UNIVERSITY OF CALIFORNIA, SAN DIEGO

Hedgehog Signaling Regulates KIT Expression in Gastrointestinal Stromal Tumors

A Thesis submitted in partial satisfaction of the requirements
for the degree Master of Science

in

Biology

by

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The Thesis of Tracy Ellen Lee is approved and it is acceptable in quality and form for publication on microfilm and electronically:

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Co-Chair

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Chair

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LIST OF ABBREVIATIONS

ATP	Adenosine-5'-triphosphate
COL1A1	Collagen type I, alpha I
Dhh	Dessert Hedgehog
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
GANT	GANT-61
GIST	Gastrointestinal Stromal Tumor
GSC	GIST Stem Cell
Gli1	Glioma-associated oncogene homolog 1
Gli2	Glioma-associated oncogene homolog 2
Gli3	Glioma-associated oncogene homolog 3
GNP	granule neuron precursors
Hh	Hedgehog
HSC	Hepatic stellate cells
IM	Imatinib
Ihh	Indian Hedgehog
ICC	Interstitial Cell of Cajal
KIT	c-kit, CD117
LDE	LDE225
mRNA	Messenger ribonucleic acid
PDAC	Pancreatic ductal adenocarcinoma
Ptc	Patched
PBS	Phosphate buffered saline
PDGF	Platelet-derived growth factor
PDGFB	Platelet-derived growth factor subunit B
PDGFR .	Platelet-derived growth factor receptor alpha
PCR	Polymerase chain reaction
RT-PCR	Reverse transcriptase polymerase chain reaction
Smo	Smoothened
SmoM2	Smoothened Mutant 2
Shh	Sonic Hedgehog
TKI	Tyrosine Kinase Inhibitor

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ACKNOWLEDGEMENTS

I would like to thank the members of the Sicklick lab for their collaboration, hard work, and mentorship. Without your help, it would not have been possible to successfully finish my Master's thesis. I have learned and grown so much from this experience and will always treasure it as I continue my work in the medical field. I would like to thank Dr. Jason Sicklick for being a great mentor, always supporting my research and my goal of becoming a great physician. I would like to give special acknowledgement to Amy Tang—for helping me obtain mice for my experiments, teaching me about PCR, siRNA, drug treatment, and cell culture techniques—and Stephanie Leonard for helping me with Western blotting and drug treatment techniques.

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ABSTRACT OF THE THESIS

Hedgehog Signaling Regulates KIT Expression in Gastrointestinal Stromal Tumors

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Master of Science in Biology

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Assistant Professor Jason K. Sicklick, Chair

Professor Dong-Er Zhang, Co-Chair

Gastrointestinal Stromal Tumors (GISTs) arise within the gut due to overexpression of mutated KIT genes within the Interstitial Cells of Cajal (ICC). Earlier work demonstrated that gastrointestinal mesenchymal development depends upon Hedgehog (Hh) signaling, while Gli3, a Hh transcription factor, regulates KIT mRNA expression in murine ICC-like cells. Therefore, we hypothesized that Gli transcription factors (Gli1/2/3) within the Hh pathway may regulate KIT expression in human GISTs.

We investigated the effects of regulating the activating (Gli1/Gli2) and the repressing (Gli3) members of the Gli family in two GIST lines (GIST-T1 and GIST882). In both lines, KIT mRNA expression is inverse to Gli3 expression (KIT/Gli3 ratio: T1=0.38; 882=1.98). Using genetic means, Gli3 siRNA induces a 62% increase in KIT mRNA, while Gli3 overexpression induces a 270% decrease in KIT mRNA and a 24% reduction in KIT protein levels in the $KIT^{hi}Gli3^{lo}$ line (882). Inversely, Gli1 and Gli2 siRNA knockdowns result in 55% and 19% decreases in KIT mRNA, respectively. Pharmacologic treatment with GANT61, a Gli1/2 inhibitor, decreases KIT mRNA by 50%. Given that KIT is the target of anti-GIST therapies, viability and apoptosis assays show that increasing KIT (i.e. Gli3 siRNA) slightly increases cell viability (5%) while decreasing KIT decreases viability (Gli2 siRNA: 10%) and increases apoptosis (Gli3 overexpression: 20%).

Herein, we provide the first evidence that Gli1/2/3 transcriptionally regulates expression of KIT in GIST. Gli1/2 activates KIT expression, while Gli3 represses KIT expression. Taken together, these transcription factors may serve as novel targets for treating KIT-driven GISTs.

Introduction

Background

Gastrointestinal Stromal Tumor (GIST) is the most common mesenchymal tumor of the gastrointestinal tract with ~6,000 new cases annually in the United States. The worldwide incidence is up to 20 cases per million people annually.[9,10] GISTs arise from the Interstitial cells of Cajal (ICC, Figure 1) within the gut mesenchyme and act as pacemaker cells for peristaltic contraction.[2] GISTs can occur in the stomach (60%) and small intestine (35%), as well as the esophagus and rectum. (Figure 2)[5,11] KIT (c-KIT, CD117), a receptor tyrosine kinase and proto-oncogene, was the first protein identified as a diagnostic tool for GIST because it was found to be expressed in ICCs and GIST, but also mutated in GISTs.

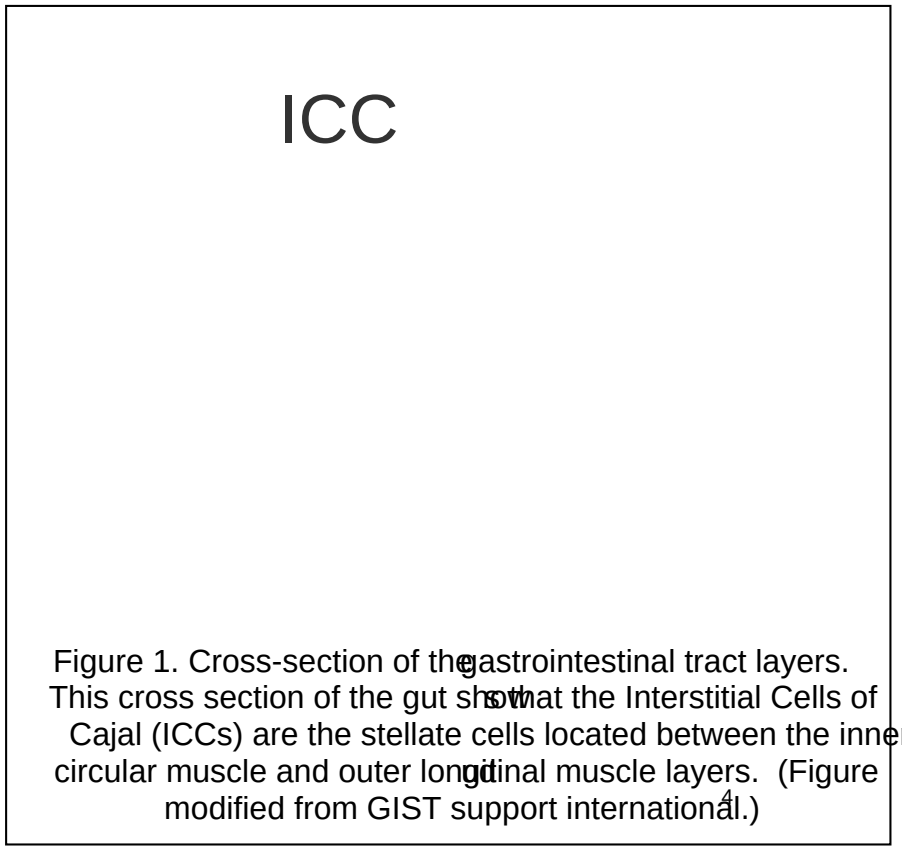

Figure 1. Cross-section of the gastrointestinal tract layers. This cross section of the gut shows that the Interstitial Cells of Cajal (ICCs) are the stellate cells located between the inner circular muscle and outer longitudinal muscle layers. (Figure modified from GIST support international.)[4]

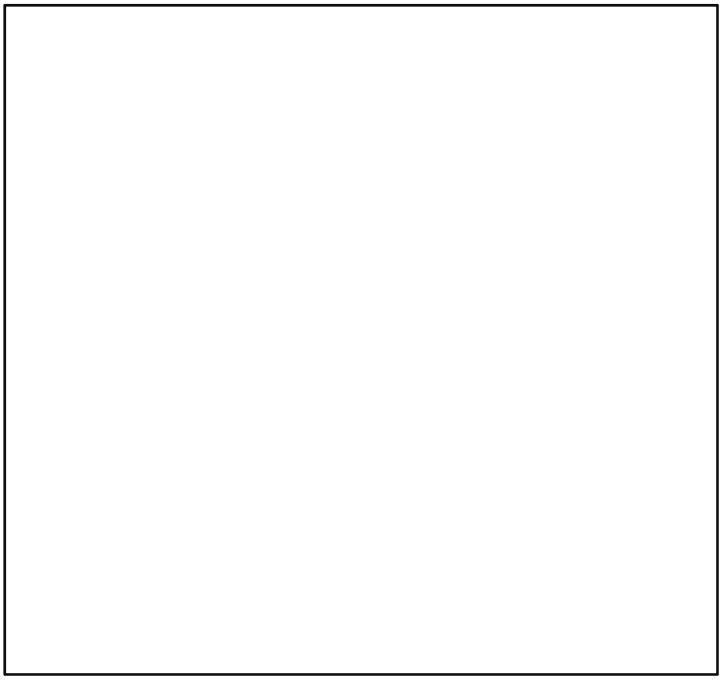

Figure 2. Primary GIST anatomic locations and relative frequencies.[5]

