activation. .......................................................... 14

Figure 1.4 mGluR structure and conformational activation. ........................................... 15

Figure 3.1 workflow of miRNAs screening in FET cells using TGF\(\beta\). ................................. 27

Figure 4.1. GRM3 expression is elevated significantly in colon cancer specimens. .................. 31

Figure 4.2. GRM3 expression is upregulated in colon cancer cells. ........................................ 34

Figure 4.3. GRM3 mediates tumor growth in vivo. ......................................................... 37

Figure 4.4. The GRM3 antagonist inhibits tumor growth in vivo. .......................................... 40

Figure 4.5. GRM3 antagonizes TGF\(\beta\)-mediated activation of PKA/AKT. ................................. 44

Figure 4.6. GRM3 is a direct target of miR-487b-3p. ......................................................... 48

Figure 4.7. MiR-487b-3p mimics the effect of GRM3 knockdown in colon cancer cells. ...... 51

Figure 4.8. MiR-487b-3p suppresses GRM3 expression and inhibits tumor growth in vivo. .. 53

Figure 4.9. Expression of miR-487b-3p is decreased in colon cancer specimens. ................... 55

Fig. 4.10. miR-487b-5p dose not target GRM3 ....................................................................... 57

Fig. 4.11. Comparison of miR-487b-3p expression between normal colon and colon tumor samples ........................................................................................................... 58

Fig. 4.12. GRM4 mRNA expression in HCECs and colon cancer cell lines ......................... 59
Fig. 4.13. Comparison of percentage of GRM3 positive cells in well-, moderately- and poorly-differentiated colon tumors. .......................................................... 60

Fig. 4.14. miR-487b-3p and knockdown of GRM3 inhibit ERK activation .................. 61

Fig. 4.15. GRM3 has no effect on BMP signaling .................................................. 62

Figure 5.1. VGLUT3 expression is elevated in colon cancer cells and patient specimens..... 69

Figure 5.2. Knockdown of VGLUT3 sensitizes colon cancer cells to stress-induced apoptosis and enhances TGFβ effect................................................................. 72

Figure 3. VGLUT3 mediates tumor growth of colon cancer cells in vivo .................. 74

Figure 5.4. VGLUT3 is a direct target of miR-656-3p ........................................... 78

Figure 5.5. MiR-656-3p mimics the effect of VGLUT3 knockdown in colon cancer cells.... 80

Figure 5.6. MiR-656b-3p suppresses VGLUT3 expression and inhibits tumor growth in vivo. ........................................................................................................ 83

Figure 5.7. Expression of miR-656-3p is decreased in colon cancer specimens .......... 85

Figure 5.8. A proposed model of crosstalk between miR-656-3p, VGLUT3 and TGFβ signaling ................................................................................................................................. 87

Fig. 5.9. Correlation of VGLUT3 with tumor grade, stages and differentiation .......... 88

Fig. 5.10. VGLUT3 has no effect on ERK and PKA activation ................................. 89

Fig. 5.11. Glutamate secretion was evaluated in the control and VGLUT3 knockdown cells. 90
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AGO</td>
<td>Argonaute family</td>
</tr>
<tr>
<td>AMPA</td>
<td>a-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid</td>
</tr>
<tr>
<td>ATD</td>
<td>the amino terminal domain</td>
</tr>
<tr>
<td>BMP</td>
<td>bone morphogenetic protein</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic AMP</td>
</tr>
<tr>
<td>CRC</td>
<td>colorectal cancer</td>
</tr>
<tr>
<td>CRD</td>
<td>cysteine-rich domain</td>
</tr>
<tr>
<td>CTD</td>
<td>C-terminal domain</td>
</tr>
<tr>
<td>CTGF</td>
<td>connective tissue growth factors</td>
</tr>
<tr>
<td>DNRII</td>
<td>dominant negative TGFβ RII</td>
</tr>
<tr>
<td>EAATs</td>
<td>excitatory amino acid transporter</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>GFDS</td>
<td>growth factor deprivation stress</td>
</tr>
<tr>
<td>GPCRs</td>
<td>G-protein-coupled receptors</td>
</tr>
<tr>
<td>GRKs</td>
<td>GPCR kinases</td>
</tr>
<tr>
<td>HCEC</td>
<td>immortalized human colon epithelial cells</td>
</tr>
<tr>
<td>hTERT</td>
<td>human telomerase reverse transcriptase</td>
</tr>
<tr>
<td>iGluR</td>
<td>ionotropic receptors</td>
</tr>
<tr>
<td>IP3</td>
<td>inositol 1,4,5-trisphosphate</td>
</tr>
<tr>
<td>LBD</td>
<td>ligand binding domain</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
</tr>
<tr>
<td>---------</td>
<td>-----------</td>
</tr>
<tr>
<td>mGluR</td>
<td>metabotropic receptors</td>
</tr>
<tr>
<td>miRNAs</td>
<td>MicroRNAs</td>
</tr>
<tr>
<td>MMPs</td>
<td>the matrix metalloproteinase</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>PKA</td>
<td>of protein kinase A</td>
</tr>
<tr>
<td>PNS</td>
<td>peripheral nervous system</td>
</tr>
<tr>
<td>Pol II</td>
<td>RNA polymerase II</td>
</tr>
<tr>
<td>RISC</td>
<td>RNA-induced silencing complex</td>
</tr>
<tr>
<td>SBE</td>
<td>SMAD binding element</td>
</tr>
<tr>
<td>SHIP</td>
<td>Src homology 2 domain-containing 5’ inositol phosphatase</td>
</tr>
<tr>
<td>SNPs</td>
<td>polymorphisms</td>
</tr>
<tr>
<td>SXC</td>
<td>independent cystine/glutamate exchanger, System $x_c^-$</td>
</tr>
<tr>
<td>TGFβ</td>
<td>transforming growth factor beta</td>
</tr>
<tr>
<td>TMAs</td>
<td>Tissue micro-arrays</td>
</tr>
<tr>
<td>TMD</td>
<td>transmembrane domains</td>
</tr>
<tr>
<td>UTR</td>
<td>3’-untranslated regions</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
<tr>
<td>VGLUTs</td>
<td>vesicular glutamate transporters</td>
</tr>
</tbody>
</table>
CHAPTER 1: INTRODUCTION

Colorectal cancer

Colorectal cancer (CRC) is a common and fatal disease. Nearly 150,000 U.S. residents are diagnosed annually with CRC, and approximately one-third of CRC patients die from the disease. CRC begins as a benign adenomatous polyp, which develops into an advanced adenoma with high grade dysplasia and then progresses to an invasive cancer. Stage I and II tumors which are confined within the wall of the colon are curable by surgery. Combination of surgery and adjuvant chemotherapy can increase 3-year disease free survival for up to 73% of cases of stage III tumor. But stage IV tumors which metastasize to distant sites are usually incurable. Therefore, understanding the mechanisms of CRC development and metastasis and identifying novel targets for CRC treatment are of importance and challenge.

TGFβ and cancer

The transforming growth factor-beta (TGFβ) was discovered more than three decades ago and was isolated as a secreted factor from sarcoma virus-infected cells. TGFβs are 25-kDa cytokines that play a pivotal role during mammalian development and act as a regulator of wound healing, fibrosis, angiogenesis and differentiation of the cells. More than 40 different members have been identified in this family. In mammalian cells, there are three TGFβ isoforms (TGFβ1, TGFβ2, and TGFβ3), among which TGFβ 1 is the most abundant and universally expressed isoform. TGFβ signaling is initiated by TGFβ binding to TGFβ receptor type II (TGFβRII) which binds and activates TGFβ receptor type I (TGFβRI). Activated TGFβRI subsequently phosphorylates SMAD2 and SMAD3 at the carboxyl terminal serines. Following phosphorylation, SMAD2 and SMAD3 form heteroligomeric complexes with the co-SMAD,
SMAD4, and accumulate in the nucleus. SMAD proteins recognize DNA sequence CAGAC, called SMAD binding element (SBE). They also bind to some other GC rich sequences, but the binding for those DNA sequences is weak. SMADs usually associate with other transcriptional cofactors to achieve specific regulation of TGFβ-mediated transcription response (Fig 1.1) \(^6\). Other members in the TGFβ superfamily, such as bone morphogenetic proteins (BMPs), active their signaling through other R-SMAD proteins (such as SMAD1, 5 and 8) \(^7\).

**Fig 1.1**

![Figure 1.1. Schematic diagram of TGFβ signaling pathway.](image)

On the cell membrane, TGFβ binds to the receptor II which in turn attracts and phosphorylates TGFβ receptor I. Activated receptor I phosphorylates Smad2/3 which recruit smad4 in the nuclear. In the nucleus, Smad complex bind to the cofactor and regulate target genes engaged in both tumor promotion and tumor repression.
TGFβ/SMAD signaling is regulated by multiple mechanisms to maintain tightly controlled TGFβ response. SMAD7 is the first described inhibitory SMAD which negatively regulates TGFβ response by preventing access of SMAD2/3 to the kinase domain of receptor I. SMAD7 also recruits phosphatases and ubiquitin ligases (smurf1/2) to the active TGFβ receptor I to terminate TGFβ signaling. Another inhibitory SMAD, SMAD6, competes with SMAD4 for binding to receptor activated SMAD7.

In addition to mediating the transcription of its target genes, TGFβ can also activate GTPases (Rac1, Cdc42, RhoA and Ras), MAP kinases (JNK, p38 MAPK and ERK1/ERK2), growth and survival promoting kinases (PI3K, AKT/PKB and mTOR) and tyrosine kinases (FAK, Src and Abl) in different cell context.

In normal condition, TGFβ is released by blood platelets and various stromal components. TGFβ is frequently present in tumor environment. Tumor infiltrating cells such as leukocytes, macrophages, and bone marrow-derived endothelial, mesenchymal, and myeloid precursor cells can secret TGFβ, tumor cells themselves and tumor stroma cells are sources of TGFβ too. In tumors, TGFβ is a potent inducer of growth inhibition at early stages of tumorigenesis. For example, when the tumor is still benign, TGFβ acts directly on tumor cells to suppress tumor outgrowth by inducing expression of CDK inhibitors p15INK4B and/or p21CIP1. The induction of p15 and p21 by TGFβ is through SMADs and other transcriptional factors like FoxO forkhead and Sp1. Suppression of oncogenes like c-Myc and ID family of helix-loop-helix transcription factors (ID1,ID2,ID3) is also observed in many cell types that are growth inhibited by TGFβ. TGFβ has also been shown to induce apoptosis in epithelium, liver, immune system and tumors. In hepatocarcinomas, TGFβ induces apoptosis by increasing the
interaction of Daxx protein with TGFβ RII, which further actives JNK and Fas-mediated apoptotic pathways. In many types of cells, TGFβ induces pro-apoptotic Bcl-2 family members, Bmf and Bim, which activate Bax and induce apoptosis. In some other types of cells, TGFβ suppresses the expression of anti-apoptotic proteins Bcl-XL and Bcl-2. In immune cells, TGFβ shows a strong pro-apoptotic effect by inducing expression of Src homology 2 domain-containing 5’ inositol phosphatase (SHIP), which causes cell death of immune cells. In colon cancer cells, TGFβ antagonizes the survival pathways through downregulation of PI3K-AKT and Bcl-XL. In addition, TGFβ prevents cellular immortalization by regulating the expression of human telomerase reverse transcriptase (hTERT) in both normal and cancer cells.

Mutations in the TGFβ signaling pathway occur in at least half of colorectal cancer, commonly inactivating TGFβ RII, SMAD2, SMAD3 or SMAD4, which leads to its inability to inhibit cell growth and induce apoptosis. Mutations of TGFβ RII lead to the formation of truncated or kinase inactive mutant forms of the receptor. Mutations of SMADs are mostly due to deletions, frameshift, nonsense and missense mutations. Overexpression of SMAD repressors also contributes to the inhibition of SMAD signaling. For example, SnoN, Ski, and inhibitory SMAD family members SMAD7 have been found to be overexpressed in many different types of cancers. Besides the change on TGFβ receptors and SMADs, the alteration of other signaling pathways can also affect TGFβ signaling during tumor progression. For instance, the Ras-Raf-MAPK pathway regulates the activation of SMAD3 by regulating its phosphorylation.
As tumor progresses, the cytostatic effects of TGFβ are often lost, and instead, TGFβ is shown to stimulate tumor progression by inducing epithelial to mesenchymal transition (EMT), stimulating angiogenesis and immunosuppression \(^{31}\). In some cancers, TGFβ secretion is correlated with high tumor grade and metastasis \(^{32}\). As mentioned above, TGFβ secreted by tumor cells and tumor stroma cells has a huge effect on immune system. It inhibits expression and activation of interleukin-2 and its receptors and blocks its stimulation on T cells \(^{33}\). TGFβ also exerts inhibitory effect on proliferation and differentiation of T lymphocytes, lymphokine-activated killer cells, nature killer cells, neutrophils, macrophages and B cells \(^{34}\). It has been shown that TGFβ inhibits the expression of tumor cell surface major histocompatibility complex class II antigen in a SMAD3 dependent manner \(^{35}\). Taken together, TGFβ in the tumor environment helps tumor cells to escape host immunosurveillance and contributes to tumor progression.

Angiogenesis is essential to allow blood vessels to deliver oxygen and nutrients to tumor cells. TGFβ signaling has been shown to be correlated with increased micro vessel density in many types of cancers \(^{36}^{37}\). The most common role of TGFβ on angiogenesis is to stimulate the expression of vascular endothelial growth factor (VEGF) and connective tissue growth factors (CTGF) \(^{38}^{39}\). In addition, the matrix metalloproteinase (MMPs) induced by TGFβ allows endothelia cells to be released from the basement and migrate to the tumor site \(^{40}\). Moreover, TGFβ represses the expression of angiopoiétin-1 to allow the tumor-associated blood vessels to become more permeable \(^{41}\).

EMT is an important process that cancer cells use to migrate and invade \(^{42}\). The role of TGFβ on EMT has been well characterized. The TGFβ-SMADs canonical pathway
transcriptionally activates EMT regulatory factors, such as SNAIL, SLUG, ZEB-2 and TWIST, resulting in the loss of epithelial cell polarity and the increase of migration and invasion \(^{43}\). TGFβ induced EMT changes the profile of adhesion molecules and promote cancer cells to detach from their primary site and disseminate throughout the stroma.

TGFβ significantly affects colon cancer biological behaviors. Moderate and well differentiated colon tumors were growth inhibited by TGFβ, while, metastatic colon tumors responded to TGFβ by invasion and proliferation\(^{44}\). In the well differentiated colon tumors, TGFβ activates its downstream targets which are important cell cycle checkpoint genes, including p21, p17 and p15 \(^{44}\). TGFβ also interacts with PI3K-AKT pathways and induce apoptosis in colon cancer cells \(^{21}\). TGFβ RII mutation is the most common alteration in colon cancers \(^{45}\). Loss of TGFβ RII function grants a growth advantage for cancer cells by cooperating with mutant Kras and Wnt signaling \(^{46}\). SMAD4 is also a frequently mutated gene in TGFβ pathway. SMAD4 is a key mediator for TGFβ induced antiproliferative response, but TGFβ induced EMT is not dependent on SMAD4 \(^{47}\). This indicate that loss of SMAD4 function might be an important event during colon cancer progression.