During embryonic development, ICC cell[12] cells express little or no KIT. However, they eventually mature to develop strong KIT expression. Through unclear mechanisms, ICCs may acquire activating gene mutations in kinases, including KIT, which lead to constitutive signal transduction, and in turn, GIST formation.[2,3,13] Approximately 70–80% of non-familial GISTs are caused by gain-of-function mutations in the KIT (c-KIT) gene (most often exons 11 and 9), whereas 5–10% are caused by activating mutations, deletions, or insertions that activate the platelet-derived growth factor receptor alpha (PDGFR.) gene (Figure 3).[2,3] Despite this genetic heterogeneity, more than 95% of GISTs express the KIT biomarker.[14,15] Given KIT's importance in GIST sarcomagenesis, all FDA-approved anti-GIST therapies, including imatinib (Gleevec, Novartis, Basel, Switzerland), are predicated upon targeting KIT. As a result, GIST tumor biology has

served as the paradigm for highlighting how genotype may predict phenotype, as well as how targeted therapies may be employed to treat cancers in a personalized manner.

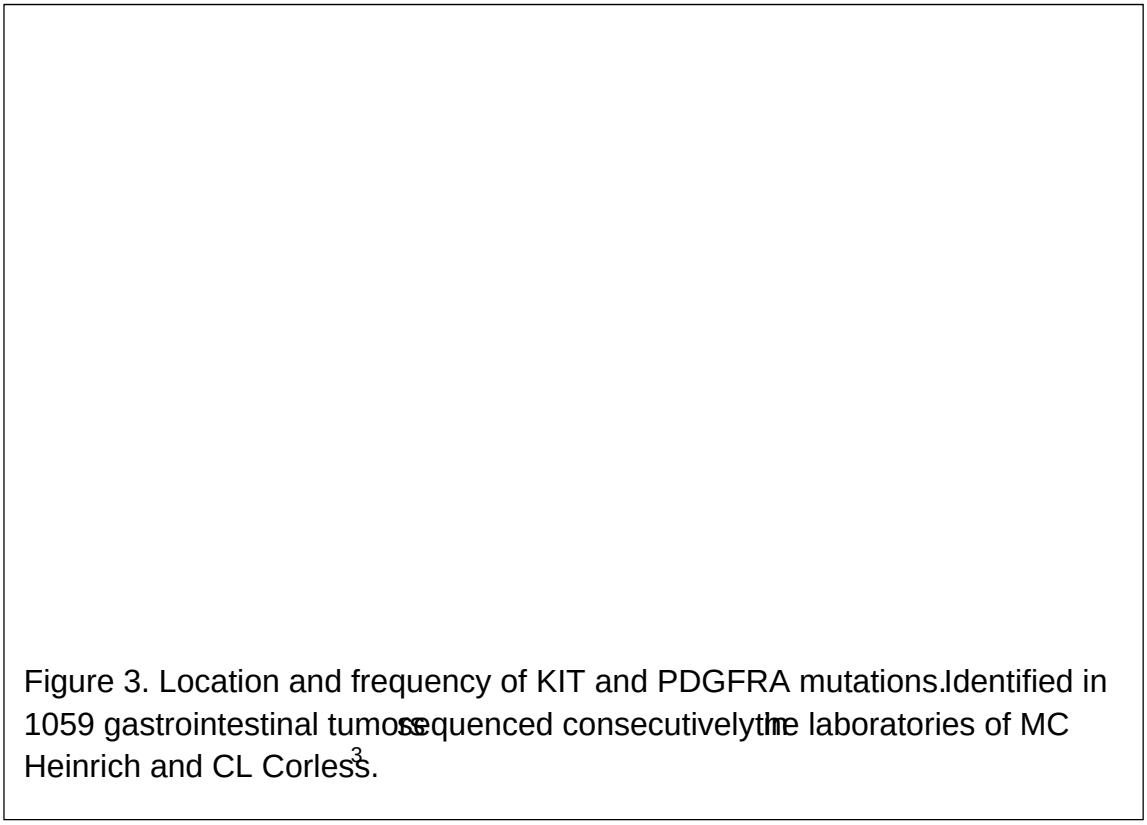

Figure 3. Location and frequency of KIT and PDGFRA mutations. Identified in 1059 gastrointestinal tumors sequenced consecutively in the laboratories of MC Heinrich and CL Corless.[3]

Gene Mutations and Therapeutic Targets in GIST

Targeting KIT with imatinib (IM) has led to improved survival in patients with unresectable or metastatic GIST.[16-19] In the adjuvant setting, IM significantly reduced GIST recurrence rates following 1 or 3 years of therapy, as compared to treatment with placebo or 1 year of therapy, respectively.[20,21] However, in both randomized, prospective trials, patients began to develop recurrences approximately 9 months after stopping therapy, irrespective of the length of treatment. This suggests that there is a population of IM-resistant cells present within these tumors. In general, this is thought to be caused by primary resistance associated by specific mutations (i.e., PDGFR^{D842V} or BRAF^{V600E}) or

secondary resistance caused by acquired mutations in KIT's ATP binding pocket (i.e., exon 13-14) or the kinase activation loop (i.e., exon 17-18, Figure 4).[2] However, a third possibility exists. Like ICC stem cells, GIST stem cells (GSC) have little or no KIT expression.[12] Therefore, these stem cells may have IM-resistance that is independent of the aforementioned mechanisms. Consequently, pathways that regulate GSCs via KIT-independent mechanism are of distinct interest. Gleaning insight from embryonic gut developmental, the Hedgehog (Hh) pathway has been shown to regulate the viability and differentiation of adult gastrointestinal stem cells.[22,23]

Figure 4. Secondary mutations in KIT and their drug sensitivities. A comparison of the relative in vitro potency of imatinib (IM) and sunitinib (SU, second-line therapy) versus secondary mutations that are associated with resistance to IM as assessed by in vitro expression studies is shown. JM= juxtamembrane.[2]

Gut development depends upon Hedgehog signaling

Several developmental signaling pathways, including the Wnt, Notched, and Hedgehog (Hh) pathways have been implicated in embryogenesis. Numerous studies have demonstrated that the Hh pathway is essential for the embryonic development of organs (i.e., gastrointestinal tract, liver, pancreas, and lung; figure 5), as well as axis formation (i.e., left-right and anterior-posterior) of the limbs and neutral tube. Normal patterning within the endoderm and surrounding mesoderm regulates morphogenesis and initiating of organogenesis, including laying down boundaries of the organ primordia. At the cellular level, the Hh pathway works via paracrine or autocrine signaling to regulate cellular differentiation, proliferation, and survival in order to regulate organogenesis in time and space.[22,24-26]

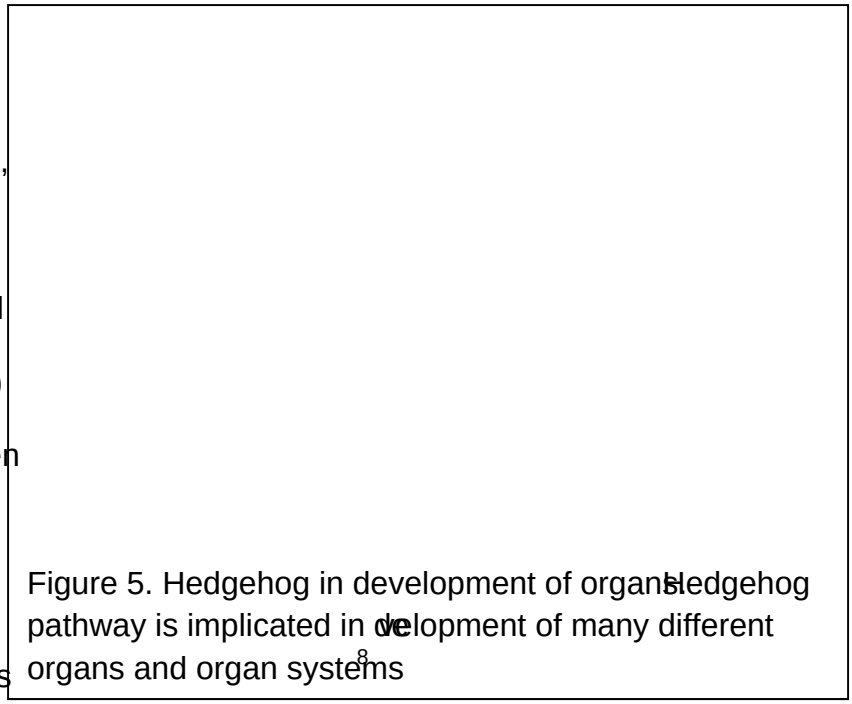

Figure 5. Hedgehog in development of organs. Hedgehog pathway is implicated in development of many different organs and organ systems[8]

The Hh pathway was discovered in Drosophila by Nüsslein- Volhard and Wieshchaus, in which they reported that Hh controls the development of the denticles or a segmented anterior-posterior body axis (Figure 6).[6] The Hh pathway is also essential for

mesenchymal development and proliferation, inhibition of mesenchyme differentiation, and development of normal patterning in the GI tract in humans along the crypt-villus axis, the anterior-posterior axis, dorsal-ventral axis, and the radial axis at the tissue level.[22,24-27]

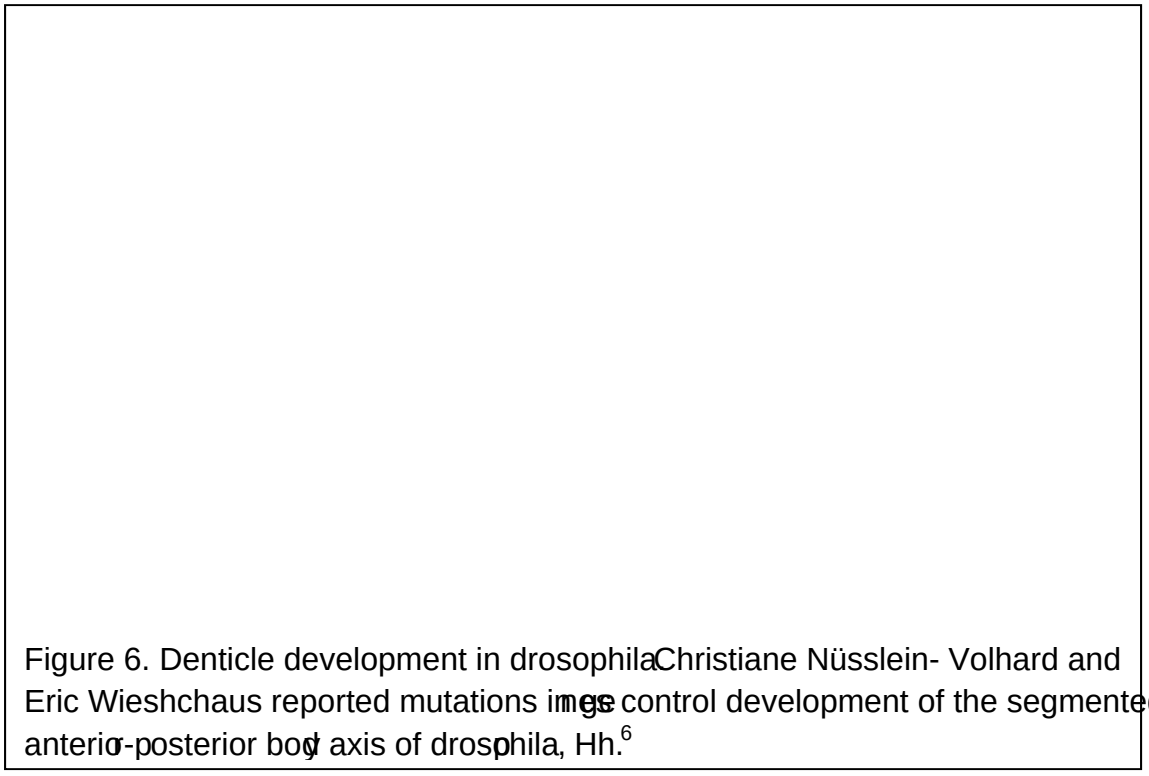

Figure 6. Denticle development in drosophila. Christiane Nüsslein- Volhard and Eric Wieshchaus reported mutations in genes that control development of the segmented anterior-posterior body axis of drosophila, Hh.[6]

Furthermore, pancreas development is dependent on signaling between the mesoderm and the two regions of ventral and dorsal foregut endoderm, which expresses Shh and Ihh until embryonic day 8.5.[28] The Hh pathway components in various mesenchymal and stromal cells of the abdomen have been observed, such as in pancreatic stromal cells and hepatic stellate cells. Pancreatic ductal adenocarcinoma (PDAC) tumors and cells lines show an increase in and Shh Ihh not seen in adult pancreatic cells, which activate the Hh pathway in neighboring non-malignant stromal cells. This leads to

a paracrine feedback loop to the malignant pancreatic cells, which prevents vascularization of the surrounding stroma. PDAC comes from exocrine cells that produce digestive enzymes, which make up the majority of cells in the pancreas.[8,29] Likewise, the activity and regulation of hepatic stellate cells (HSC) are mediated by Hh pathway.[28] In addition, both PDAC cells and HSCs have a neural component and a mesenchymal component. Pancreatic exocrine cells regulate the ability to release digestive enzymes to the gut for digestion. HSCs become fibroblast cells during liver injury to repair the liver.[8,28]

Besides the gut, Hh signaling is also found in the development of the neural tube and the brain. The main role of the Hh pathways in neural tube development is as a morphogen. The main role of Hh ligands in cerebellar granule neuron precursors (GNPs) is as a mitogen or a survival factor. In medulloblastoma, there is an increase in Hh pathway expression, which leads to uncontrolled GNP proliferation.[8,29] Similar to HSCs and pancreatic stromal cells in pancreatic cancer, glioblastoma also has a paracrine signaling pattern in which the tumor-associated endothelial cells and the surrounding normal astrocytes have increased Hh ligand secretion to Hh responsive tumor cells.[29]

In addition, Hh is implicated in maintaining the stem cell niches in the stomach and aiding in enterocyte differentiation in the bowel. This can occur in a long-range or short-range pattern through epithelial-mesenchymal interactions. Work by Mao and colleagues have recently demonstrated that Hh signaling is essential for normal mammalian embryonic gut development by promoting survival and proliferation of the

mesenchyme.[22] In collaboration with Mao, we have determined that Hh signaling regulates the development of the ICC population and thus, GISTs.

Hedgehog Signaling Pathway

The Hh pathway (Figure 7) can be activated via ligand-dependent or ligand-independent mechanisms. The Hh ligands, Sonic (Shh), Indian (Ihh), and Desert (Dhh), are secreted ligands with different, but often overlapping expression and ultimately bind to the Patched (Ptc) receptor.[27] The Hh ligands are produced in the secreting cells and are modified by cleaving the full length form and adding cholesterol to it. The cholesterol acts as a stabilizing molecule, thus, restricting the range of Hh activity. In order for the Hh ligands to have long range activity, Dispatched (Disp) – a transmembrane transporter-like protein similar to Ptc—is needed to release the Hh ligands and transport it to the target cells.[25,27] Ptc is a 12-transmembrane domain tumor suppressor and an integral membrane protein. In the absence of the Hh ligands, Ptc acts as a tumor suppressor protein by binding to, and repressing, the activity of Smoothened (Smo). Smoothened is a 7-transmembrane domain proto-protein and is a member of the G-protein coupled receptor (GPCR) family. Smo is essential for all aspects of Hh signal transduction because it activates downstream transcription factors. Conversely, when the Hh ligands bind to Ptc, the Ptc-Smo complex undergoes a conformation change without dissociation and Smo becomes activated. This multi-step signal transduction leads to activation of the Gli family zinc finger transcription factors (in mammalian cells). Generally, Gli1 and Gli2 serve as transcriptional activators while Gli3 functions as a transcriptional repressor. However, the Gli family proteins have a gradient of signaling. Gli2 and Gli3 both have an

N-terminal repressor domain and a C-terminal activator domain. Gli1 lacks the similar N-terminal repressor domain and acts as the terminal and critical transcriptional activator of the pathway. Transcription of Gli1 is induced by Hh signaling, thus, being consistently transcribed by the Hh-activated cells. Gli2 can act as either an activator or a repressor depending on the binding of the Hh ligands. In contrast, Gli3 can act as a weak activator, but mostly acts as a repressor because the C-terminal domain of the full-length protein is proteolytically cleaved into the repressor.[25,30] Thus, high Gli1/ Gli2 expression and low Gli3 expression in cells can be correlated with more active Hh signaling than the opposite expression pattern in other cells. Activation of Gli proteins causes them to enter the nucleus and turn on transcription of Hh target genes such as Ptc1, Gli1, and Hhip (Hedgehog-interacting protein).[7] These genes are feedback and feed-forward components of the Hh pathway. Ptc causes feedback inhibition of the Hh pathway by sequestering the Hh ligands. Hip is a membrane-bound protein that competes with Hh ligands for Ptc binding to attenuate the signal transduction.[25,27]