**MiRNAs**

MicroRNAs (miRNAs) are approximately 19–25 nucleotides long (with an average of 22 nucleotides), noncoding RNA molecules that post-transcriptionally regulate gene expression by binding either to the 3’untranslated regions (3’UTR), 5’untranslated regions (5’UTR) or the coding sequence of protein-encoding mRNAs \(^{48}\). The region at the 5’ end of miRNAs that spans from nucleotide position 2 to 7 is termed the 'miRNA seed'. It is crucial for target recognition. The downstream nucleotides of miRNA (particularly nucleotide 8 and less importantly
nucleotides 13–16) also contribute to base pairing with the targets. miRNAs regulate approximately 30–50% human genes and majority of genetic pathways. miRNA genes are transcribed by RNA polymerase II (Pol II). The long primary transcript has a local hairpin structure where miRNA sequences are embedded. Majority of human miRNAs are encoded by introns of noncoding or coding transcripts, but some miRNAs are encoded by exonic regions. The transcription of miRNAs is controlled by RNA Pol II associated transcription factors and epigenetic regulators such as P53, MYC and other DNA/RNA binding proteins. Following transcription, the primary miRNAs are processed through several steps to become mature miRNAs. The maturation of primary miRNAs is initiated by a nuclear RNase III, called Drosha, which forms a microprocessor with a cofactor DGCR8 and crops the stem–loop to release a small hairpin-shaped RNA of ~65 nucleotides in length (pre-miRNA). Pre-miRNAs are exported to the cytoplasm after processed by Microprocessor. The protein exportin 5 (EXP5; encoded by XPO5) forms a transport complex with GTP-binding nuclear protein RAN•GTP, which exports the pre-miRNA out of the nuclear. In the cytoplasm, pre-miRNAs are cleaved by RNase III type endonuclease called Dicer, liberating a small RNA duplex. The small RNA duplex is subsequently incorporated into an AGO protein to form an effector complex called RNA-induced silencing complex (RISC). As a result, the passenger strand is quickly removed and a mature RNA-induced silencing complex is formed. MiRNA biogenesis is shown in Fig 1.2.
Fig 1.2

Fig 1.2. Biogenesis of miRNAs.

Pri-miRNA are transcribed by RNA polymerase II, and processed to pre-miRNA in the nucleus. RNA splicing is also a source of pre-miRNAs. Pre-miRNAs are transported into the cytosol, where they are pressed by Dicer to become mature miRNAs and regulate target gene expression.
miRNAs and cancer

A significant number of miRNAs are located at genomic regions linked to cancer\textsuperscript{56}. The first evidence of aberrant miRNA expression in human cancers was described in B-cell chronic lymphocytic leukemia. A chromosomal deletion at the 13q14 locus resulted in the loss or reduction of miR-15 and miR-16 expression, which are inhibitors of oncogenetic protein BCL-2\textsuperscript{57}. miRNA expression can be altered through different mechanisms, including chromosomal abnormalities, epigenetic changes, mutations and single nucleotide polymorphisms (SNPs), and defects in the miRNA biogenesis machinery\textsuperscript{58}. Many miRNAs reside at fragile site of the chromosome where sister chromatid exchange, translocation, deletion, amplification happen very frequently. miR-143 and miR145 has been found to be decreased in B cell chronic lymphocytic leukemias and lung cancers, while miR-19-92, located at chromosome 13q31, is found overexpressed due to chromosomal amplification in those cancers\textsuperscript{57,59}. In addition, epigenetic changes affect miRNAs expression as well. MiR21, miR-203 and miR-205 were found to be overexpressed in ovarian carcinomas through hypomethylation mechanism\textsuperscript{60}. On the contrary, decreased miR-124a expression in colon, breast, and lung cancers was attributed to DNA hypermethylation\textsuperscript{61}.

miRNAs have been demonstrated to function as oncogenes or tumor suppressors\textsuperscript{62}. The overexpression of miR-155 is associated with several types of lymphomas. Transgenic mice overexpressing miR-155 developed a preleukemic lymphoproliferative disease that progressed to B-cell leukemia and high grade lymphoma\textsuperscript{63}. Down regulation of some miRNAs might be advantageous for cancer cells. For example, miR-17 was found to be downregulated in breast cancers and ectopic expression of this miRNA reduced cancer cell proliferation\textsuperscript{64}.
In cancers, miRNAs affect cell growth, proliferation, apoptosis, invasion and metastasis by regulating genes in different signaling pathways \(^6^5\). miR-221/222, miR-17-92 and miR-106-25 have been reported to target negative cell-cycle regulators and promote cancer cell proliferation \(^6^6\). miR15a/16 was shown to target cyclin D1, cyclin D3, cyclin E1 and CDK6, and their downregulation increased expression of multiple cell cycle-promoting genes \(^6^7\).

Furthermore, the accumulated data on miRNA expression in tumors demonstrate that miRNAs are promising candidates for prognostic and/or diagnostic markers. It has been shown that the miR-222, miR-106a, and miR-17-92 cluster is associated with the degree of differentiation of hepatocellular carcinomas \(^6^8\). High miR-21 expression was present in colon adenomas and in tumors with more advanced TNM staging \(^6^9\). Another study showed that high level of MiR-92a was associated with decreased survival in patients with small-cell lung cancer \(^7^0\). And the expression of MiR-210 was found to be associated with poor clinical outcome of breast cancer \(^7^1\).

**MiRNAs and TGFβ**

miRNAs and the TGFβ pathway are tightly interacted with each other. Transcription of primary miRNAs can be dynamically regulated in response to stimulation of many growth factors including TGFβ \(^7^2\). TGFβ promotes miRNA processing by regulating miRNA DROSHA microprocessor complex in a SMAD-depend manner \(^7^3\).

Upon TGFβ treatment, alterations in expression of numerous miRNAs have been reported in different types of cells. Expression of the miR-200 family (miR-200a, miR-200b, miR-200c, miR-141 and miR-429) and miR-205 was markedly downregulated upon TGFβ treatment. In addition, TGFβ suppresses expression of many other miRNAs, miR-145, Let-7d, miR-155, \(\ldots\)
miR-34, miR-27b, miR-29. On the contrary, there are quite a few miRNAs whose expression is upregulated by TGFβ, for example, miR-21, miR-142, miR-146, miR17/92, etc. 74.

The TGFβ pathway is regulated by miRNAs at multiple levels. Expression of most, if not all, members of the canonical TGFβ signaling pathway may be influenced by miRNAs. The miR-23b cluster (including miR-23b, miR-27b and miR-24-1) suppress TGFβ signaling by downregulating expression of SMAD3, SMAD4, and SMAD5 75. In the heart, downregulation of miR-133 and miR-590 enhances TGFβ signaling through TGFβ RI and RII 76. In the liver, miR-21 enhances TGFβ signaling by targeting the negative regulator SMAD7 77. While miR-26a was found to target SMAD1, miR-141 and miR-200a directly inhibit TGFβ RII expression in rat proximal tubular epithelial cells 78,79.

miRNAs can also downregulate TGFβ target genes, which ultimately affects the outcome of TGFβ effect. The miR-106b/25 cluster (miR-106b, miR-93 and miR-25) was reported to interfere with the tumor suppression effect of TGFβ by targeting the cell cycle inhibitor p21Waf1/Cip1 and the pro-apoptotic protein BCL2L11 (BIM) in gastric cancer 80. BIM is also targeted by miR-17/92 in B cells 81.

Glutamate and its signaling

Glutamate is a nonessential amino acid, a major bioenergetic substrate for proliferating in normal and neoplastic cells. In the nervous system, glutamate is one of the key neurotransmitters and it participates in several physiological processes such as learning, memory, behavior and long-term potentiation and synaptic plasticity 82,83. Glutamate has been shown to regulate proliferation, migration and survival of neuronal progenitors and immature neurons during development of nervous system 84-86. Moreover, its function in regulating cell
proliferation and migration has also been described in human brain, breast, lung and colon cancer cell lines. Recent studies have demonstrated that human cancer cells release high level of glutamate, which triggers invasive tumor growth. Serum glutamate level was found correlated with Gleason Score in prostate cancer patients. An analysis of freshly frozen human samples also demonstrated that glutamate level was strikingly increased in chronic pancreatitis and pancreatic ductal adenocarcinoma tissues compared to normal pancreatic tissue.

Glutamate is stored in synaptic vesicles and released upon stimulation. In mammals, glutamatergic system is maintained by a group of glutamate transporters, which include two super families: the excitatory amino acid transporter (EAATs) located at the plasma membrane and the vesicular glutamate transporters (VGLUTs) located at the membrane of synaptic vesicles. The family of VGLUTs is comprised of three highly homologous proteins VGLUT1-3. They transport glutamate from cytoplasm into the vesicles to be released. Expression of VGLUTs is mostly complementary with limited overlap. VGLUT1 and VGLUT2 are mainly expressed in glutamatergic neurons whereas VGLUT3, encoded by SLC17A8 gene, is present in a limited number of glutamatergic neurons in multiple brain regions and in a population of symmetrical synapses. VGLUT3 is the only vesicular glutamate transporter detected in the dendrites of striatal neurons. In addition to CNS, VGLUTs can be found in peripheral nervous system (PNS) and in the glutamate-secreting non-neuronal cells of different organs, such as the pineal gland, islets of Langerhans, intestine and stomach, bone and testes.

Glutamate signaling is initiated when glutamate binds to different type of receptors. Glutamate receptors are divided into two groups, ionotropic glutamate receptors (iGluR) and
metabotropic glutamate receptors (mGluR)\textsuperscript{101}. \textit{iGluRs} are quaternary ligand-gate ion channels that allow cation influx upon glutamate binding\textsuperscript{101}. Based on structural similarities \textit{iGluRs} are classified into three groups, N-methyl-D-aspartate (NMDA) receptor, \textit{\textalpha}-{amino\texttext{-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor}, and kainate receptor subfamilies. mGluRs belong to the superfamiliy of G-protein coupled receptors, they are classified into three subgroups. mGluR1 and mGluR5, coupled to the phospholipase C through G\textsubscript{q} G-protein, belong to group I; group II consists of mGluR2 and mGluR3, whereas group III comprises of mGluR4, mGluR6, mGluR7 and mGluR8, both of which are negatively coupled to adenylate cyclase through G\textsubscript{i} G-protein\textsuperscript{102}.

\textit{iGluRs} have four conserved domains, including the extracellular amino-terminal (ATD), ligand-binding (LBD), transmembrane (TMD), and intracellular carboxy-terminal (CTD) domains. Glutamate or agonist binds to the LBD, resulting in a conformational change of the TMD, which forms a channel in the membrane and allows calcium to pass into the cells (Fig 1.3)\textsuperscript{103}. 
iGluRs share the same basic structure: the N terminus (N), the amino terminal domain (ATD), the ligand binding domain (LBD), the four transmembrane domains (TMD; numbered 1-4) and the C-terminal domain (CTD). Panel A shows the resting conformation of iGluR. The entire protein consists of 4 subunits, two of them can create a channel through the plasma membrane. When glutamates bind to LBD (Panel B), a conformational change in TMD domain 1 causes opening of the channel where Ca2+ passes into the cell by diffusion.
All mGluRs share a basic structure which includes a large extracellular ATD at the N-terminus, a cysteine-rich domain (CRD), a seven alpha-helical TMD and an intracellular CTD. GluRs function when dimerized at the CRD domain, with two glutamate molecules binding to the ATD (Fig 1.4) \(^{103}\).

**Fig 1.4**

Figure 1.4 mGluR structure and conformational activation.

mGluR family members share the same basic structure: the N terminus (N), the amino terminal domain (ATD), the cysteine rich domain (CRD), the seven transmembrane domains (TMD; numbered) and the C-terminal domain (CTD). Panel A shows the resting conformation of mGluR1a. Cysteine residues in the CRD aid in dimerization, as indicated by the S-S bond. When glutamate bind to ATD domain (Panel B), a conformational change in the ATD causes activation of bound G proteins.
Group I mGluRs couple to \( G_{q/11} \) and activate phospholipase \( C_\beta \), resulting in the hydrolysis of phosphotinositides and generation of inositol 1,4,5-trisphosphate (IP3) and diacyl-glycerol. This classical pathway leads to calcium mobilization and activation of protein kinase C (PKC) \(^{104} \). Recently, it is been recognized that this receptor can activate many other protein kinase pathways such as casein kinase 1, cyclindependent protein kinase 5, Jun kinase, components of the mitogen-activated protein kinase/extracellular receptor kinase (MAPK/ERK) and the mammalian target of rapamycin (MTOR)/p70 S6 kinase pathway \(^{105-109} \). Group II and group III receptors are coupled with \( G_{i/o} \) which inhibits adenylyl cyclase. In addition, they have been shown to activate MAPK and PI3K-AKT pathways \(^{107} \).

In recent years, many studies have shown that mGluRs are the predominant mediators of glutamatergic signaling in cancers \(^{103} \). Function of mGluRs in cancer progression is first studied in tumors of central nervous system \(^{110} \). Many evidences have also shown that mGluRs are involved in other types of cancers \(^{111-114} \). Both group I and II mGluRs have been implicated to play a role in glioma. Inhibiting mGluR1 inhibits U87 glioma cell growth and induces apoptosis both in vitro and in vivo by mitigating the activation of the PI3K/Akt/mTOR pathway \(^{115} \). Another study showed that an mGluR2/3 antagonist suppressed glioma cell growth and removal of the antagonist restored cell growth \(^{113} \). In addition, mGluRs are also studied in other non-neuronal cancers. Chen group found that the aberrant expression of mGluR1 is the driving force of melanomagenesis in transgenic mouse models, and mGluR1 mediated activation of the MAPK and PI3K/AKT pathways is critical in melanoma pathogenesis \(^{116} \). An exon capture sequencing of GPCRs study showed that in malignant melanoma specimens, somatic mutations of mGluR3 (GRM3) (E767K, S610L,G571E and E870K) can regulate the phosphorylation of
MEK1/2 and induce micro metastasis of melanoma\textsuperscript{117}. In colorectal cancer, mGluR4 has been found to be correlated with 5-Fu resistance and poor prognosis\textsuperscript{118}. The increasing evidence is showing that glutamate signaling could be a potential target for cancer treatment.
CHARAPTER 2: MATERIAL AND METHODS

Cell lines and reagents

The immortalized human colon epithelial cells (HCEC) were provided by Dr. Jerry Shay. The human colon cancer HCT116, RKO, FET, CBS, HCT116b and GEO cells were cultured in McCoy’s 5A medium (Sigma) with 10 ng/ml epidermal growth factor (EGF), 20 µg/ml insulin, and 4 µg/ml transferrin. When subjected to GFDS, cells were cultured in medium without EGF, insulin and transferrin. Cells were maintained at 37°C in a humidified incubator with 5% CO₂ and checked periodically for mycoplasma contamination. TGFβ and LY341495 were obtained from R&D Systems. The miR-656-3p and miR-487b-3p inhibitor were obtained from and Qiagen Inc. respectively. Antibody information is included in Supplemental Table 2.1.