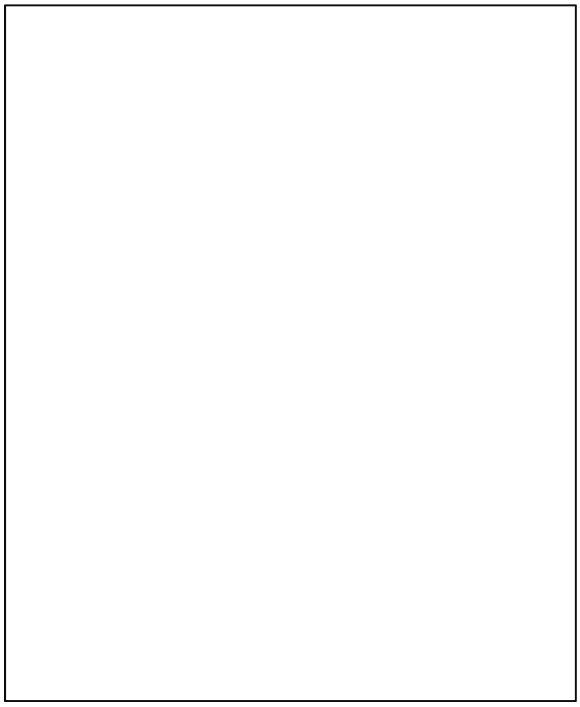

Figure 7. The hedgehog pathway. This figure shows the Hedgehog pathway starting with Shh binding to Patched (Ptc) and causing Ptc to release its inhibition of Smoothened (Smo). Smo, through other molecules, ultimately causes activation of downstream transcription factors, the Gli family proteins. Gli1 and Gli2 are transcriptional activators and Gli3 is a transcriptional repressor.

Additionally, the activating members (Gli1 and Gli2) and the repression members (Gli3) of the Gli transcription factor family have the same DNA consensus binding site (GACCACCCA).[31,32] Some of the positions on the consensus sequence are absolutely conserved, while others can have one or more nucleotide differences.[33]

Role for Hedgehog in Cancer

The role of Hh has been implicated in various cancers. Since Hh is known to be a developmental pathway, cancer cells can hijack the pathway in order to proliferate, to recruit blood vessels, and to enter surrounding tissue. In some malignancies, Hh signaling is caused by loss-of-function mutations in the tumor suppressor gene, Ptc (i.e., Gorlin's syndrome and medulloblastoma), while others are caused by gain-of-function mutations in the Smo proto-oncogene, in which the pathway is independent of Hh or Ptc binding (i.e., basal cell carcinoma).[29] Gorlin's syndrome causes a predisposition to basal cell carcinoma (BCC), medulloblastoma and rhabdomyosarcoma.[30] In other cancers, tumorigenesis may be driven by overexpression of pathway activators including Hh ligands (i.e., esopheageal, pancreatic cancer), Smo (i.e., hepatocellular carcinoma) or Gli1/2 and act an autocrine manner. In upper GI tract carcinomas, there is presence of Shh and Ihh mRNA in almost all cell lines, while Ptc and Gli mRNA are only coexpressed in about 75% of the cell lines.[24] Shh and Ihh mRNA were detected in all colon cancer cell lines tested, but only a small portion of cell lines had mRNA upregulation for the other Hh pathway components.[24,30] However, it was shown that inactivation of the Hh pathway in mice with adenomas causes a decrease in the tumor size.[34] Pancreatic adenocarcinomas show increased expression of the Hh pathway.

Furthermore, there have been studies showing the epithelial-mesenchymal interaction of the tumors that involve epithelial cells as well as stromal cells. Various studies have supported model of ligand-dependent autocrine/juxtacrine Hh signaling that supports the growth of pancreatic adenocarcinomas. While recent studies challenge that model, a new model was proposed that favors a contribution by paracrine Hh pathway activation in the tumor stroma to pancreatic cancer development instead.[29,35] In addition, the epithelial-stromal interaction has been shown in hepatic stellate cells within cirrhotic lives which drive the development of hepatocellular carcinoma.[36,37] Moreover, this signaling pathway is involved in genesis of a variety of mesenchymal cancers including rhabdomyosarcoma.[38]

Link between Hedgehog and GIST

Emerging data suggests that Hh signaling may play a role in the pathogenesis of GIST. Mao and colleagues have also shown that overexpression of mutant Smo (e.g., SmoM2) within the developing gut mesenchyme leads to the genesis of gastric and intestinal submucosal tumor-like proliferation.[22] In collaboration with Mao, a similar phenotype was seen with Gli2 overexpression in the gut mesenchyme. Tumors from these mice

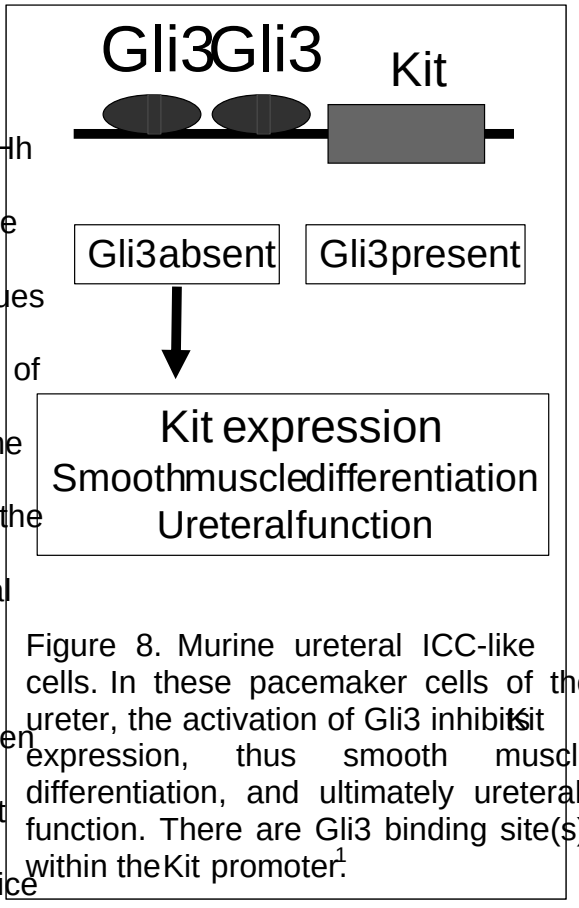

Figure 8. Murine ureteral ICC-like cells. In these pacemaker cells of the ureter, the activation of Gli3 inhibits Kit expression, thus smooth muscle differentiation, and ultimately ureteral function. There are Gli3 binding site(s) within the Kit promoter.[1]

stained positive for KIT (unpublished data). Moreover, earlier work by Yoshizaki and colleagues investigated submucosal tumors and suggested that gastric GISTs may express certain Hh components, including Shh, Ptc, Smo, and Gli1.[39] During the course of our work, Pelczar et al. reported that Ptc inactivation leads to the development of PDGFRα+, but KIT-, GIST-like lesions in mice in late 2012.[40] However, the authors were unable to reconcile the KIT negativity. Cain and colleagues have demonstrated that Gli3, the Hh transcription factor, represses the expression of KIT in murine ureteral ICC-like cells (Figure 8).[1] Taken together, these studies would point to a correlation between Hh and GISTs in both KIT-positive and KIT-negative GISTs.

Preliminary Data from the Sicklick Lab

In order to establish the presence of the Hh signaling pathway in human GISTs and human GIST cell lines, microarray analyses and quantitative RT-PCR was performed in collaboration with Novartis. The expression of two biomarkers (KIT and DOG-1) and

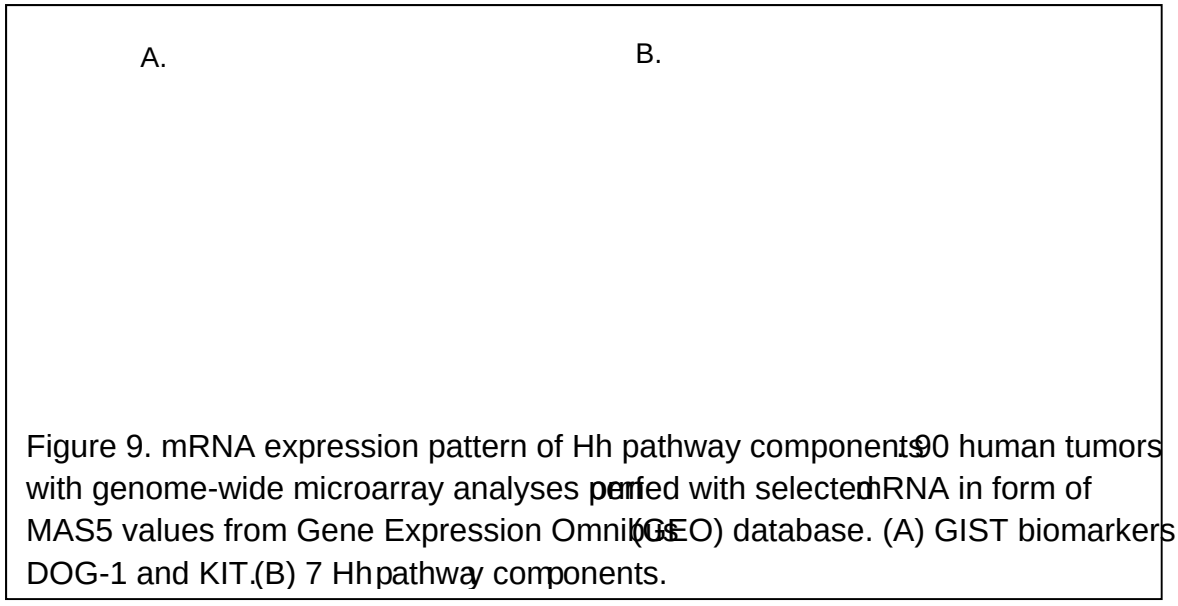

Figure 9. mRNA expression pattern of Hh pathway components in 90 human tumors with genome-wide microarray analyses performed with selected mRNA in form of MAS5 values from Gene Expression Omnibus (GEO) database. (A) GIST biomarkers DOG-1 and KIT. (B) 7 Hh pathway components.

seven Hh pathway components (Shh, Ihh, Ptc, Smo, Gli1, Gli2, Gli3) were evaluated in 93 human tumors (55 gastric, 14 small bowel, 1 extra-intestinal GIST, 1 peritoneal metastasis, and 22 GISTs with an unknown location). All samples highly expressed the two biomarkers, KIT and DOG-1 (Figure 9A). In addition, all samples had increased expression of Ptc, Smo, Gli2, and Gli3 (Figure 9B). An early event in GIST pathogenesis is the development of chromosomal aberrations.[41,42] Using an Affymerix SNP array analysis, Astolfi and colleagues have reported that there were chromosomal alterations, including amplifications and deletions, in all the mutant GIST patients, whereas most of the wild-type GIST patients did not have chromosomal alteration. In addition, there were known oncogenes and tumor suppressors in the 3 most commonly mutated chromosomes. In chromosome 7, there is statistically significant amplification in copy number of Gli3.[42] Our collaboration with Novartis confirmed these findings (data not shown). To determine if the expression of Hh signaling components is partially attributable to these occurrences, Affymetrix SNP array analyses were performed. Copy number analyses show that there is increased copy number in Smo and Gli3, but decreased or no change in copy number of Ptc and Gli2. Thus, the expression of Smo and Gli3 may be partially attributable to increased gene copy numbers, while the expression of Ptc and Gli2 are more likely to reflect increased proto-oncogene expression.

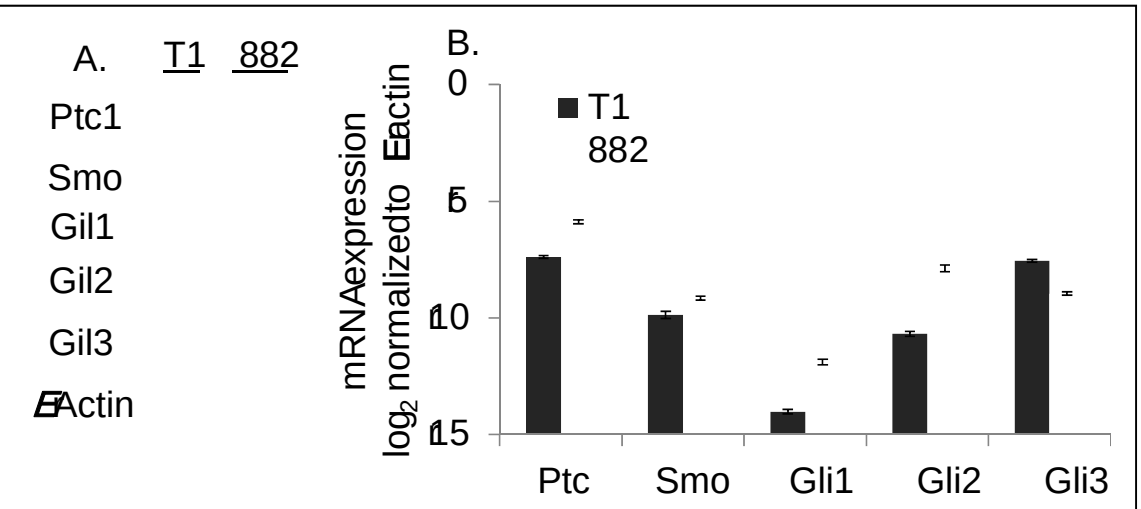

Figure 10. Human GIST lines express hedgehog components. Quantitative RT-PCR followed by gel electrophoresis of two human GIST lines. (A) Gel electrophoresis of Hh pathway components. (B) Quantitative RT-PCR using the ΔΔCT method relative to β-actin control shows mean ± SD.

To further validate the microarray analysis, quantitative RT-PCR analyses were performed in two parental GIST lines. GIST-T1 has a KIT $^{V559'}$ (exon 11) and GIST882 has a KIT ^{K642E} mutation (exon 13). While both lines lack expression of Shh and Ihh, it was confirmed that the lines expressed high, although distinct, levels of Ptc, Smo, Gli2, and Gli3 mRNA with Ptc having the highest expression level followed by Gli3, Gli2, Smo, then Gli1 with the lowest expression level (Figure 10). GIST882 had 3.3-fold higher Ptc, 2.0-fold higher Smo, and 6.0-fold higher Gli2 mRNA expression levels relative to GIST-T1. In contrast, GIST882 had 2.2-fold lower Gli3 mRNA expression relative to GIST-T1. Thus, GIST-T1 more robustly expresses the Gli repressor (i.e., Gli3), while GIST882 more robustly expresses the Gli activator (i.e., Gli2). These RT-PCR findings confirmed the microarray results.

Further analysis of the KIT mRNA expression relative to the Gli3 mRNA expression was performed using quantitative RT-PCR of the Hedgehog pathway components in two GIST cell lines: GIST-T1 and GIST882 (Figure 11). This analysis shows an inverse relationship between Gli3 and KIT. The GIST-T1 cell line is a $KIT^{lo}Gli3^{hi}$ line with a KIT/Gli3 ratio of 0.38, whereas the GIST882 cell line is a $KIT^{hi}Gli3^{lo}$ with a KIT/Gli3 ratio of 1.98.

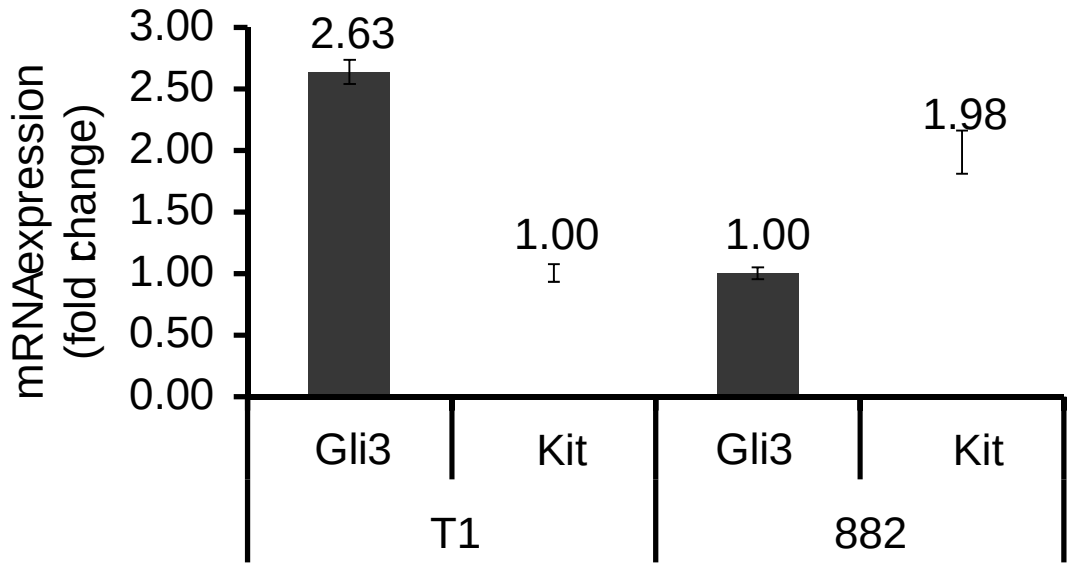

Figure 11. KIT mRNA expression is inverse to Gli3 mRNA expression. Quantitative RT-PCR of the Hedgehog pathway components in two GIST cell lines: GIST-T1 and GIST882 shows an inverse relationship between Gli3 and KIT. GIST-T1 line is a $KIT^{lo}Gli3^{hi}$ line with a KIT/Gli3 ratio of 0.38, whereas GIST882 is a $KIT^{hi}Gli3^{lo}$ and a KIT/Gli3 ratio of 1.98.