Western blot analysis, RT-PCR and Q-PCR assays

Whole cell lysates were prepared in RIPA buffer (1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 10 mM Tris–HCl, pH 7.5, 5 mM EDTA and a protease inhibitor cocktail from Sigma-Aldrich). Equivalent amounts of protein were separated by SDS–PAGE and transferred to a PVDF membrane (Millipore). Proteins were detected using an enhanced chemiluminescence system (Amersham Biosciences).

Expression of miR-656-3p and miR-487b-3p were determined by miScript primer assays and miScript SYBR® Green PCR Kit from Qiagen Inc. RNU6-2 was used as an endogenous reference gene.

Q-PCR analysis of GRM3 mRNA was performed using SYBR Green qPCR Mastermixes (Qiagen). The primer sequences are GCACCTCAACAGGTTCACTGTG-F and
Expression of VGLUT1-3 and GRM2-4 mRNA was determined by RT-PCR analysis. The primer sequences are included in Table 2.2.

**Apoptosis assays**

Apoptosis was detected using a DNA fragmentation ELISA kit (Roche). DNA fragmentation ELISA assays were performed according to manufacturer’s protocol. Briefly, cells were seeded in 96-well plates and subjected to growth factor deprivation stress (GFDS) or treated with TGFβ. The cells were stained with MTT to determine cell numbers or lysed for the ELISA assays to determine apoptosis. The relative apoptosis was determined by dividing ELISA values by MTT values of each sample.

**Soft agarose assays**

Cells were seeded in culture medium containing low melting point agarose (Thermo Scientific) at a density of 3,000 cells per well in 6-well plates. Two weeks later, colonies were stained with 1% iodonitrotetrazolium violet (Sigma-Aldrich). Visible colonies were counted.

**Plasmids construction and lentiviral infection**

The miR-656 and miR-487b precursor-expressing lentiviral vector, pCDH-CMV, was from SBI. ShRNAs targeting GRM3 or VGLUT3 were cloned into FSIPPW lentiviral vector. The targeting sequences of VGLUT3 are VGLUT3 sh2 5'-GCTGTAGGATTTAGTGGCTTCGCTA-3', VGLUT3 sh3 5'-CCCAGAATTGTG AAGTCCAGAAGAA-3'. pCDF1-VGLUT3 was purchased from Harvard plasmid, provided by David E Root group. Clone: HsCD00421938, PMID 21706014. The targeting sequences of GRM3 was described previously 117: sh1, 5'-CAGAACATGGAAATAACCATT-3'and sh2, 5'-GCCTGTTCCTATTAACGAAA-3
SMAD2 shRNA and SMAD3 shRNA are 5'-GCACCTGCTCTGAATATTTG-3' and 5'-GGATTGAGCTGCACCTTGAA TG-3' respectively. PKAc shRNA was described previously\textsuperscript{121}. pCDF1-GRM3 WT was a gift from Yardena Samuels (Addgene #31798).

293 packaging cells were co-transfected with pPackH1 packaging plasmid mix (SBI) and the lentiviral vectors using Fugene HD (Promega). Viruses were harvested 48 hours later and used to infect target cells.

**Luciferase Assays**

The predicted miR-656-3p recognition sites in the 3’UTR of VGLUT3 and corresponding mutated sequences were synthesized and cloned into psiCHECK\textsuperscript{TM}-2 (Promega) downstream of Renilla reporter gene. The predicted miR-487b-3p recognition sites in the 3’UTR of GRM3 was synthesized and cloned in the same vector. The reporter was transfected into cells and luciferase activity was measured 48 hrs later using Dual-Luciferase Reporter Assay (Promega). Values were normalized with firefly luciferase activity.

**In vivo xenograft model**

Animal experiments were approved by University of Nebraska Medical Center (UNMC) Institutional Animal Care and Use Committee. CBS cells (4 x 10\textsuperscript{6}) expressing a control vector, miR-487b precursor, a scrambled shRNA or GRM3 shRNAs and HCT116 cells (2 x 10\textsuperscript{6}) expressing a control vector, miR-656 precursor, a scrambled shRNA or VGLUT3 shRNAs were injected into the flank of 4-6 week old male athymic nude mice on both sides (Harlan Laboratories). There were 8 mice and 16 tumors in each experimental group. Tumor volumes (V) were calculated by the formula V = W\textsuperscript{2} × L × 0.5, where W represents the largest tumor diameter and L represents the next largest tumor diameter. Upon termination of the experiments,
tumors were dissected out and photographed on the same scale.

**IHC staining of GRM3/VGLUT3 in xenograft tumors and human patient samples**

Human sample study was performed with the approval of Institutional Review Board. TMAs consisting of triplicates of 1 mm cores of colon adenocarcinomas and adjacent normal tissues of patients treated at UNMC in 2008 and 2009 were obtained from tissue core.

IHC staining was performed in paraffin slides using Novolink™ Min Polymer Detection System (Leica) following manufacture’s instruction. Briefly, slides were subjected to antigen retrieval using Novocastra Epitope Retrieval Solutions, pH6, followed by incubation with an anti-VGLUT3 or anti-GRM3 antibody overnight at 4 °C. Slides were developed with DAB after incubation with Novolink polymer. Finally, slides were counterstained with hematoxylin. The slides were scanned with IScan Coreo (Ventana Medical Systems, Inc). The staining intensity of epithelial cells was quantified with Imagescope Software (Aperio Technologies, Inc.).

**TUNEL assay and Ki67 staining**

Formalin-fixed paraffin embedded (FFPE) tissue blocks of xenograft tumors were stained for Ki67 using Novolink™ Min Polymer Detection System (Leica). TUNEL staining was performed using Trevigen In Situ Apoptosis Detection Kit following manufacturer’s instruction. Three tumors from each group were analyzed. Ten histologically similar fields were randomly selected from each slide for analysis. Apoptosis and proliferation of tumor cells was determined quantitatively by counting the numbers and calculating the percentage of positively stained cells for TUNEL and Ki67 at 20x magnification respectively.
In Situ hybridization

The double DIG labeled probe for miR-487b-3p and miR-656-3p and hybridization kit were purchased from Exiqon. Hybridization was performed following Exiqon’s protocol. Briefly, tissues were deparaffinized and digested with proteinase K (15 μg/ml; Exiqon) for 10 min at 37°C. Slides were incubated in hybridization buffer with 40 nM probe (miR-487b-3p 5DigN/AAGTGGATGACCTGTACGATT/3Dig_N/, miR-656-3p 5DigN/AGAGGTTGACTGTAATAAT/3Dig_N/) in a humidified chamber at 45°C overnight. Slides were then blocked with anti-digoxigenin-alkaline phosphatase antibodies (Roche) at 1:800 dilution for 1 hr and stained with AP substrate (NBT/BCIP tablet, Roche) at 30°C for 6 hrs. The nuclei were counterstained with nuclear fast red (Sigma). The slides were scanned with IScan Coreo (Ventana Medical Systems, Inc). The staining intensity of epithelial cells was determined using Image-Pro Plus (Media Cybernetics. Inc).

Transwell assays

Cells were seeded onto the upper surface of 8-μm pore, 6.5 mm polycarbonate filters (Corning Costar Corp.) in medium without growth factors or serum, allowed to migrate towards medium with 10% FBS for 18 hrs and stained with MTT. The cells on the upper surface of the filter were removed with a cotton swab, and those migrated to the underside were dissolved in DMSO. Absorbance was read at 570 nm.

Statistical analysis

Statistical analyses were performed using Bonferroni two-sided t-test, two-way ANOVA or Student’s t-test. *P < 0.05, ** P < 0.01, *** P < 0.001.
### Table 2.1 Antibodies

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Companies</th>
<th>Western blots</th>
<th>IHC</th>
</tr>
</thead>
<tbody>
<tr>
<td>anti-PARP</td>
<td>Cell Signaling # 9542S</td>
<td>1:1000</td>
<td></td>
</tr>
<tr>
<td>anti-pAKT S473</td>
<td>Cell Signaling # 9271S</td>
<td>1:1000</td>
<td></td>
</tr>
<tr>
<td>anti-CREB</td>
<td>Cell Signaling # 9197S</td>
<td>1:1000</td>
<td></td>
</tr>
<tr>
<td>anti-pCREB</td>
<td>Cell Signaling # 9198S</td>
<td>1:1000</td>
<td></td>
</tr>
<tr>
<td>anti-AKT</td>
<td>Cell Signaling # 4691S</td>
<td>1:1000</td>
<td></td>
</tr>
<tr>
<td>anti-SMAD2</td>
<td>Cell Signaling # 5339S</td>
<td>1:1000</td>
<td></td>
</tr>
<tr>
<td>anti-SMAD3</td>
<td>Cell Signaling # 9523S</td>
<td>1:1000</td>
<td></td>
</tr>
<tr>
<td>anti-pSMAD1/5</td>
<td>Cell Signaling # 9516S</td>
<td>1:1000</td>
<td></td>
</tr>
<tr>
<td>anti-ERK</td>
<td>Santa Cruz sc-514302</td>
<td>1:1000</td>
<td></td>
</tr>
<tr>
<td>anti-pERK</td>
<td>Santa Cruz sc-7383</td>
<td>1:1000</td>
<td></td>
</tr>
<tr>
<td>anti-Actin</td>
<td>Thermo Scientific MA5-15739</td>
<td>1:5000</td>
<td></td>
</tr>
<tr>
<td>anti-GAPDH</td>
<td>Thermo Scientific MA1-16757</td>
<td>1:5000</td>
<td></td>
</tr>
<tr>
<td>anti-Ki67</td>
<td>Thermo Scientific MA5-14520</td>
<td>1:1000</td>
<td></td>
</tr>
<tr>
<td>anti-GRM3</td>
<td>Alomone labs AGC-012</td>
<td>1:1000</td>
<td>1:500</td>
</tr>
<tr>
<td>anti-VGLUT3</td>
<td>Alomone labs AGC-037</td>
<td>1:1000</td>
<td>1:500</td>
</tr>
</tbody>
</table>
### Table 2.2. Primers

<table>
<thead>
<tr>
<th>Primers</th>
<th>sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>GRM2</td>
<td>5'-GAGAAGGTGGGGCCGTCATGAG-3</td>
</tr>
<tr>
<td></td>
<td>5'-CGCTGCCTGCCCAGCATAGGT-3</td>
</tr>
<tr>
<td>GRM3</td>
<td>5'-GCTCAAACATCGCAAGTCCTA-3</td>
</tr>
<tr>
<td></td>
<td>5'-TGTCATGGCCAGGCTTTCGTCTGTC-3</td>
</tr>
<tr>
<td>VGLUT3</td>
<td>5'-TGGGCTGGGATTCTGCATT-3</td>
</tr>
<tr>
<td></td>
<td>5'-TGCAAGAGACCCACACTTAGAC-3</td>
</tr>
<tr>
<td>VGLUT2</td>
<td>5'-TGGCAACCACCTCTTTTGT-3</td>
</tr>
<tr>
<td></td>
<td>5'-CATAGTGGACTAGGCCAGCG-3</td>
</tr>
<tr>
<td>VGLUT1</td>
<td>5'-CAGGAAGGCGCGAGACATTGGAG-3</td>
</tr>
<tr>
<td></td>
<td>5'-AGTGAACACGGGCTGCTGAAGGGA-3</td>
</tr>
<tr>
<td>VGLUT3 sh2</td>
<td>GCTGTAGGATTTAGTGCTTCGCTA</td>
</tr>
<tr>
<td>VGLUT3 sh3</td>
<td>CCCAGAATTGTGAAGTCCAGAAGAA</td>
</tr>
</tbody>
</table>
CHAPTER 3: MIRNA LIBRARY SCREENING.

Accumulating reports have shown that miRNAs are important players in many types of human cancers. TGFβ is an important regulator in colon cancer. Functional genetic screening is a systematic, non-biased and powerful approach for gene discovery. Here we used a microarray based miRNAs screening approach and identified some nova miRNAs that involved in TGFβ function in colon cancer cells.

The functional screening is described in Figure 3.1. A library of 560 miRNAs expression vectors was introduced into FET cells, after recovery from infection, cells were seeded into 10cm dish at a density of 9000 cell per well in the presence of 10ng/ml TGFβ (eight replicates). The empty vector-transduced cells were plated at the same density as a negative control, and TGFβ response defect FET Dominant negative RII cells as positive control. Two weeks after treatment, colonies were harvested and a second round of TGFβ selection was performed. By the end of two rounds selection, survived cells were harvested and genome DNA was extracted from each plate. The integrated miRNAs were amplified from genomic DNA by PCR using specific primers. The PCR product was purified and labeled with cy3 as end sample. PCR product from vector control cells which underwent two round of colony formation without any TGFβ treatment will be labeled with cy5 as initial sample. Equal amount of initial sample and end sample were mixed and hybridized to the customized microarray. Microarray data was analyzed by Agilent, the average fold change of each miRNAs compared to control was calculated, p value was calculated by Student t test. As shown in Table 3.1, some miRNAs are enriched while some others are significantly dropped out after TGFβ selection. We then focused on the dropped out miRNAs which can potentially make FET cells sensitive to TGFβ for future
studies.

We then used DIANA miPath to look for the common pathways that are regulated by the miRNAs we identified. Interestingly we found that glutamatergic pathway is highly regulated by those miRNAs. Many studies have shown the importance of glutamatergic pathway in cancers. GRM3 and VGLUT3 are two important components in glutamatergic pathway, they are also targets of the miRNAs we identified. So, we hypothesize that, GRM3 and VGLUT3 might be very important regulators of colon cancer tumorigenesis and TGFβ function.
**Figure 3.1** workflow of miRNAs screening in FET cells using TGFβ.

**Table 3.1**

<table>
<thead>
<tr>
<th>Decreased miRNA</th>
<th>P value</th>
<th>fold change</th>
<th>Increased miRNA</th>
<th>P value</th>
<th>fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>mir_937</td>
<td>0.00000</td>
<td>-88.20</td>
<td>mir_938</td>
<td>0.00237</td>
<td>3.06</td>
</tr>
<tr>
<td>mir_499</td>
<td>0.00000</td>
<td>-67.49</td>
<td>mir_612</td>
<td>0.00111</td>
<td>3.02</td>
</tr>
<tr>
<td>mir_656</td>
<td>0.00000</td>
<td>-51.68</td>
<td>mir_23a</td>
<td>0.00747</td>
<td>3.02</td>
</tr>
<tr>
<td>mir_199a_2</td>
<td>0.00001</td>
<td>-43.32</td>
<td>mir_526a_1</td>
<td>0.00112</td>
<td>2.80</td>
</tr>
<tr>
<td>mir_34a</td>
<td>0.00097</td>
<td>-36.50</td>
<td>mir_520h</td>
<td>0.00015</td>
<td>2.65</td>
</tr>
<tr>
<td>mir_655</td>
<td>0.00000</td>
<td>-31.25</td>
<td>mir_492</td>
<td>0.00406</td>
<td>2.61</td>
</tr>
<tr>
<td>mir_129_2</td>
<td>0.00391</td>
<td>-28.69</td>
<td>mir_562</td>
<td>0.01228</td>
<td>2.59</td>
</tr>
<tr>
<td>mir_424</td>
<td>0.00000</td>
<td>-22.57</td>
<td>mir_520e</td>
<td>0.00381</td>
<td>2.57</td>
</tr>
<tr>
<td>mir_206</td>
<td>0.00005</td>
<td>-21.00</td>
<td>mir_520g</td>
<td>0.00074</td>
<td>2.38</td>
</tr>
<tr>
<td>mir_634</td>
<td>0.0024</td>
<td>-17.73</td>
<td>mir_767</td>
<td>0.00049</td>
<td>2.27</td>
</tr>
<tr>
<td>mir_182_a</td>
<td>0.00081</td>
<td>-14.94</td>
<td>mir_483</td>
<td>0.00002</td>
<td>2.26</td>
</tr>
<tr>
<td>mir_487b_a</td>
<td>0.00050</td>
<td>-16.88</td>
<td>let_7i</td>
<td>0.00202</td>
<td>2.15</td>
</tr>
</tbody>
</table>

**Table 3.1** List of increased and decreased miRNAs from screening
CHAPTER 4: THE MIR-487B-3P/GRM3/TGFβ SIGNALING AXIS IS AN IMPORTANT REGULATOR OF COLON CANCER TUMORIGENESIS

INTRODUCTION

Glutamate functions as a major excitatory neurotransmitter in mammalian central nervous system. Glutamate signaling is mediated by two classes of glutamate receptors, ionotropic and metabotropic receptors. Metabotropic receptors (mGluR), a group C family of G-protein-coupled receptors (GPCRs), consist of eight members, classified into three subtypes. Group I receptors (mGluR1 and 5) are coupled to phospholipase C leading to activation of protein kinase C, whereas group II (mGluR2 and 3) and group III (mGluR4, 6, 7 and 8) receptors are negatively coupled to adenylyl cyclase, inhibiting production of cyclic AMP (cAMP). Although glutamatergic system is mainly restricted to the CNS, expression of functional glutamate receptors has been reported in non-neuronal peripheral cells such as skin and pancreatic islets. Furthermore, studies have revealed that glutamate signaling is dysregulated and may play a role in cancer.

GRM3 is the gene encoding mGluR3, which is frequently mutated in melanoma. Mutant GRM3 selectively activates MEK, leading to increased anchorage-independent growth and migration. Activation of GRM3 has been reported to BMP signaling and sustain tumorigenic potential of glioma-initiating cells. Pharmacological blockade of GRM3 reduced growth of glioma cells in vitro and in vivo. These studies suggest that GRM3 plays a role in cancer and could be a potential target for cancer treatment.

TGFβ signaling plays a dual role in cancer. While studies show that TGFβ promotes metastasis and is associated with worse prognosis, others demonstrate that it suppresses
tumorigenicity and metastasis \(132-135\) and that loss or reduction of TGFβ signaling is associated with development of metastasis \(136,137\). In genetically engineered mouse models, inactivation of TGFβ signaling increases malignancy and invasiveness of intestinal tumors of Apc mutant mice \(138-141\).

MiRNAs are a group of small non-protein coding RNAs evolutionarily conserved \(142\). MiRNAs suppress expression of gene targets at the posttranscriptional level through sequence-specific interaction with the 3’-untranslated regions (UTR), leading to translation inhibition or mRNA degradation \(48\). Alterations in miRNA expression are found to be associated with many human cancers \(79\).

Here we demonstrate that GRM3 expression is significantly upregulated in majority of human colonic adenocarcinomas tested and colon cancer cell lines. Knockdown of GRM3 expression or pharmacological blockade of GRM3 in colon cancer cells reduces cell survival and anchorage-independent growth \textit{in vitro} and inhibits tumor growth \textit{in vivo}. Mechanistically, GRM3 antagonizes TGFβ-mediated activation of protein kinase A (PKA) and inhibition of AKT activation. In addition, TGFβ signaling increases GRM3 protein stability and knockdown of GRM3 expression enhances TGFβ-mediated tumor suppressor function. Further studies indicate that GRM3 is a direct target of miR-487b-3p and that miR-487b-3p mimics the effects of GRM3 knockdown in colon cancer cells \textit{in vitro} and \textit{in vivo}. Expression of miR-487b-3p is decreased in colon adenocarcinomas and inversely correlates with GRM3 expression. Taken together, these studies indicate that the miR-487b-3p/GRM3/TGFβ signaling axis is an important regulator of colon cancer tumorigenesis and that upregulation of GRM3 is a functionally important molecular event in colon cancer. Therefore, GRM3 is a promising
molecular target for colon cancer treatment. This is particularly interesting and important from a therapeutic standpoint because numerous metabotropic glutamate receptor antagonists are available\textsuperscript{143}(37), many of which have been found unsuitable for treatment of neuropsychiatric disorders due to their inability to readily penetrate blood brain barriers. Since GRM3 is upregulated in colon cancer, but rarely expressed in normal peripheral tissues, targeting GRM3 with such agents would not likely cause adverse neurological or peripheral side effects, making GRM3 an attractive and specific molecular target for colon cancer treatment.

**RESULTS**

**Expression of GRM3 is markedly increased in colon cancer specimens.**

Although it has been implicated that GRM3 is an important player in melanoma and glioma\textsuperscript{107,128,120}, it is unknown whether GRM3 plays a role in colon cancer. GRM3 expression was therefore examined in human specimens using immunohistochemistry (IHC) analysis. Verification of the anti-GRM3 antibody is shown in Fig. 3c. Tissue micro-arrays (TMAs) consisting of 29 normal colon and 65 colon adenocarcinomas were analyzed. Mouse brain tissue was used as a positive control. GRM3 expression was very low in normal colon epithelium, but increased significantly in colon tumors (Fig. 4.1a). Quantification showed that the average intensity of GRM3 staining and percentage of GRM3 positive cells were approximately 5.5-fold and 3.8-fold higher respectively in tumors than in normal colon (Fig. 4.1b & 4.1c). In addition, GRM3 expression was increased in more than 90% of colon tumors examined (Fig. 4.1b & 4.1c). These results demonstrate that GRM3 expression is upregulated in majority of colon adenocarcinomas.
Figure 4.1. GRM3 expression is elevated significantly in colon cancer specimens.

a. Immunohistochemistry staining of GRM3 was performed in normal colon and colon adenocarcinomas. Mouse brain tissue stained with the anti-GRM3 antibody in the absence or
presence of a specific blocking peptide was used as a positive and negative control respectively. Representative images are shown. Scale bars, 100 µm. b&c, Quantification of GRM3 staining intensity (b) and percentage of GRM3 positive cells (c) was performed. The values of individual samples are shown. Error bars indicate SEM of the values. *** \( P < 0.001 \).
GRM3 is critical for tumor growth in vivo.

These observations prompted us to investigate whether GRM3 plays a functional role in colon cancer. A panel of human colon cancer cell lines and an immortalized human colon epithelial cell line, HCEC\textsuperscript{119}, were used. HCT116 and RKO cells are defective in TGF\(\beta\) signaling due to lack of TGF\(\beta\) RII\textsuperscript{144}. HCT116b cells were isolated from the same colon tumor as HCT116, but displayed much lower metastatic potential\textsuperscript{145}. FET cells, isolated from a well differentiated colon tumor, are sensitive to TGF\(\beta\)-mediated growth inhibition and apoptosis\textsuperscript{21}. CBS and GEO cells are partially responsive to TGF\(\beta\) due to low TGF\(\beta\) RII and RI expression, respectively\textsuperscript{146}. HT29 cells do not express SMAD4 due to mutations\textsuperscript{147}. All cell lines bear either KRAS or BRAF mutations, and all except RKO\textsuperscript{148} have mutated APC or \(\beta\)-catenin.

GRM3 expression was much higher in colon cancer cells than in HCECs (Fig. 4.2a, left), consistent with the results from human specimens. However, GRM3 mRNA levels were similar between HCECs and most of colon cancer cell lines (Fig. 4.2a, middle and right), suggesting that upregulation of GRM3 may be through post-transcriptional mechanism(s). Of note, expression of GRM2, the other member of group II metabotropic glutamate receptors, was almost undetectable in all cell lines (Fig. 4.2a, middle). Mouse brain tissue was used as a positive control. These results indicate that expression of GRM3 but not GRM2 is increased in colon cancer cells.

To determine GRM3 function, its expression was knocked down in FET, CBS and HCT116, three colon cancer cell lines with different genetic background. Each of two independent shRNAs (sh1 and sh2) reduced GRM3 expression by more than 90\% as compared to a scrambled shRNA and had no effect on GRM2 expression (Fig. 4.2b, data not shown).
Knockdown of GRM3 increased sensitivity to growth factor deprivation stress (GFDS)-induced apoptosis, reflected by enhanced PARP cleavage (Fig. 4.2c) and increased apoptosis in DNA fragmentation assays (Fig. 4.2d). In addition, GRM3 knockdown decreased anchorage-independent growth (Fig. 4.2e,) and inhibited motility and migration (Fig. 4.2f).

Figure 4.2. GRM3 expression is upregulated in colon cancer cells.

a, GRM3 expression was determined in colon cancer cell lines and HCECs by western blot
analysis (left). GRM2 and GRM3 mRNA expression was determined by RT-PCR assays. Mouse brain tissue was used as a positive control (right). GRM3 mRNA expression was determined by Q-PCR assays (lower). b, GRM3 expression was knocked down by two shRNAs. c&d, Control or GRM3 knockdown cells were subjected to GFDS. Cleaved PARP (c) and apoptosis (d) were determined. e, Colony numbers were determined in soft agarose assays of control or GRM3 knockdown cells. f, Cell motility and migration were determined in Transwell assays of control or GRM3 knockdown HCT116 cells. The data are presented as the mean ± SD of three replications. ** $P < 0.01$. 
We next examined the effect of GRM3 knockdown *in vivo*. Mice were subcutaneously injected with CBS cells stably expressing a scrambled shRNA or GRM3 shRNAs. Xenograft tumor growth curves showed that tumors of GRM3 shRNA-expressing cells (designated GRM3 shRNA tumors) grew at a significantly lower rate than those of control cells (designated control tumors) (Fig. 4.3a). As a result, GRM3 shRNA tumors were much smaller than control tumors (Fig. 4.3b). IHC staining confirmed GRM3 knockdown in GRM3 shRNA tumors and verified the specificity of the anti-GRM3 antibody (Fig. 4.3c). TUNEL and Ki67 staining showed much more apoptotic cells and fewer proliferative cells in GRM3 shRNA tumors than in control tumors (Fig. 4.3d). These results indicate that knockdown of GRM3 inhibits tumor growth *in vivo* and that this inhibitory effect is a combined result of increased apoptosis and suppressed proliferation.
Figure 4.3. GRM3 mediates tumor growth in vivo. a, Xenograft tumor growth curves of CBS control and GRM3 shRNA-expressing cells are shown. n=16. b, Images of tumors at the endpoint of experiments are shown. The pictures were taken on the same scale with the ruler with each tumor (Fig. S9). c, Representative images of GRM3 staining in xenograft tumors are shown (left). Quantification of staining intensity and percentage of GRM3 positive cells was
performed (right). Representative images of TUNEL and Ki67 staining are shown (upper). Percentage of positive staining cells was determined (lower). Scale bars, 100 µm. The data are presented as the mean ± SD. *$P < 0.05$, **$P < 0.01$. 
A GRM3 antagonist mimics GRM3 knockdown in vitro and in vivo.

LY341495 is a potent and selective antagonist of GRM2/3. As shown in Fig. 4a, LY341495 reduced colony formation of HCT116 cells in soft agar. When mice subcutaneously injected with HCT116 cells were treated with LY341495, tumors grew at a slower rate than those treated with vehicle control (Fig. 4b). Therefore, tumors were markedly smaller in LY341495-treated group than in control group (Fig. 4c). TUNEL and Ki67 staining showed that LY341495 increased apoptotic cells and decreased proliferative cells (Fig. 4d). These results indicate that pharmacological blockade of GRM3 reduces anchorage-independent growth in vitro and tumor growth in vivo.
Figure 4.4. The GRM3 antagonist inhibits tumor growth in vivo. a, Colony numbers were determined in soft agarose assays of control or LY341495-treated HCT116 cells. b, Xenograft tumor growth curves of HCT116 cells treated with LY341495 or vehicle are shown. n=14. c, Images of tumors at the endpoint of experiments are shown. d, Representative images of TUNEL and Ki67 staining are shown (left). Percentage of positive staining cells was
determined (right). Scale bars, 100 µm. The data are presented as the mean ± SD. *$P < 0.05$, 

**$P < 0.01$.**
GRM3 antagonizes TGFβ to regulate cell survival through the PKA/AKT signaling axis.

GRM3 is negatively coupled to adenylyl cyclase, inhibiting production of cAMP. Since cAMP activates PKA, which inhibits AKT activation, activation of PKA and AKT was determined. PKA activity assays showed that knockdown of GRM3 increased PKA activity (Fig. 4.5a). Phosphorylation of CREB, a target of PKA, was also used to indicate PKA activity. Knockdown of GRM3 increased pCREB and decreased pAKT (Fig. 4.5b, left and Fig. 4.5c). Treatment with LY341495 or forskolin, a PKA activator, showed similar effect (Fig. 4.5b, middle). Complementarily, overexpression of GRM3 reduced pCREB and increased pAKT (Fig. 4.5b, right). These results indicate that GRM3 inhibits PKA and activates AKT in colon cancer cells.

To determine whether PKA is involved in GRM3-mediated cell survival, the PKA catalytic α subunit (PKACα) was knocked down in FET cells. GRM3 knockdown-induced PKA activation and AKT inhibition were abrogated (Fig. 4.5d, right). Consequently, knockdown of PKACα attenuated GRM3 knockdown-mediated increase of apoptosis under GFDS (Fig. 4.5e). These results indicate that GRM3 knockdown inhibits AKT and suppresses cell survival through PKA activation.

Unlike GRM3’s coupling to cAMP, TGFβ activates PKA independent of cAMP, which inhibits AKT and suppresses cell survival. We confirmed TGFβ effect on pCREB and pAKT in FET cells (Fig. 4.5d, left). Since GRM3 knockdown and TGFβ activate PKA independently, we determined whether combination of both would further activate PKA and inhibit AKT. The results showed that GRM3 knockdown and TGFβ treatment concomitantly led to additional increase in pCREB and decrease in pAKT (Fig. 4.5d, left). As a result, when cells were treated
with TGFβ under GFDS for 14 hours, TGFβ had little effect in control cells but induced apoptosis in GRM3 knockdown cells (Fig. 4.5f). In addition, GRM3 knockdown sensitized FET cells to TGFβ-mediated inhibition of anchorage-independent growth (Fig. 4.5g). These results indicate that GRM3 antagonizes TGFβ-mediated tumor suppressor function and that GRM3 knockdown enhances TGFβ effects.