The Hh pathway expression in three small bowel GISTs (duodenal, jejunal, and ileal) with KIT exon 9, 11, and 9 mutations, respectively, were analyzed to further evaluate additional human tumors (Figure 12). This analysis confirmed the relative expression pattern of Ptc > Gli3 > Gli2 > Smo > Gli1 seen in the GIST cell lines. Taken

together, the human tumor data parallels the cell line findings in that there is high Ptc, Gli3, Gli2, and Smo mRNA expression, but low Gli1 mRNA. Shh and Ihh mRNA expression appear to be absent. Therefore, Hh signaling in human GIST appears to be driven via ligand-independent mechanisms.

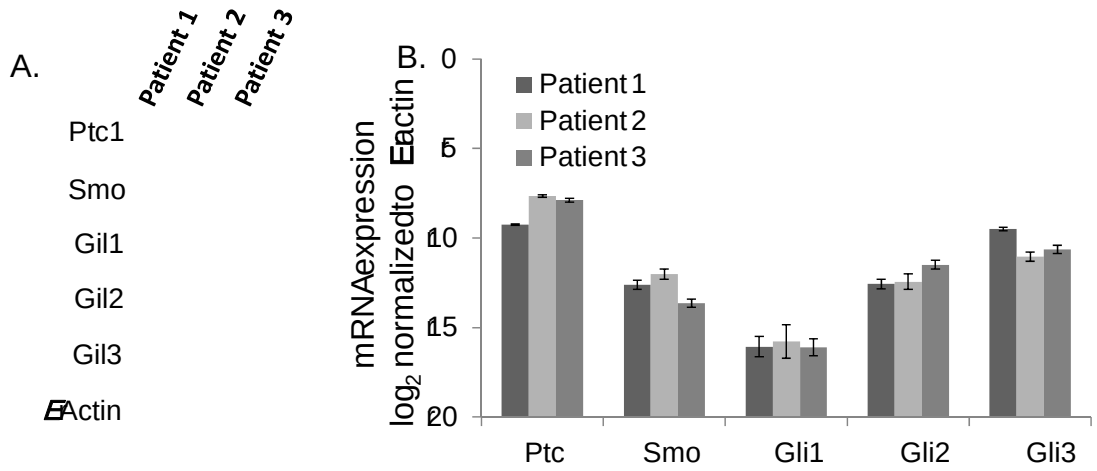

Figure 12. Human GIST tumors express hedgehog components. Expression of Hh signaling components, based upon RT-PCR and qPCR analyses in tumor tissue from 3 GIST patients from Moores UCSD Cancer Center. (A) Representative gel electrophoresis of PCR products and (B) bar graph shown as mean ± SD relative to Actin.

In summary, the preliminary studies show that Hh is present and active in 2 GIST cell lines and 93 human tumors. The aim of this study is to investigate using murine models of GISTs and to understand the Gli family transcription factors regulation on the KIT biomarkers in human GISTs. Understanding the regulation of KIT will elucidate novel targets for treating these KIT-driven tumors.

Material and Methods

I. Animal Studies

Mutant $Kit^{V558\Delta/+}$ mice[43] and $Kit^{+/K641E}$ transgenic mice[44] were kindly provided by P. Besmer (Memorial Sloan-Kettering Cancer Center, New York) and by B. Rubin (Cleveland Clinic, OH), respectively. Mice were housed in a barrier facility on a 12-hour light-dark cycle, and autoclaved laboratory rodent chow and water were available ad libitum. All experimental procedures were approved by the Institutional Animal Care and Use Committee at UCSD. Animal studies were conducted in accordance with NIH guidelines for the Care and Use of Animals.

II. Cell Culture

We obtained the GIST-T1 line from Dr. T. Taguchi, Kochi Medical School, Japan,[45] and the GIST882 lines from Dr. JA. Fletcher, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, as well as Dr. S. Singer, Memorial Sloan-Kettering Cancer Center, New York.[46] GIST-T1 cells were cultured in Dulbecco's modified Eagle's medium with 10% FBS, while GIST882 cells were cultured in RPMI 1640 with 20% FBS. All cell culture media were supplemented with 1% penicillin/streptomycin (Mediatech, Manassas, VA) and 2 mM glutamine (Mediatech). All cell lines were maintained in a humidified incubator with 5% CO_2 at 37°C.

III. Electroporation

Using the Amaxa® Cell Line Nucleofector® Kit V (Lonza), GIST882 cells were electroporated using 2 million cells per condition per assay according to manufacturer instructions. The siRNA oligos (Dharmacon) were designed and electroporated for

knockdown experiments: 2 nM Gli1, Gli2 or Gli3. Scrambled oligos were used as a negative control. In vitro overexpression was generated by electroporating with either 4 μg of Gli3 plasmid DNA or pGIPz empty vector. Cells were incubated for 24-48 hours.

IV. Cell Viability Assay and Apoptosis Assay

After electroporation, the GIST cells were plated at 20,000 cells per well in 6 replicates on 96-well plates and incubated for 24-48 hours. They were then analyzed for viability using Cell Counting Kit-8 (Dojindo Molecular Technologies) after 1.5 hour incubation with the reagent. Caspase activity was measured with Apo-ONE Homogeneous Capase-3/7 Assay (Promega) after 30 minute incubation with the reagent.

V. Two-step Real-time Quantitative RT-PCR

Mouse tumor tissue was homogenized in TRIzol reagent (Life Technologies, Carlsbad, CA) with a tissue homogenizer for 1 min at maximum speed. Total RNA from tissue homogenates or cell pellets was extracted using TRIzol reagent (Life Technologies) and reverse transcribed to cDNA in 96 plates with iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA) according to the manufacturers' instructions. Quantitative real-time PCR was conducted with SsoFast EvaGreen Supermix (Bio-Rad Laboratories) on a CFX96 real-time system (Bio-Rad Laboratories) using primers specific for genes of interest and the β-actin housekeeping gene. Primers were designed using Genbank sequences or as described previously[47-55], and primer sequences for human and mouse genes assayed were listed in Tables 1 and 2, respectively. Samples were run in triplicate with the following PCR parameters: denaturing at 95°C for 30

seconds followed by 40 cycles of 5-second denaturation at 95°C and 5-second annealing–extension at the optimal primer annealing temperatures. The CFX96 system automatically generated threshold cycle (Ct) values after each run. Target gene levels in cells are presented as a ratio to levels of β-actin, according to the ΔΔCt method, or as a ratio to levels detected in the corresponding control cells, according to the ΔΔCt method.[37] These fold changes were determined using point and interval estimates. Final PCR products were analyzed by gel electrophoresis separated on a 2.0% agarose gel run in 1X Tris-acetate-EDTA buffer.

VI. Western blot

Following electroporation, GIST882 cells were plated for 24-48 hours and harvested using 1X RIPA lysis buffer (Cell Signaling Technologies) to make protein lysates. DC Protein Assay (Bio-Rad Laboratories) was performed per manufacturer instructions to determine protein concentrations. Electrophoresis was performed using 4-12% Bis-Tris gels with 30 mg of protein and the NuPAGE® MOPS SDS Running Buffer system (Life Technologies). Protein was transferred to a PVDF membrane using the iBlot Gel Transfer System (Life Technologies). Primary antibodies for polyclonal rabbit anti-human CD-117, c-KIT antibody (Dako; 1:1000) and b-actin antibody (Cell Signaling Technology; 1:1000) were incubated for 90 minutes at room temperature. ImmunoPure goat anti-rabbit IgG (H+L) peroxidase-conjugated secondary antibody (Thermo Scientific; 1:10,000) was incubated for 30 minutes at room temperature. The blot was developed using Amersham ECL Plus Western Blotting Detection Reagents (GE Healthcare Life Sciences) per manufacturer instructions.

VII. Statistical Analysis

All statistical analysis was performed using GraphPad Prism 4 (GraphPad Software, La Jolla, CA). Data are reported as means ± standard deviation (SD). Comparisons between controls and experimental groups were analyzed for significance by the Student's t-test (2-tailed testing). Statistical significance was set as $P < 0.05$. Results are expressed as mean ± standard error mean (SEM) or standard deviation (SD) as appropriate. Comparisons between groups were performed using the Student's t-test (Stata 9.0, StataCorp, College Station, TX). Statistical significance was accepted at the 5% level and statistical trends were accepted at the 10% level.

Results

Murine GIST Models Express Hh Signaling Components

Given that the Hh pathway has been shown to regulate the development of the mouse gut mesenchyme[22] and that Ptc inactivation in the lysozyme M–expressing cells of the bowel mesenchyme results in the development of GIST-like lesions[40], we sought to determine whether two murine models of GIST express Hh signaling components (Shh, Ihh, Ptc, Smo, Gli1, Gli2, Gli3) in the same pattern as human cell lines and tumors did. Similar to the GIST-T1 line, $Kit^{V558\Delta/+}$ mice[43] has a $KIT^{V559\Delta}$ (exon 11), and like the GIST882 line, $Kit^{+/K641E}$ transgenic mice[44] has a KIT^{K641E} mutation (exon 13). Total RNA was extracted from GISTs (N=3 mice per model) and quantitative RT-PCR analyses were performed (Figure 13B). KIT and β-actin served as positive controls. Both models highly expressed several Hh pathway components, including Ihh, Ptc, Smo, Gli1 and Gli2. There was little Shh or Gli3 mRNA expression in either model. We confirmed our findings by gel electrophoresis of the PCR products (Figure 13A). We then compared the mRNA expression of these components in the two models. K641E GISTs had 3.2-fold higher expression of Ihh (P=0.01) and 1.4-fold higher expression of Smo (P=0.02) than V558Δ GISTs (Figure 13B). Given that K641E mice may carry either one or two copies of the transgene, we compared single versus double knock-in mice (Figure 13C). Comparison of Hh pathway mRNA expression in the tumors demonstrated a 1.2-fold higher Smo expression in the double knock-in tumors (P=0.02). The single knock-in tumors had a 13.9-fold higher Ihh expression (P=0.003). Taken together, these findings demonstrate that two murine models of GIST express Hh signaling components. The two

murine models of GIST appeared to have high Ihh and Gli1 levels, but low Shh and Gli3 levels—as compared to the human GIST cell lines and tumors which don't have Shh and Ihh expression, have low Gli1, and high Gli3 expression. Therefore, these two murine models of GIST do not reflect the expression of Hh pathway components seen in seven human GIST cell lines and the 93 human tumors.

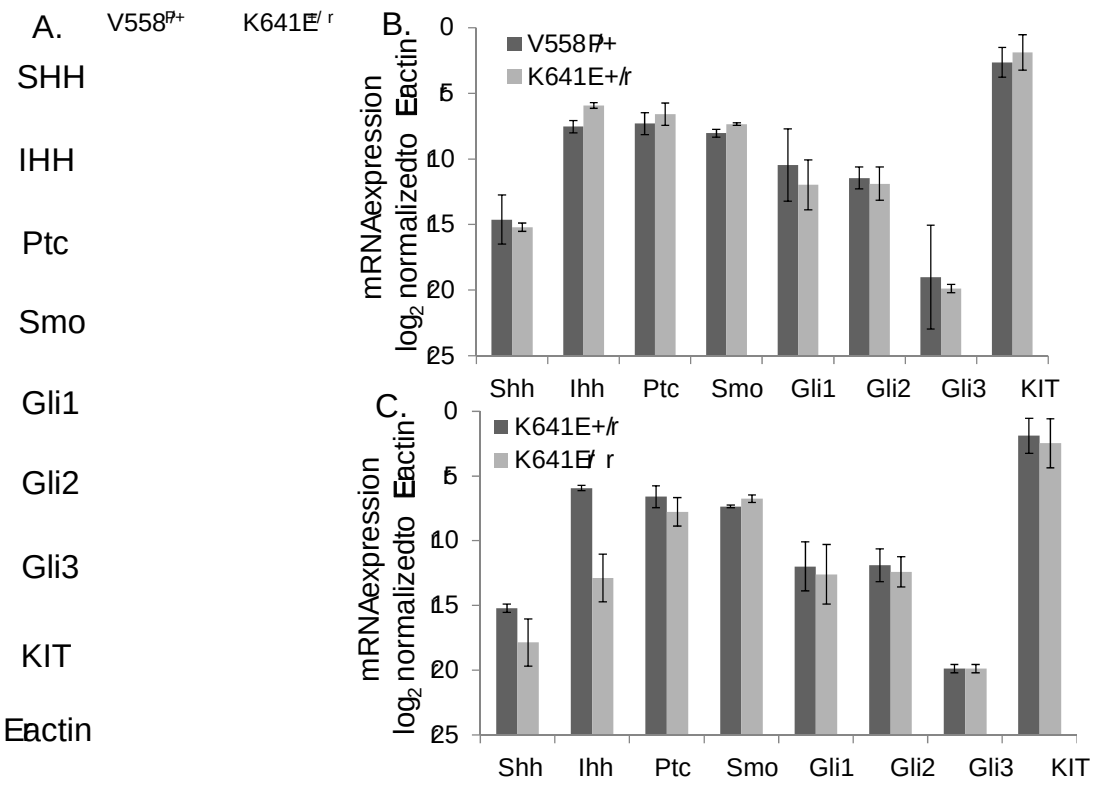

Figure 13. Murine mRNA expression profile. This figure shows the Hh pathway component mRNA expression. (A) gel electrophoresis of the RT-PCR between K641E and V558 mice (B) RT-PCR between K641E and V558 mice (C) RT-PCR between the heterozygous and homozygous K641E mice mean ± SD relative to actin.

Genetic Modulation of Gli3 Regulates KIT Expression

Since modulation with animal models was not as reliable as hoped, we decided to investigate the Hh pathway modulation along with the biomarkers, specifically KIT, with the inhibition of the Gli transcription factors in human GIST cell lines. Electroporation of the GIST-T1 cell line was very difficult, thus the GIST882 cell line was used instead. Electroporation of the GIST882 cell lines was performed using the Amaxa Cell Line Nucleofector Kit with Gli1, 2, 3, and scrambled siRNA purchased from Dharmacon, and then quantitative RT-PCR of KIT was performed. At 24 hours after electroporating cells with Gli1 and Gli2 siRNA, Gli1/2 were successfully knocked down and there was a 19% (P=0.05) and a 6% (P=0.66) decrease in KIT mRNA expression, respectively. At 48 hours, there is a 55% (P=0.08) and a 19% (P=0.15) decrease in KIT mRNA expression,

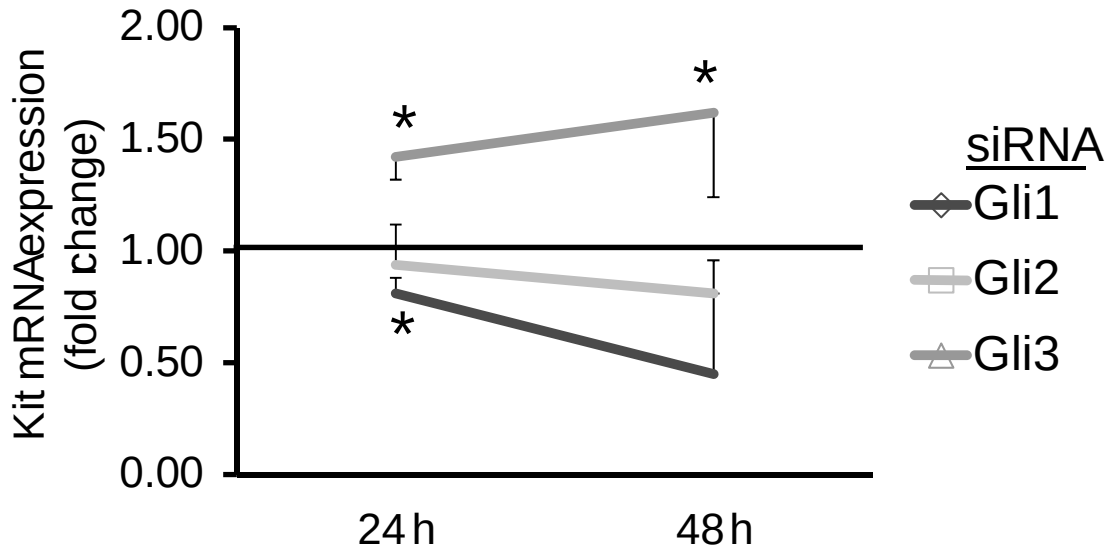

Figure 14. Genetic modulation of Gli family transcription factor regulates KIT expression. At 24 and 48 hours after electroporated cells with Gli1 and Gli2 siRNA, quantitative RT-PCR was performed for KIT mRNA.

respectively. Since the GIST882 line is a KIT^{hi}Gli3^{lo} line, we also evaluated the levels of KIT with Gli3 siRNA knockdown. At 24 hours, there was a 42% (P=0.01) increase in KIT mRNA expression, whereas at 48 hours, there was a 62% (P=0.04) increase in KIT mRNA expression (Figure 14).