To determine the mechanism underlying collaborative effect of TGFβ and GRM3 knockdown on PKA/AKT activation, cell survival and anchorage-independent growth, we investigated whether GRM3 mediates TGFβ signaling and found that GRM3 knockdown had no effect on expression of SMAD2, SMAD3, SMAD4 or on canonical TGFβ signaling (data not shown). However, TGFβ increased GRM protein expression in FET and CBS cells (Fig. 4.5h, left and middle) while had little effect on GRM3 mRNA (Fig. 5i). Inactivation of TGFβ signaling by a dominant negative TGFβ RII (DNRII) in FET cells abrogated TGFβ effect on GRM3 expression (Fig. 4.5h, right). Time course experiments showed that TGFβ increased GRM3 expression as early as 30 minutes (Fig. 4.5j), suggesting that TGFβ may increase GRM3 protein stability. Treatment with cycloheximide indicated that TGFβ markedly increased the half-life of GRM3 protein (Fig. 4.5k). Moreover, knockdown of SMAD2 or SMAD3 attenuated TGFβ-induced GRM3 protein expression but had little effect on GRM3 mRNA levels (Fig. 4.5l), indicating that SMAD2/3 contributes to TGFβ-mediated GRM3 protein expression.
Figure 4.5. GRM3 antagonizes TGFβ-mediated activation of PKA/AKT. a, PKA activity was determined in FET, HCT116 and CBS cells. b, pCREB, and pAKT were determined in FET control and GRM3 knockdown cells (left), FET cells treated with LY341495 or forskolin (middle) and vector- or GRM3-expressing FET cells (right). c&d, pCREB, and pAKT were determined in HCT116 and CBS control and GRM3 knockdown cells (c) and FET control and PKACα KD cells each infected with GRM3 sh2 or treated with 4 ng/ml TGFβ or both (d). e, PKACα was knocked down in FET control or GRM3 knockdown cells. Cells were subjected...
to GFDS for 24 hrs. Cleaved PARP (upper) and apoptosis (lower) were determined. **f**, FET control or GRM3 knockdown cells were treated with 4 ng/ml TGFβ under GFDS for 14 hrs. Cleaved PARP (upper) and apoptosis (lower) were determined. **g**, Colony numbers were determined in soft agarose assays of FET control or GRM3 knockdown cells treated with 0.5 ng/ml TGFβ. **h**, Cells were treated with 4 ng/ml TGFβ for 6 hrs. GRM3 expression was determined by western blot assays. **i**, FET cells were treated with 4 ng/ml TGFβ for 6 hrs. GRM3 mRNA was determined by RT-PCR assays. **j**, FET cells were treated with 4 ng/ml TGFβ for the indicated time. GRM3 expression was determined by western blot assays. **k**, FET cells were treated with cycloheximide in the presence or absence of TGFβ. GRM3 expression was determined by western blot assays. **l**, Expression of Smad2 or Smad3 was knocked down individually in FET cells (left). Cells were treated with TGFβ. GRM3 protein and mRNA expression was determined by western blot assays (middle) and RT-PCR analysis (right) respectively. The data are presented as the mean ± SD of three replications. *P < 0.05, **P < 0.01.
MiR-487b-3p regulates GRM3 expression in colon cancer cells.

Although TGFβ increases GRM3 expression (Fig. 4.5h), it cannot fully explain ubiquitous upregulation of GRM3 expression in colon cancer cell lines regardless of their response to TGFβ signaling (Fig. 4.2a, left). Since GRM3 expression may be regulated post-transcriptionally (Fig. 4.2a), we turned our attention to miRNAs, which function as post-transcriptional regulators of mRNA expression and/or translation. In the previous study we have identified several miRNAs that are potential regulators of TGFβ and colon cancer. We performed an in silico search for putative miRNA-binding sites in the 3’UTR of human GRM3 mRNA using TargetScan152, PicTar153 and miRanda-mirSVR154. Among miRNA candidates conserved between human and mouse, miR-487b-3p was identified as a potential regulator of GRM3. Q-PCR assays indicated that expression of miR-487b-3p was much lower in colon cancer cell lines than in HCECs (Fig. 4.6a), suggesting that decreased miR-487b-3p expression may contribute to upregulated GRM3 expression in colon cancer cells.

To demonstrate that miR-487b-3p regulates GRM3 expression, miR-487b precursor was stably infected into FET and CBS cells. As a result, expression of mature miR-487b-3p was significantly increased (Fig. 4.6b, left). Complementarily, a chemically synthesized miR-487b-3p inhibitor markedly reduced endogenous miR-487b-3p expression (Fig. 4.6b, right). Overexpression of miR-487b-3p decreased GRM3 protein expression whereas the inhibitor increased GRM3 protein expression (Fig. 4.6c, left). However, GRM3 mRNA expression was not affected (Fig. 4.6c, right). These results indicate that miR-487b-3p suppresses GRM3 expression by inhibiting its translation. To determine whether GRM3 is a direct target of miR-487b-3p, 3’-UTRs of GRM3 containing potential miR-487b-3p recognition element or mutated
seed sequence (Fig. 4.6d, left) was cloned into a luciferase construct. Luciferase assays revealed that miR-487b-3p repressed wild type but not mutant 3'-UTRs of GRM3 (Fig. 4.6d, right). These results indicate that GRM3 is a direct target of miR-487b-3p. Of note, miR-487b-5p did not target GRM3 (Fig. 4.10).

**MiR-487b-3p regulates PKA/AKT activation and mimics GRM3 knockdown effect in colon cancer cells.**

Similar to GRM3 knockdown, miR-487b-3p enhanced PKA activity (Fig. 4.6e), increased pCREB and decreased pAKT (Fig. 4.6f). MiR-487b-3p-expressing cells displayed increased apoptosis under GFDS (Fig. 4.6g) and decreased anchorage-independent growth (Fig. 4.6h and ), indicating that miR-487b-3p mimicked the effect of GRM3 knockdown. In contrast, the miR-487b-3p inhibitor decreased GFDS-induced apoptosis (Fig. 4.6i).

To determine whether miR-487b-3p-mediated effect could be reversed by restoration of GRM3 expression, GRM3 cDNA was infected into miR-487b-3p-expressing cells (Fig. 4.6j). Ectopically expressed GRM3 is resistant to miR-487b-3p inhibition due to the lack of 3’-UTR. Restored GRM3 expression reversed miR-487b-3p-mediated increase of apoptosis (Fig. 4.6k). These results indicate that miR-487b-3p sensitizes colon cancer cells to GFDS-induced apoptosis through down-regulation of GRM3.
Figure 4.6. GRM3 is a direct target of miR-487b-3p. a, Expression of miR-487b-3p was determined in HCECs and colon cancer cells by Q-PCR assays. b, Expression of miR-487b-3p was determined after infection with miR-487b precursor (left) or transfection with the miR-487b inhibitor (right). c, GRM3 expression was determined by western blot assays (left) and by RT-PCR assays (right) after infected with miR-487b precursor or transfected with the miR-487b-3p inhibitor. d, The predicted miR-487b-3p recognition site in the 3’UTR of GRM3 and
corresponding mutated sequences are indicated by lines (left). After transfection with psiCheck-2 constructs containing the wild type (Wt) or mutant (Mut) sequences, luciferase assays were performed in FET control and miR-487b-3p expressing cells (right). e, PKA activity was determined in colon cancer cells. f, pCREB and pAKT were determined in vector- or miR-487b-3p-expressing FET and CBS cells. g, Vector- or miR-487b-3p-expressing FET and CBS cells were subject to GFDS for 24 and 48 hrs respectively. Cleaved PARP (upper) and apoptosis (lower) were determined. h, Colony numbers were determined in soft agarose assays of vector- or miR-487b-3p-expressing FET and CBS cells. i, FET and HCT116b cells transfected with the miR-487b-3p inhibitor were subjected to GFDS for 24 or 48 hrs respectively. Cleaved PARP (upper) and apoptosis (lower) were determined. j&k, GRM3 was ectopically expressed in miR-487b-3p-expressing FET and CBS cells (j), which were subjected to GFDS for 24 and 48 hrs respectively. Cleaved PARP (k, upper) and apoptosis (k, lower) were determined. The data are presented as the mean ± SD of three replications. ** P < 0.01, *** P < 0.001.
To demonstrate that miR-487b-3p enhances TGFβ-mediated tumor suppressor function, FET cells were treated with TGFβ under GFDS for 14 hours. TGFβ induced apoptosis in miR-487b-3p-expressing cells but not in control cells (Fig. 4.7a). In addition, TGFβ inhibited anchorage-independent growth by 96% in miR-487b-3p-expressing cells but only by 50% in control cells (Fig. 4.7b). Restoration of GRM3 expression reversed miR-487b-3p-mediated sensitization to TGFβ-induced apoptosis (Fig. 4.7c). Complementarily, FET cells were treated with TGFβ under GFDS for 24 hours. While TGFβ induced apoptosis in control cells, the miR-487b-3p inhibitor attenuated TGFβ-induced apoptosis (Fig. 4.7d). These results indicated that miR-487b-3p enhances TGFβ-induced apoptosis by repressing GRM3 expression and that inhibition of miR-487b-3p antagonizes TGFβ effect.
Fig 4.7

Figure 4.7. MiR-487b-3p mimics the effect of GRM3 knockdown in colon cancer cells. a, Vector- or miR-487b-3p-expressing FET cells were treated with 4 ng/ml TGFβ under GFDS for 14 hrs. Cleaved PARP (left) and apoptosis (right) were determined. b, Colony numbers were determined in soft agarose assays of vector- or miR-487b-3p-expressing FET cells treated with 0.5 ng/ml TGFβ. c, MiR-487b-3p-expressing FET cells were infected with GRM3 cDNA and treated with 4 ng/ml TGFβ under GFDS for 14 hrs. Cleaved PARP (left) and apoptosis (right) were determined. d, FET cells transfected with the miR-487b-3p inhibitor were treated with 4 ng/ml TGFβ under GFDS for 24 hrs. Cleaved PARP (left) and apoptosis (right) were determined. The data are presented as the mean ± SD of three replications. * P < 0.05, ** P < 0.01.
MiR-487b-3p inhibits tumor growth in vivo.

To determine the function of miR-487b-3p in vivo, athymic nude mice were subcutaneously inoculated with CBS control or miR-487b-3p-expressing cells. Xenograft tumor growth curves indicated that tumors of control cells (designated control tumors) grew at a significantly higher rate than those of miR-487b-3p-expressing cells (designated miR-487b-3p tumors) (Fig. 4.8a). Consequently, control tumors were much bigger than miR-487b-3p tumors (Fig. 4.8b). Analysis of GRM3 expression showed that the intensity of GRM3 staining and percentage of GRM3 positive cells were notably lower in miR-487b-3p tumors than in control tumors (Fig. 4.8c). Moreover, TUNEL and Ki67 staining indicated more apoptotic cells and fewer proliferative cells in miR-487b-3p tumors than in control tumors (Figs. 4.8d & 4.8e). These results demonstrate that miR-487b-3p suppresses GRM3 expression and inhibit tumor growth in vivo.
Figure 4.8. MiR-487b-3p suppresses GRM3 expression and inhibits tumor growth in vivo. a, Xenograft tumor growth curves of CBS control and miR-487b-3p-expressing cells is shown. n=16. b, Representative images of tumors at the endpoint of experiments are shown. The pictures were taken on the same scale with the ruler with each tumor (Fig. S10). c, Representative images of GRM3 staining in xenograft tumors are shown (upper). Quantification of staining intensity and percentage of GRM3 positive cells was performed (lower). d&e, Representative images of TUNEL (d) and Ki67 (e) staining are shown (left). Percentage of positive TUNEL (d) and Ki67 (e) staining cells were determined (right). Scale bars, 100 µm. The data are presented as the mean ± SD. *P < 0.05, **P < 0.01. f, A proposed model of crosstalk between miR-487b-3p, GRM3 and TGFβ signaling in regulation of PKA/AKT activation, cell survival, proliferation and tumorigenesis of colon cancer cells.
Expression of miR-487b-3p is decreased in colon cancer specimens.

To explore clinical relevance of miR-487b-3p, its expression was determined by *in situ* hybridization in the same sets of TMAs utilized in Figure 1. The staining intensity of miR-487b-3p was much stronger in miR-487b-3p-expressing tumors than in control tumors (Fig. 4.9a), verifying the specificity of the probe. Analysis of TMAs indicated that miR-487b-3p expression was very high in normal colon epithelium, but decreased markedly in colon tumors (Fig. 4.9b). Quantification of staining showed that the average intensity was significantly lower in colon adenocarcinomas than in normal colon (Fig. 4.9c), which is consistent with results from TCGA databases (Fig. 4.11). In addition, the correlation study revealed a significant inverse correlation between miR-487b-3p and GRM3 expression (Fig. 9d, *** *P* = 0.0003). These results demonstrate that miR-487b-3p expression is decreased in colon adenocarcinomas and that miR-487b-3p expression is inversely correlated with GRM3 expression.

Taken together, our studies suggest a novel model of crosstalk between miR-487b-3p, GRM3 and TGFβ signaling (Fig. 4.8f). In this model, while miR-487b-3p inhibits GRM3 translation, TGFβ increases GRM3 protein stability as a negative feedback mechanism to antagonize TGFβ-induced PKA activation, AKT inhibition and suppression of cell survival, proliferation and tumorigenesis. Therefore, inhibition of GRM3 or expression of miR-487b-3p prevents this negative feedback and enhances TGFβ-mediated tumor suppressor function.
Figure 4.9. Expression of miR-487b-3p is decreased in colon cancer specimens. *In situ* hybridization analysis of miR-487b-3p was performed in xenograft tumors and human specimens. **a**, Representative images of miR-487b-3p staining in xenograft tumors are shown. Scale bars, 100 μm. **b**, Normal colon in the absence of the probe was used as a negative control. Representative images of miR-487b-3p staining in normal colon and colon tumors are shown.
Scale bars, 100 µm. c, Quantification of the staining intensity was performed. The values of individual samples are shown. Error bars indicate SEM of the values. ** *P* < 0.01. d, Correlation of expression of miR-487b-3p and GRM3 was determined using Pearson's test (r = -0.439, *** *P* = 0.0003; n = 64). The slope was generated by lineage regression analysis.
Fig 4.10

FET cells were transfected with miR-487b-5p mimic. GRM3 expression was determined by western blot analysis.

**Fig. 4.10.** FET cells were transfected with miR-487b-5p mimic. GRM3 expression was determined by western blot analysis.
Fig. 4.11. Comparison of miR-487b-3p expression between normal colon and colon tumor samples was performed by analyzing UNC-H_miRNA 8X15K level 3 dataset from TCGA database. The difference between two groups was evaluated by 2 tail student t test (*** $P < 0.001$).
Fig 4.12

Fig. 4.12. RT-PCR analysis of GRM4 mRNA expression in HCECs and colon cancer cell lines.

Mouse brain tissues were used as a positive control.
Fig 4.13

a

![Graph showing comparison of percentage of GRM3 positive cells in well-, moderately- and poorly-differentiated colon tumors. The values of individual samples are shown. Error bars indicate SEM of the values. * P < 0.05.]

b

![Immunohistochemistry staining of GRM3 performed in normal pancreas and pancreatic tumors. Representative images are shown. Scale bars, 100 µm.]

c

![Graph showing quantification of GRM3 staining intensity (left) and percentage of GRM3 positive cells (right) performed. The values of individual samples are shown. Error bars indicate SEM of the values. *** P < 0.001.]

Fig. 4.13. a, Comparison of percentage of GRM3 positive cells in well-, moderately- and poorly-differentiated colon tumors. The values of individual samples are shown. Error bars indicate SEM of the values. * P < 0.05. b, Immunohistochemistry staining of GRM3 was performed in normal pancreas and pancreatic tumors. Representative images are shown. Scale bars, 100 µm. c, Quantification of GRM3 staining intensity (left) and percentage of GRM3 positive cells (right) was performed. The values of individual samples are shown. Error bars indicate SEM of the values. *** P < 0.001.
Fig. 4.14. Western blot analysis of pERK and ERK in GRM3 knockdown (a) and miR-487b-3p-expressing cells (b).
Fig 4.15

Fig. 4.15. a. Control and GRM3 knockdown cells were treated with 5 ng/ml BMP. Phosphorylation of Smad1/5 was determined. b, FET and HCT116 cells were treated with 5 ng/ml BMP. Expression of GRM3 was determined.
DISCUSSION

Although GRM3 protein expression is considerably higher in colon adenocarcinomas than in normal colon (Fig. 4.1), analysis of TCGA databases reveals that GRM3 mRNA remains unchanged (data not shown). It suggests that GRM3 upregulation is mediated at the posttranscriptional level. While transcriptional regulation of GRM3 has been reported, our findings that miR-487b-3p directly targets GRM3 to suppress its translation and that TGFβ increases GRM3 protein stability provide novel mechanisms of posttranscriptional regulation of GRM3 in colon cancer. Of note, GRM2, the other group II metabotropic glutamate receptor, and GRM4, reported to be present in colon cancer cells, are barely expressed in colon cancer cells used in the study (Fig. 4.2a and Fig. 4.12), suggesting that they may not play a role in colon cancer.

When correlating GRM3 expression with clinicopathological features of tumors, no significant correlation was found between GRM3 expression and tumor grades or stages (data not shown). However, percentage of GRM3 positive cells is noticeably higher in poorly-differentiated tumors than moderately-differentiated ones (Fig. 4.13a). Due to small sample size of well-differentiated tumors, the difference between well-differentiated tumors and moderately- or poor-differentiated ones is not significant. In addition, GRM3 expression is significantly higher in pancreatic tumors than in normal pancreas (Fig. 4.13b and 4.13c), indicating that upregulation of GRM3 expression is not specific to colon cancer.

It has been shown that mGluRs, such as GRM1 and mutant GRM3, activate the MEK pathway in nervous and melanoma cells. In this study, we show that GRM3 knockdown activates PKA and inhibits AKT in colon cancer cells. In addition, GRM3 knockdown or
overexpression of miR-487b-3p also decreases ERK activity (Fig. 4.14). It indicates that GRM3 can activate multiple cancer-related signaling pathways and that the components of the glutamatergic system are active in colon cancer cells. Although we show that GRM2 expression is not upregulated in colon cancer cells, it remains to be determined whether other glutamate receptors or other elements of glutamate signaling are aberrantly expressed in colon cancer.

Our studies indicate that GRM3 antagonizes TGFβ-mediated tumor suppressor effect and that TGFβ increases GRM3 protein stability in a Smad2/3-dependent manner. These results suggest that there is a negative feedback regulation between GRM3 and TGFβ signaling (Fig. 4.8f). This is of significance since TGFβ is a major tumor suppressor in colon cancer. The negative feedback regulation between TGFβ and GRM3 could potentially prevent efficient intervention of tumor growth and progression by TGFβ signaling. In addition, it will be interesting to investigate whether TGFβ increases GRM3 expression in cancers where TGFβ functions as a tumor promoter (i.e. breast cancer). If so, given that GRM3 activates AKT and MEK signaling pathways, GRM3 could potentially contribute to TGFβ-mediated tumor promoting function (i.e. EMT, etc). Therefore, inhibition of GRM3 could not only enhance TGFβ-mediated tumor suppressor function but also counteract TGFβ-mediated tumor promoting function, making targeting GRM3 an efficient anti-cancer approach despite of dual function of TGFβ signaling. Furthermore, GRM3 can also function independently of TGFβ signaling since GRM3 knockdown in HCT116 cells with defective TGFβ signaling increased apoptosis and reduced anchorage-independent growth (Fig. 4.2). Hence, GRM3 can be a promising molecular target in colon cancer defective of TGFβ signaling, which occurs in 30-40% of colon cancer patients.
It has been reported that GRM3 negatively regulates BMP signaling in glioma cells \(^{128}\). However, we found that GRM3 knockdown did not activate BMP signaling in colon cancer cells (Fig. 4.15a). In addition, unlike TGF\(\beta\), BMP treatment did not increase GRM3 expression (Fig. 4.15b). These results suggest that BMP signaling is not likely involved in the effects mediated by GRM3 knockdown and in the regulation of GRM3 expression.

Taken together, we have identified a signaling axis, miR-487b-3p/GRM3/TGF\(\beta\)/PKA/AKT, as an important regulator of tumorigenesis in colon cancer. Our studies suggest that GRM3 could be a novel molecular target for colon cancer treatment. However, one concern to target glutamate signaling for cancer treatment is whether it would affect brain or neuron function. A significant amount of work has gone into designing and testing of drugs to modulate glutamatergic system to treat neurological and psychiatric disorders. Pharmaceutical companies have generated libraries of compounds that are not optimal for treating neuropsychiatric disorders due to problems such as inability to readily penetrate blood brain barriers. However, those compounds could be ideal agents for other applications, for example, anti-cancer therapy. Since GRM3 is mainly upregulated in colon cancer but rarely expressed in normal peripheral tissues, targeting GRM3 with such agents would not likely cause adverse neurological or peripheral side effects, making GRM3 an attractive and specific target for colon cancer treatment.
CHAPTER 5: MICRORNA-656-3P/VGLUT3 CROSSTALKS WITH TGFβ SIGNALING TO REGULATE COLON CANCER TUMORIGENESIS

INTRODUCTION

Colon cancer is the second leading cause of cancer death in the US, primarily due to metastatic disease \(^1\). Treatment of metastatic colon cancer with molecule-targeting drugs has not had a large clinical impact; therefore, there is an urgent need to identify new molecule targets and develop novel target-specific therapies.

Glutamate is the predominant excitatory neurotransmitter in the central nervous system. In nervous glutamate is sorted in the synaptic vesicle before release to synaptic cleft \(^92\). In mammals, two group of glutamate transport are responsible for maintaining glutamatergic system, Sodium-dependent excitatory Amino Acid Transporters (EAATs) and the VGLUTs. The family of VGLUTs is comprised of three highly homologous proteins VGLUT1-3. They transport glutamate from the cytoplasm into the synaptic vesicles \(^93\). In addition to the CNS, VGLUTs can be found in peripheral nervous system and in glutamate-secreting non-neuronal cells of different organs, such as the pineal gland \(^97\), bone \(^99\) and testes \(^100\). It has been postulated that the peripheral glutamatergic system may use glutamate as an intercellular messenger to communicate with neighboring cells \(^158\).

In this study, we show for the first time that VGLUT3 expression is upregulated in human colonic adenocarcinomas and colon cancer cell lines. Knockdown of VGLUT3 expression in colon cancer cells reduces cell survival and anchorage-independent growth \(in\ vitr o\) and inhibits tumor growth \(in\ vivo\). In addition, VGLUT3 antagonizes TGFβ-mediated inhibition of AKT activation and suppression of cell survival and clonogenicity. Furthermore, VGLUT3 is a direct
target of miR-656-3p, expression of which mimics the effects of VGLUT3 knockdown in colon cancer cells \textit{in vitro} and \textit{in vivo}. Expression of miR-656-3p is downregulated in colon adenocarcinomas and inversely correlates with VGLUT3 expression. Taken together, these studies indicate that VGLUT3 maintains AKT activation and counteracts TGFβ signaling to regulate colon cancer tumorigenesis. It suggests that upregulation of VGLUT3 expression is a functionally important molecular event during colon cancer development and that VGLUT3 is a potential molecular target for colon cancer treatment. This is especially interesting and important from a therapeutic standpoint. Given that VGLUT3 is mainly present in CNS and upregulated in colon cancer, but rarely expressed in normal peripheral tissues, targeting VGLUT3 with agents that cannot penetrate blood brain barrier would therefore not likely cause adverse neurological or peripheral side effects, making VGLUT3 an attractive and specific target for colon cancer treatment.

\textbf{RESULTS}

\textit{Expression of VGLUT3 is significantly increased in colon cancer specimens.}

Aberrant glutamatergic signaling has been reported to play a role in cancer\textsuperscript{118,127}. VGLUTs are critical components in glutamatergic system. However, little is known of their expression and function in cancer. To determine whether VGLUTs may play a functional role in colon cancer, we examined expression of VGLUT1-3 in a panel of human colon cancer cell lines. HCT116 and RKO cells are defective in TGFβ signaling due to the lack of TGFβ RII expression\textsuperscript{144}. HCT116b cells were isolated from the same primary colon tumor as HCT116 cells, but displayed much lower metastatic potential \textsuperscript{145}. FET cells, isolated from a well differentiated early stage colon tumor, are sensitive to TGFβ-mediated growth inhibition and
apoptosis. CBS and GEO cells are partially responsive to TGFβ due to low TGFβ RII and RI expression, respectively. All cell lines bear either KRAS or BRAF mutations, and all of them except RKO have mutated APC or β-catenin. An immortalized human colon epithelial cell line, HCEC, was included as a control. RT-PCR assays indicated that VGLUT3 mRNA was ubiquitously expressed in HCECs and colon cancer cell lines whereas expression of VGLUT1 and VGLUT2 mRNA was undetectable (Fig. 5.1A, left). Western blot analysis showed that VGLUT3 protein expression was markedly higher in colon cancer cells than in HCECs (Fig. 5.1A, right). These results indicate that VGLUT1 and VGLUT2 are not expressed in HCECs and colon cancer cells and that VGLUT3 expression is upregulated in colon cancer cells as compared to immortalized colon epithelial cells. Given that VGLUT3 mRNA levels were similar between HCECs and most of colon cancer cell lines (Fig. 5.1A, left), it suggests that upregulation of VGLUT3 may be through post-transcriptional mechanism(s).

To determine the clinical relevance and significance of VGLUT3 upregulation, we extended our studies to patient specimens. VGLUT3 expression was determined in normal colon and colonic adenocarcinomas using immunohistochemistry (IHC) staining. Verification of the anti-VGLUT3 antibody is shown in Fig. 5.3C. Tissue micro-arrays (TMA) consisting of 35 normal colon and 66 colon tumors were analyzed. Mouse brain tissue was used as a positive control. VGLUT3 expression was very low in normal colon epithelium, but increased significantly in colon tumors (Fig. 5.1B). Quantification of the staining showed that the average intensity of VGLUT3 staining and percentage of VGLUT3 positive cells were approximately 2.7-fold and 2.2-fold higher in tumor samples than in normal colon respectively (Fig. 5.1C). These results demonstrate that VGLUT3 expression is upregulated in colon tumors, suggesting
that VGLUT3 may play a functional role in colon cancer. When correlating VGLUT3 expression with clinicopathological features of tumors, no significant correlation was found between VGLUT3 expression and tumor grade, stage and differentiation (Figure 5.9). However, due to small sample size of certain groups, it is difficult to draw a clear conclusion.

**Fig 5.1**

![Image of Figure 5.1 showing VGLUT3 expression in colon cancer cells and patient specimens.](image)

**Figure 5.1. VGLUT3 expression is elevated in colon cancer cells and patient specimens.**

Expression of VGLUT1-3 mRNA was determined in a panel of colon cancer cell lines and HECEs by RT-PCR assays. Mouse brain tissues were used as a positive control (left). VGLUT3 protein expression was determined by western blot analysis (right). B, Immunohistochemistry
staining of VGLUT3 was performed in normal colon and colon adenocarcinomas. Mouse brain tissues stained with the anti-VGLUT3 antibody in the absence or presence of a specific blocking peptide were used as a positive and negative control respectively. Representative images are shown. Scale bars, 100 µm. C, Quantification of VGLUT3 staining intensity (left) and percentage of VGLUT3 positive cells (right) was performed. The values of individual samples are shown. Error bars indicate SEM of the values. * $P < 0.05$, ** $P < 0.01$. 
Knockdown of VGLUT3 expression decreases cell survival and clonogenicity of colon cancer cells and enhances TGFβ effect.

To determine whether upregulation of VGLUT3 plays an important role in colon cancer, VGLUT3 expression was knocked down in FET, CBS and HCT116, three colon cancer cell lines with different genetic background. Each of two independent shRNAs (sh2 and sh3) reduced VGLUT3 expression markedly as compared to a scrambled shRNA control, but had no effect on VGLUT1 and VGLUT2 expression (Fig. 5.2A and Fig. 5.10A). Knockdown of VGLUT3 expression led to increased sensitivity to growth factor deprivation stress (GFDS)-induced apoptosis, reflected by enhanced PARP cleavage (Fig. 5.2B) and 61% to 2.3-fold increase of apoptosis detected in DNA fragmentation assays (Fig. 5.2C). In addition, soft agar assays showed that reduction of VGLUT3 expression led to 34-46% decrease in colony formation under anchorage-independent conditions (Fig. 5.2D). These results demonstrate that downregulation of VGLUT3 expression decreases cell survival and clonogenicity in vitro.

We next determined the underlying mechanisms of VGLUT3 function. It has been shown that glutamatergic signaling mediates the MAPK/ERK and PI3K/AKT pathways. Therefore, activation of ERK and AKT was determined using phosphorylation of ERK (pERK) and phosphorylation of AKT (pAKT) as respective indicators. Knockdown of VGLUT3 in FET, CBS and HCT116 cells resulted in decreased pAKT (Fig. 5.2E and 2F). However, it had no effect on pERK (Fig. 5.10B). These results indicate that VGLUT3 expression is involved in the activation of AKT, which is an important survival factor in colon cancer cells.

It has been shown that TGFβ signaling activates PKA, which inhibits AKT activation and cell survival in colon cancer cells. Although knockdown of VGLUT3 expression decreased
pAKT, it did not affect CREB phosphorylation (Fig. 5.10C), indicating that the effect of VGLUT3 on AKT activation is independent of PKA. Since knockdown of VGLUT3 and TGFβ signaling inhibit activation of AKT independently, we determined whether combination of both would further repress AKT activation. The results showed that concomitant VGLUT3 knockdown and TGFβ treatment led to a greater decrease in pAKT than seen with either treatment alone (Fig. 5.2E). When cells were treated with TGFβ under GFDS for 14 hours, TGFβ had little effect in control cells but induced apoptosis in VGLUT3 knockdown cells (Fig. 5.2G and 2H). In addition, knockdown of VGLUT3 also sensitized FET cells to TGFβ-mediated inhibition of anchorage-independent growth (Fig. 5.2I). Taken together, these results indicate that, in addition to activating AKT, VGLUT3 also antagonizes TGFβ-mediated tumor suppressor function and that knockdown of VGLUT3 enhances TGFβ effects.

**Fig 5.2**
Figure 5.2. Knockdown of VGLUT3 sensitizes colon cancer cells to stress-induced apoptosis and enhances TGFβ effect. A, VGLUT3 expression was knocked down by two shRNAs in FET, CBS and HCT116 cells. B & C, Control or VGLUT3 knockdown cells were subjected to GFDS for indicated time. Western blot analysis of PARP cleavage (B) and DNA fragmentation assays (C) were performed. D, Colony numbers were determined in soft agarose assays of control or VGLUT3 knockdown cells. E, Western blot analysis showed pAKT and AKT in FET control and VGLUT3 knockdown cells treated with 4 ng/ml of TGFβ. F, Expression of pAKT and AKT in CBS and HCT116 control and VGLUT3 knockdown cells. G&H, FET control or VGLUT3 knockdown cells were treated with 4 ng/ml TGFβ under GFDS for 14 hrs. Cleaved PARP (G) and apoptosis (H) were determined. I, Colony numbers were determined in soft agarose assays of FET control or VGLUT3 knockdown cells treated with 0.5 ng/ml TGFβ. The data are presented as the mean ± SD of three replications. * P < 0.05, ** P < 0.01.
VGLUT3 plays a critical role in tumor growth in vivo.

To determine VGLUT3 function in tumor growth in vivo, athymic nude mice were injected with HCT116 cells stably expressing a scrambled shRNA or VGLUT3 shRNAs. Xenograft tumor growth curves showed that tumors of VGLUT3 shRNA-expressing cells (designated VGLUT3 shRNA tumors) grew at a significantly lower rate than those of control cells (designated control tumors) (Fig. 5.3A). Consequently, VGLUT3 shRNA tumors were much smaller than control tumors (Fig. 5.3B). IHC staining confirmed knockdown of VGLUT3 expression in VGLUT3 shRNA tumors and verified the specificity of the anti-VGLUT3 antibody (Fig. 5.3C). TUNEL and Ki67 staining showed much more apoptotic cells and fewer proliferative cells in VGLUT3 shRNA tumors than in control tumors (Fig. 5.3D). These results indicate that knockdown of VGLUT3 inhibits tumor growth in vivo and that this inhibitory effect is a combined result of increased apoptosis and suppressed proliferation.

Fig 5.3
Figure 3. VGLUT3 mediates tumor growth of colon cancer cells in vivo. A, Xenograft tumor growth curves of HCT116 control and VGLUT3 shRNA-expressing cells are shown. n=16. B, Images of tumors at the endpoint of experiments are shown. Scale bars, 1 cm. C, Representative images of VGLUT3 staining in xenograft tumors are shown (upper). Quantification of staining intensity was performed (lower). D, Representative images of TUNEL and Ki67 staining are shown (upper). Percentage of positive staining cells was determined (lower). Scale bars, 100 µm. The data are presented as the mean ± SD. *P < 0.05, ** P < 0.01, *** P < 0.001.
MiR-656-3p regulates VGLUT3 expression in colon cancer cells.

Since VGLUT3 expression may be regulated post-transcriptionally, miRNAs, functioning as post-transcriptional regulators of mRNA expression and/or translation, could regulate its expression. We therefore performed an in silico search for putative miRNA-binding sites in the 3’-UTR of the human VGLUT3 mRNA using TargetScan\textsuperscript{152}, PicTar\textsuperscript{153} and miRanda-mirSVR\textsuperscript{154}. Among miRNA candidates conserved between human and mouse, miR-656-3p was identified as a potential regulator of VGLUT3. Q-PCR assays indicated that expression of miR-656-3p was much lower in colon cancer cell lines than in HCECs (Fig. 5.4A), suggesting that decreased miR-656-3p expression may contribute to upregulated VGLUT3 expression in colon cancer cells.

To demonstrate that miR-656-3p regulates VGLUT3 expression, the miR-656 precursor was stably infected into FET, CBS and HCT116 cells. As a result, expression of mature miR-656-3p was significantly increased (Fig. 5.4B). Complementarily, a chemically synthesized miR-656-3p inhibitor markedly reduced endogenous miR-656-3p expression by 40% and 43% in FET and HCT116 cells respectively (Fig. 5.4B). Overexpression of miR-656-3p decreased VGLUT3 protein expression (Fig. 5.4C) whereas the miR-656-3p inhibitor increased VGLUT3 protein expression (Fig. 5.4D). However, VGLUT3 mRNA expression was not affected (Fig. 5.4E). These results indicate that miR-656-3p suppresses VGLUT3 expression by inhibiting its translation. To determine whether VGLUT3 is a direct target of miR-656-3p, three sequences in the 3’-UTRs of VGLUT3 containing the potential miR-656-3p recognition element (Fig. 5.4F) was cloned into a luciferase construct. Luciferase assays revealed that miR-656-3p repressed site 1 but not site 2 or site 3 of 3’-UTRs of VGLUT3 (Fig. 5.4G). These results
indicate that miR-656-3p directly targets VGLUT3 partially through binding to site 1 to inhibit its translation.

**MiR-656-3p inhibits AKT activation and mediates stress-induced apoptosis and clonogenicity in colon cancer cells.**

Similar to VGLUT3 knockdown, miR-656-3p decreased pAKT (Fig. 5.4C) but had no effect on ERK or PKA activation (Fig. 5.10D and 5.10E). MiR-656-3p-expressing cells displayed increased PARP cleavage and 48-54% increase of apoptosis under GFDS (Fig. 5.5A). In addition, anchorage-independent growth was reduced by 34-60% in miR-656-3p-expressing cells (Fig. 5.5B). These results indicate that miR-656-3p mimics the effect of VGLUT3 knockdown. In contrast, the miR-656-3p inhibitor, which effectively inhibited miR-656-3p expression in FET and HCT116 cells (Fig. 5.4B), reduced PARP cleavage and decreased GFDS-induced apoptosis by 34% and 54% respectively (Fig. 5.5C).
Fig 5.4

Figure 5.4. VGLUT3 is a direct target of miR-656-3p. A, Expression of miR-656-3p was determined in HCECs and colon cancer cells by Q-PCR assays. B, Expression of miR-656-3p was determined after infection with miR-656 precursor or transfection with the miR-656-3p inhibitor. C&D, VGLUT3 expression was determined by western blot assays after infected with the miR-656 precursor (C) or transfected with the miR-656-3p inhibitor (D). Expression of AKT and pAKT was also determined. E, mRNA expression of VGLUT3 was determined by RT-PCR assays after infected with miR-656 precursor or transfected with the miR-656-3p inhibitor. F, The predicted miR-656-3p recognition sites in the 3’-UTR of VGLUT3 are indicated by vertical lines. G, After transfection with psiCheck-2 constructs containing site 1, 2 or 3 sequences, luciferase assays were performed in FET control and miR-656-3p expressing cells.
To determine whether miR-656-3p-mediated sensitization to GFDS-induced apoptosis could be reversed by restoration of VGLUT3 expression, VGLUT3 cDNA was infected into miR-656-3p-expressing FET and HCT116 cells (Fig. 5.5D, upper). Ectopically expressed VGLUT3 is resistant to down-regulation mediated by miR-656-3p due to the lack of 3’-UTR. Ecotopic VGLUT3 expression reversed miR-656-3p-mediated increase of apoptosis under GFDS (Fig. 5.5D). These results indicate that miR-656-3p sensitizes colon cancer cells to GFDS-induced apoptosis through the down-regulation of VGLUT3 expression.

To investigate whether miR-656-3p enhances TGFβ-mediated tumor suppressor function, FET cells were treated with TGFβ under GFDS for 14 hours. TGFβ induced apoptosis in miR-656-3p-expressing cells but not in control cells (Fig. 5.5E). In addition, TGFβ inhibited anchorage-independent growth by 61% in miR-656-3p-expressing cells but only by 30% in control cells (Fig. 5.5F). Complementarily, FET cells were treated with TGFβ under GFDS for 24 hours. While TGFβ induced apoptosis in control cells, the miR-656-3p inhibitor significantly attenuated TGFβ-induced apoptosis (Fig. 5.5G). These results indicated that miR-656-3p enhances TGFβ-induced apoptosis and that inhibition of miR-656-3p antagonizes TGFβ effect.
Figure 5.5. MiR-656-3p mimics the effect of VGLUT3 knockdown in colon cancer cells. A, Vector- or miR-656-3p-expressing FET, CBS and HCT116 cells were subject to GFDS for indicated time. Cleaved PARP (left) and apoptosis (right) were determined. B, Colony numbers were determined in soft agarose assays of vector- or miR-656-3p-expressing cells. C, FET and HCT116 cells transfected with the miR-656-3p inhibitor were subjected to GFDS for 24 or 72 hrs respectively. Western blot analysis of cleaved PARP (left) and DNA fragmentation assays (right) were determined. D, VGLUT3 was ectopically expressed in miR-656-3p-expressing FET and HCT116 cells (upper), which were subjected to GFDS for 24 and 72 hrs respectively. Cleaved PARP (upper) and apoptosis (lower) were determined. E, Vector- or miR-656-3p-expressing FET cells were treated with 4 ng/ml TGFβ under GFDS for 14 hrs. Cleaved PARP (left) and apoptosis (right) were determined. F, Colony numbers were determined in soft
agarose assays of vector- or miR-656-3p-expressing FET cells treated with 0.5 ng/ml TGFβ. G, FET cells transfected with the miR-656-3p inhibitor were treated with 4 ng/ml TGFβ under GFDS for 24 hrs. Cleaved PARP (left) and apoptosis (right) were determined. The data are presented as the mean ± SD of three replications. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. 
**MiR-656-3p inhibits tumor growth in vivo.**

We next determine the function of miR-656-3p in vivo. Athymic nude mice were subcutaneously inoculated with HCT116 control or miR-656-3p-expressing cells. Xenograft tumor growth curves showed that tumors of miR-656-3p-expressing cells (designated miR-656-3p tumors) grew at a significantly lower rate than those of control cells (designated control tumors) (Fig. 5.6A). Consequently, miR-656-3p tumors were much smaller than control tumors (Fig. 5.6B). Analysis of VGLUT3 expression showed that the intensity of VGLUT3 staining was notably lower in miR-656-3p tumors than in control tumors (Fig. 5.6C). Furthermore, TUNEL and Ki67 staining indicated more apoptotic cells and fewer proliferative cells in miR-656-3p tumors than in control tumors (Fig. 5.6D). These results demonstrate that miR-656-3p suppresses VGLUT3 expression, increases apoptosis, decreases proliferation and inhibit tumor growth in vivo.
Figure 5.6. MiR-656b-3p suppresses VGLUT3 expression and inhibits tumor growth in vivo. A, Xenograft tumor growth curves of HCT116 control and miR-656-3p-expressing cells is shown. n=16. B, Images of tumors at the endpoint of experiments are shown. Scale bars, 1 cm. C, Representative images of VGLUT3 staining in xenograft tumors are shown (left). Quantification of staining intensity was performed (right). D, Representative images of TUNEL and Ki67 staining are shown (left). Percentage of positive TUNEL and Ki67 staining cells were determined (right). Scale bars, 100 µm. The data are presented as the mean ± SD. *P < 0.05, **P < 0.01.
Expression of miR-656-3p is decreased in colon cancer specimens.

To explore clinical relevance of miR-656-3p, its expression was determined by in situ hybridization in the same sets of TMAs utilized in Figure 1. The staining intensity of miR-656-3p was much stronger in miR-656-3p-expressing tumors than in control tumors (Fig. 5.7A), verifying the specificity of the probe. Analysis of TMAs indicated that miR-656-3p expression was high in normal colon epithelium, but decreased markedly in colon tumors (Fig. 5.7B). Quantification of staining showed that the average intensity was significantly lower in colon adenocarcinomas than in normal colon (Fig. 5.7C). In addition, the correlation study revealed a moderate negative correlation between miR-656-3p and VGLUT3 expression (Fig. 5.7D, *P = 0.025). These results demonstrate that miR-656-3p expression is decreased in colon adenocarcinomas and inversely correlates with VGLUT3 expression and that downregulation of miR-656-3p in colon tumors is at least partially responsible for VGLUT3 upregulation.
Figure 5.7. Expression of miR-656-3p is decreased in colon cancer specimens. *In situ* hybridization analysis of miR-656-3p was performed in xenograft tumors and human specimens. A, Representative images of miR-656-3p staining in xenograft tumors are shown. Scale bars, 100 µm. B, Normal colon in the absence of the probe was used as a negative control. Representative images of miR-656-3p staining in normal colon and colon tumors are shown. Scale bars, 100 µm. C, Quantification of the staining intensity was performed. The values of individual samples are shown. Error bars indicate SEM of the values. *** $P < 0.001$. D,
Correlation of expression of miR-656-3p and VGLUT3 was determined in colon tumors using Pearson's test ($r = -0.3$, $* P = 0.025$; $n = 59$). The slope was generated by lineage regression analysis.
Taken together, our studies suggest a novel model of crosstalk between miR-656-3p, VGLUT3, AKT and TGFβ signaling (Fig. 5.8). In this model, miR-656 downregulates VGLUT3 expression, which leads to AKT inhibition. On the other hand, TGFβ signaling activates PKA and suppresses AKT activation. Consequently, downregulation of VGLUT3 or expression of miR-656-3p collaborates with TGFβ signaling to inhibit AKT activation and enhance TGFβ-mediated tumor suppressor function whereas upregulation of VGLUT3 expression antagonizes TGFβ signaling to promote proliferation, survival and tumorigenesis.

**Figure 5.8.** A proposed model of crosstalk between miR-656-3p, VGLUT3 and TGFβ signaling in regulation of AKT activation, cell survival, proliferation and tumorigenesis of colon cancer cells.
Fig. 5.9. Comparison of VGLUT3 intensity and percentage of VGLUT3 positive cells was made in low, moderate and high grade colon tumors (A), in different stages of colon tumors (B) and in well-, moderately- and poorly-differentiated colon tumors (C). The values of individual samples are shown. Error bars indicate SEM of the values. There is no significant difference among the groups.
**Fig 5.10.** A, RT-PCR analysis of VGLUT1-3 mRNA expression in VGLUT3 knockdown FET cells. Mouse brain tissues were used as a positive control. B&D, Western blot analysis of pERK and total ERK in VGLUT3 knockdown cells (B) and miR-656-3p expressing cells (D). C&E, Western blot analysis of pCREB and total CREB in VGLUT3 knockdown cells (C) and miR-656-3p expressing cells (E).
Fig. 5.11. Glutamate secretion was evaluated in the control and VGLUT3 knockdown cells. Cells were cultured in glutamine-free, glutamate-free and phenol red-free DMEM (Gibco) for 48 hours. Conditioned medium was collected and glutamate levels were determined using AMPLEX Red Glutamic Acid assay kit (Invitrogen) and analyzed on a fluorescence plate reader. The average concentrations of glutamate per 10,000 cells are shown. Error bars indicate SD of the values. There is no significant difference among the groups.
DISCUSSION

Glutamatergic signaling has been extensively studied in nerve cells, where they modulate excitatory synaptic transmission. VGLUT3, a vesicular glutamate transporter, is an unusual member of the VGLUT family. It is expressed not only in glutamatergic neurons but also in non-glutamatergic neurons such as serotonergic and cholinergic neurons and in astrocytes as well. In addition, its subcellular localization is not restricted to synaptic boutons, but also includes the cell soma and dendrites. In this study, we discover that expression of VGLUT3 is upregulated significantly in colon tumors as compared to normal colon. Additional findings indicate that miR-656-3p directly targets VGLUT3 to suppress its translation and that downregulation of miR-656-3p in colon tumors is at least partially responsible for VGLUT3 upregulation. Therefore, our studies provide a novel mechanism by which VGLUT3 expression is regulated in colon cancer. Of note, VGLUT1 and VGLUT2, two other vesicular glutamate transporters, are barely expressed in colon cancer cells (Fig. 5.1A), suggesting that they may not play a role in colon cancer.

It has been reported that VGLUTs transport cytoplasmic glutamate into the synaptic vesicles, which is then released by glutamatergic neurons. Outside the nervous system, VGLUTs can be found in the glutamate-secreting non-neuronal cells of different organs, such as the pineal gland, bone and testes. It has been proposed that this peripheral glutamatergic system may use glutamate as a messenger to transduce signaling in an autocrine or paracrine manner. We have shown that GRM3, a group II metabotropic glutamate receptor, is significantly upregulated in colon cancer and that knockdown or inhibition of GRM3 activation in colon cancer cells reduces cell survival and anchorage-independent growth *in vitro*
and inhibits tumor growth in vivo \(^{160}\). These studies suggest that peripheral glutamatergic signaling may contribute to colon cancer development. Therefore, a potential function of VGLUT3 in colon cancer would be to facilitate glutamate secretion, which then activates GRM3 or other glutamate receptors to promote tumor growth. However, knockdown of VGLUT3 expression did not affect glutamate secretion in FET and HCT116 cells (Fig. 5.11), indicating that VGLUT3 is not involved in glutamate secretion of colon cancer cells. In addition, glutamate signaling, for example GRM3 activation, has been shown to activate downstream signaling pathways including PI3K/AKT and MAPK/ERK \(^{103,160}\). Although knockdown of VGLUT3 inhibits AKT activation, it has no effect on ERK activity (Fig. 5.10B). It remains to be determined whether VGLUT3 contributes to glutamatergic signaling and how VGLUT3 regulates AKT activation in colon cancer cells.

Our studies indicate that VGLUT3 antagonizes TGFβ-mediated tumor suppressor effect. This is of significance since TGFβ is an important tumor suppressor in colon cancer \(^{132-135}\). Upregulation of VGLUT3 could potentially prevent efficient intervention of tumor growth and progression by TGFβ signaling. TGFβ has been shown to activate PKA, which subsequently inhibits AKT activation in colon cancer cells \(^{159}\). However, knockdown of VGLUT3 has no effect on PKA signaling, but represses AKT activation. Therefore, VGLUT3 and TGFβ signaling counteract each other to regulate AKT activity (Fig. 5.8). Furthermore, VGLUT3 can also function independently of TGFβ signaling since knockdown of VGLUT3 in HCT116 cells with defective TGFβ signaling led to decreased AKT activation, increased apoptosis and reduced anchorage-independent growth (Fig. 5.2). Therefore, VGLUT3 is a potential molecular target in colon cancer regardless of the status of TGFβ signaling. In addition, VGLUT3
expression is also markedly higher in pancreatic tumors than in normal pancreas (data not shown), indicating that upregulation of VGLUT3 expression is not specific to colon cancer and that VGLUT3 could be a prospective therapeutic target in other cancers as well.

Taken together, we have identified a signaling axis, miR-656-3p/VGLUT3/AKT, as an important regulator of tumorigenesis in colon cancer. Our studies suggest that VGLUT3 could be a novel molecular target for colon cancer treatment. Therefore, development of VGLUT3 inhibitors that cannot cross blood brain barriers is of clinical significance. Since VGLUT3 is mainly expressed in CNS and upregulated in colon cancer but rarely expressed in normal peripheral tissues, targeting VGLUT3 with those inhibitors will not likely cause adverse neurological or peripheral side effects, making VGLUT3 an attractive and specific target for colon cancer treatment.
CHAPTER 6: DISCUSSION AND FUTURE DIRECTIONS

miRNAs and TGFβ

In this study, we identified several miRNAs whose expression is either downregulated or upregulated after TGFβ treatment in a functional screening. We confirmed that miR-487b-3p and miR-656-3p modulated TGFβ function in colon cancer cells. TGFβ signaling plays multiple roles in different stages of cancer. During tumor progression, there are many genetic events occur in TGFβ signaling pathway, including the mutations of TGFβ receptor RII as well as SMAD proteins. However, those changes cannot fully explain the alterations in TGFβ signaling during tumor progression. miRNAs are important regulators of TGFβ, they modulate TGFβ function by targeting canonical TGFβ signaling components or regulating TGFβ target genes. Both miR-487b-3p and miR-656-3p enhance TGFβ function by inhibiting AKT activation. However, they have no effect on canonical TGFβ signaling itself. MiR-487b-3p and miR-656-3p are both located at 14q32.31 locus. By analyzing TCGA data base, we found that loss of copy number of this region was frequently occurred in colon cancer (data not shown). Those data suggest that during cancer progression, decrease of those two miRNAs may contribute to attenuated TGFβ tumor suppressing effect in colon cancer. By analyzing the target genes of those miRNAs, we will be able to identify novel regulators of TGFβ signaling and tumor growth.

Regulation of glutamate homeostasis in cancers

We have demonstrated that expression of GRM3 and VGLUT3 was increased in colon cancer patient samples and both of them played important roles in tumorigenesis of colon cancer. GRM3 and VGLUT3 are both key components of glutamatergic signaling. Two decades ago,
people noticed that glutamate level was increased in colon cancer tissues 161. Glutamate is a major product of glutaminolysis in mammalian cells. Tumor cells use glutamine as a major fuel for their bioenergetic needs and intermediates for macromolecular synthesis. When transported into the cells, glutamine is rapidly converted into glutamate by phosphate-dependent glutaminase (GLS), which consequentially, provides materials to meet cell’s energy and biosynthetic needs 162. There are many glutamate transporters that maintain intracellular and extracellular glutamate homeostasis. EAATs transport L-and D-aspartate along with glutamate from extracellular environment to the cells and maintain low level of extracellular glutamate 163. Another transporter, Na+-independent cystine/glutamate exchanger, System x_c− (SXC), uptakes extracellular cysteine and exports intracellular glutamate outside of the cells 164. In neurons, cytoplasmic glutamate is transported into vesicles by VGLUTs, and released into synaptic cleft 93. In tumors, loss of glutamate homeostasis is usually attributed to abnormal expression of glutamate transporters 165. In this study, we found VGLUT3 was upregulated in colon cancer cells. However, knockdown of VGLUT3 did not change the extracellular glutamate level. Since the other two vesicular glutamate transporters, VGLUT1 and VGULT2, are barely expressed in colon cancer cells, other types of transporters may play major role in the increased level of extracellular Glutamate. People found EAAT2 mediated glutamate transportation was defect in glioma tissues 166. In breast cancer, high level of glutamate secretion is mediated by System x_c− 167. In colon cancer cells, downregulation of EAAT1 and EAAT3 were found to be correlated with drug resistance 168. Therefore, it will be very interesting to find out whether EAATs and SXC are abnormally expressed in colon cancers. Studying their function will help understand the regulation and function of the glutamate
pathway during tumor development.

**Glutamate activates multiple pathways through multiple receptors**

Glutamate is a major excitatory neurotransmitter in central nervous system. In cancers, increasing evidence is showing its important role in tumor development. Like growth factors, glutamate acts through binding to a large family of receptors to active multiple downstream pathways. There are two types of glutamate receptors, ionotropic glutamate receptors and metabotropic glutamate receptors, both families have been proven to foster tumor growth in different cell context. We found that knockdown of GRM3 inhibits AKT activation and inhibits tumor growth of colon cancer cells both *in vitro* and *in vivo*. In addition, knockdown of GRM3 also inhibits ERK activation. Those results indicate GRM3 activates multiple downstream pathways to regulate colon cancer tumor growth. However, GRM2 and GRM4 are absent in our cell model and do not appear to play any role. Considering that there are more than 20 glutamate receptors, future efforts will determine the function of those receptors in colon cancer.

**Regulation of GRM3**

We showed that GRM3 was upregulated in colon cancer and that the upregulation was at the posttranscriptional level. We also showed that TGFβ increased GRM3 protein stability in a Smad2/3-dependent manner. How GRM3 is upregulated during tumor progression remains an interesting question. For most GPCRs, the endocytic pathway tightly controls their activity. On the cell surface, GPCRs are able to bind their ligands and activate signaling cascades inside the cells. After that, receptors are first phosphorylated by GPCR kinases (GRKs) and then internalized, which is mediated by arrestins and clathrin-coated pits. Some of the receptors are
recycled back to cell membrane and others are targeted to the lysosome for degradation, which results in the complete termination of receptor activity. Whether GRM3 turnover is dysregulated in colon cancer remains to be investigated. Defects of the recycling machinery will lead to increased expression of GRM3 on the cell surface and prolonged receptor activity.

**Other insights of VGLUT3 and GRM3**

VGLUTs are responsible for transporting glutamate into synaptic vesicles. In this study, we found that VGLUT3 was upregulated in colon cancer, leading to increased AKT activation. It suggests the importance of glutamate transportation in cancer development. Surprisingly, knockdown of VGLUT3 in colon cancer cells does not affect the extracellular glutamate level, indicating that there may be other mechanisms involved in the function of VGLUT3. In pancreatic β cells, it was found that VGLUT3 mediated glutamate uptake into insulin containing secretory vesicles, which modulated insulin release. Those findings reveal that VGLUT3 may co-transport other growth factors through the glutamate-containing vesicles. Once released to the extracellular environment, those growth factors may bind to their receptors and active various downstream signaling in cancer cells.

Like other G protein coupled receptors, GRM3 converts extracellular signals into intracellular responses. However, some G protein coupled receptors are found to be localized on intracellular membranes, and their function is distinct from their cell surface counterparts. For example, more than 80% of GRM5 was found at intracellular membrane in the rat and monkey substantia nigra. Only intracellular mGluR5 is able to phosphorylate ERK1/2 and Elk-1. Those findings emphasize that glutamate may activate distinct signaling pathways by binding to intracellular glutamate receptors. So far, there is no evidence showing that GRM3 is
exclusively expressed on the cell membrane. Therefore, it will be very interesting to determine whether GRM3 is also expressed on the membrane of other intracellular organelles. The change of intracellular glutamate may trigger distinct signaling pathways. Moreover, in that case, VGLUTs may also play a role in this process by transporting glutamate from cytoplasm to subcellular compartments, where glutamate binds to their receptors and activates intracellular glutamate signaling.

**Pharmacology of glutamate pathway inhibitors in cancer treatment**

In recent years, increasing evidence shows the importance of glutamate signaling in tumor development. Many glutamate receptor antagonists have been implicated to have potential for cancer treatment. AMPAR/KR inhibitors, ZK 200775 and GYKI 52466 showed anti-proliferative effect in glioma models. An NMDAR antagonist, memantine has been employed in a Phase II clinical trial now. The glutamate release inhibitor riluzole which is approved by FDA for treating amyotrophic lateral sclerosis showed promising inhibitory effect in glioma, breast cancer and prostate cancers. mGluRs are considered more susceptible for anti-cancer drug design due to their structure–function relationship. However, clinical studies of mGluR antagonists are still lacking. In this study, we showed that a GRM3 inhibitor, LY341495, inhibited tumor growth of colon cancer cells in vivo. Future studies are needed to validate the anti-cancer effect of GRM3 antagonists in clinical trials. Since GRM3 activates multiple signaling pathways, combination of GRM3 antagonists with other drugs may help increase their anti-tumor efficiency.
Literature Cited


10. Kavsak, P. et al. Smad7 binds to Smurf2 to form an E3 ubiquitin ligase that targets the TGFβ receptor for degradation. Molecular cell 6, 1365-1375 (2000).


20. Valderrama-Carvajal, H. *et al.* Activin/TGF-β induce apoptosis through Smad-


29. Kleeff, J. *et al.* The TGF-β signaling inhibitor Smad 7 enhances tumorigenicity in


37. Hasegawa, Y. *et al.* Transforming growth factor-β1 level correlates with angiogenesis, tumor progression, and prognosis in patients with nonsmall cell lung carcinoma.


46. Trobridge, P. et al. TGF-β receptor inactivation and mutant Kras induce intestinal neoplasms in mice via a β-catenin-independent pathway. Gastroenterology 136, 1680-


Calin, G.A. *et al.* Human microRNA genes are frequently located at fragile sites and


93. Liguz-Lecznar, M. & Skangiel-Kramska, J. Vesicular glutamate transporters (VGLUTs): the three musketeers of glutamatergic system. *Acta neurobiologiae*


106. Saugstad, J.A. & Ingram, S.L. Group I metabotropic glutamate receptors (mGlu1 and mGlu5). in *The Glutamate Receptors* 387-463 (Springer, 2008).


109. Page, G. *et al.* Group I metabotropic glutamate receptors activate the p70S6 kinase via both mammalian target of rapamycin (mTOR) and extracellular signal-regulated kinase (ERK 1/2) signaling pathways in rat striatal and hippocampal synaptoneurosomes.


(2005).


135. Forrester, E. et al. Effect of conditional knockout of the type II TGF-β receptor gene in


143. Schoepf, D.D., Jane, D.E. & Monn, J.A. Pharmacological agents acting at subtypes of


152. Grimson, A. *et al.* MicroRNA targeting specificity in mammals: determinants beyond


161. Okada, A. *et al.* Increased aspartate and glutamate levels in both gastric and colon