Subsequently, since the GIST882 line is a Gli3^{lo} line, we decided to investigate

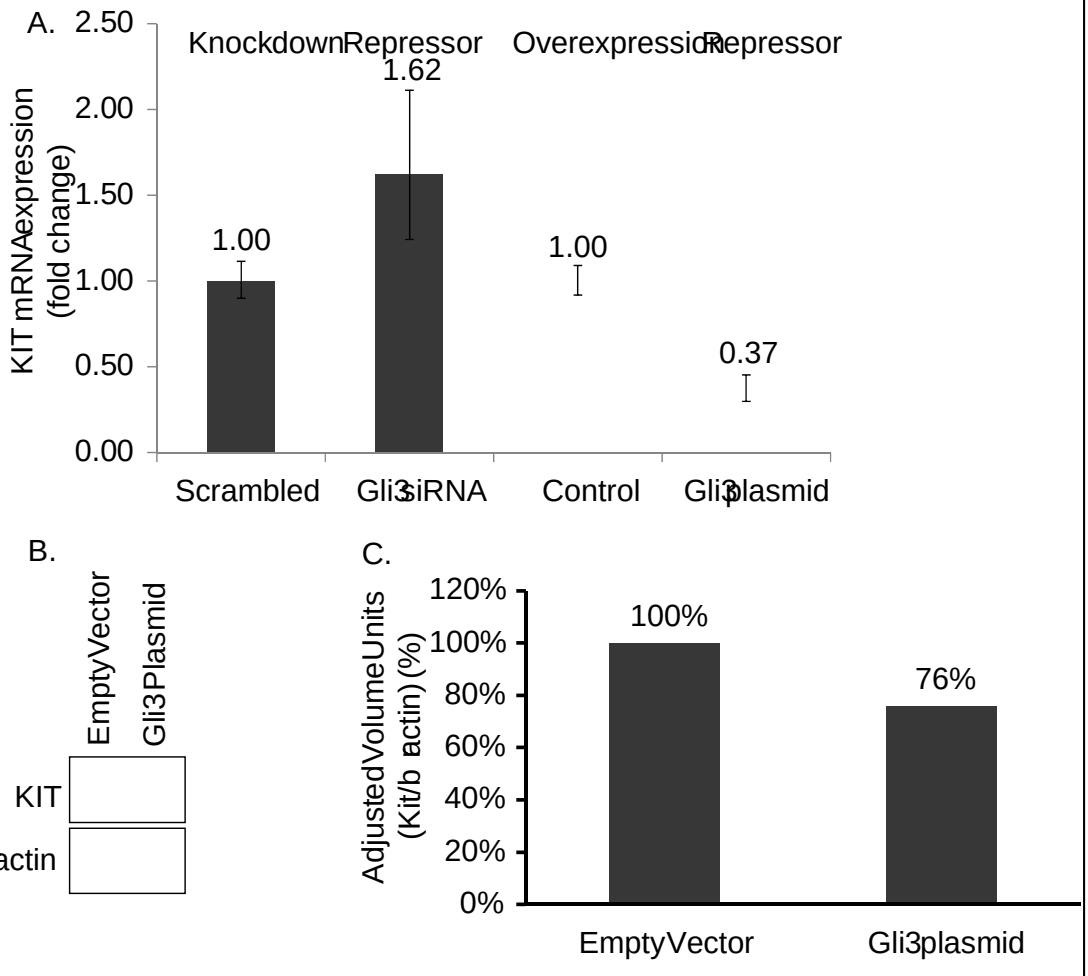

Figure 15. Genetic modulation of Gli3 regulates KIT expression. A. Quantitative RT-PCR of the KIT oncogene after electroporation of Gli3 siRNA or a Gli3 overexpression plasmid as compared to appropriate controls. B. Western blot analysis after electroporation of Gli3 plasmid probing for total c-kit and Eactin, as a loading control. C. Quantitative analysis of the KIT/Eactin ratio from Western blot analysis in (B).

the effects of Gli3 overexpression on the KIT oncogene after electroporation of a Gli3 (repressor) plasmid and an empty vector. Quantitative RT-PCR of the KIT oncogene shows that there was a 62% (P=0.04) increase in KIT mRNA expression after knocking down Gli3, while a 270% (P=0.002) decrease in KIT mRNA expression after overexpressing Gli3 at 48 hours after electroporation (Figure 15A).

While our findings suggested a role for Gli3 modulation on KIT expression, we sought to investigate this relationship on the protein level. We started by using a Western blot analysis after electroporation of Gli3 plasmid and the empty vector as a control. We probed for the total KIT protein and β-actin as a loading control. The analysis showed a decrease in KIT protein expression compared to empty vector (Figure 15B). Quantitative analysis of the KIT/β-actin ratio in the gel shows a 24% decrease in the KIT protein levels following Gli3 overexpression (Figure 15C). Taken together, genetic modulation of Gli3 expression and possibly Gli1 and Gli2 expression regulates KIT mRNA and protein expression.

Genetic Modulation of Gli3 Regulates GIST Viability and Apoptosis

KIT is known to be mutated in 95% of GISTs[14,15] and our findings have corroborated a regulation of KIT by Gli3 and possibly Gli1/2. Thus, we investigated the viability and apoptosis of GISTs via Gli modulation. Electroporation of Gli2 siRNA and Gli3 siRNA was followed by Cell Counting kit-8 viability assay. The viability assay showed that Gli2 siRNA decreases cell viability by 10% (P=0.0006), whereas electroporating Gli3 siRNA increases cell viability by 5% (P=0.09) as compared to scrambled siRNA (Figure 16A). Subsequently, we wanted to investigate whether Gli3

overexpression affected apoptosis. Gli3 overexpression, which implies the cells are Gli3^{hi}KIT^{lo}, increases caspase-3/7 activity by 20% ($P=0.0009$) as compared to an empty vector control (Figure 16B). Taken together, a genetic modulation causing a decrease in Gli2 may cause the cells to lose viability, whereas an increase in Gli3 may cause the cells to increase apoptotic mechanisms.

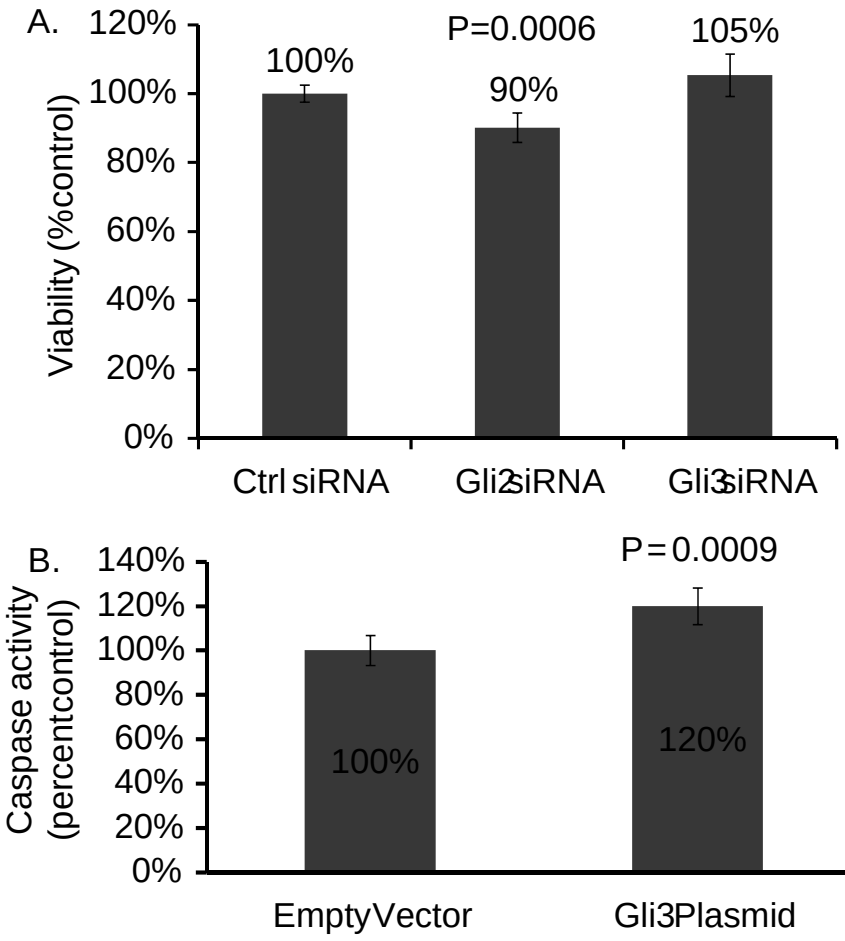

Figure 16. Genetic modulation of Gli3 regulates GIST viability and apoptosis. A. Viability assay was performed using the Cell Counting Kit 8 with Gli2 and Gli3 siRNA as compared to scrambled siRNA. B. Caspase 3/7 activity assay was performed using Gli3 overexpression plasmid compared to an empty vector control.

Pharmacologic inhibition of Gli1/2 reduces KIT expression

While our findings started to show a relationship between the Gli family transcription factors and KIT expression, in collaboration with members of the Sicklick laboratory, we sought to verify this relationship through modulation of the Gli genes through pharmacologic inhibition. GANT61 is a Gli1 and Gli2 inhibitor. GIST882 cells were treated with GANT61 (10μM) followed by real-time RT-PCR to observe the effects on Gli1 and Gli2 pan-inhibition via pharmacologic means on KIT mRNA expression. This treatment was shown to reduce KIT mRNA expression by 50% (P=0.035) when compared to GIST882 cells treated with 0.1% DMSO as a control (Figure 17). Thus, we verified that inhibition of Gli1 and Gli2 has a direct relationship with KIT mRNA expression.

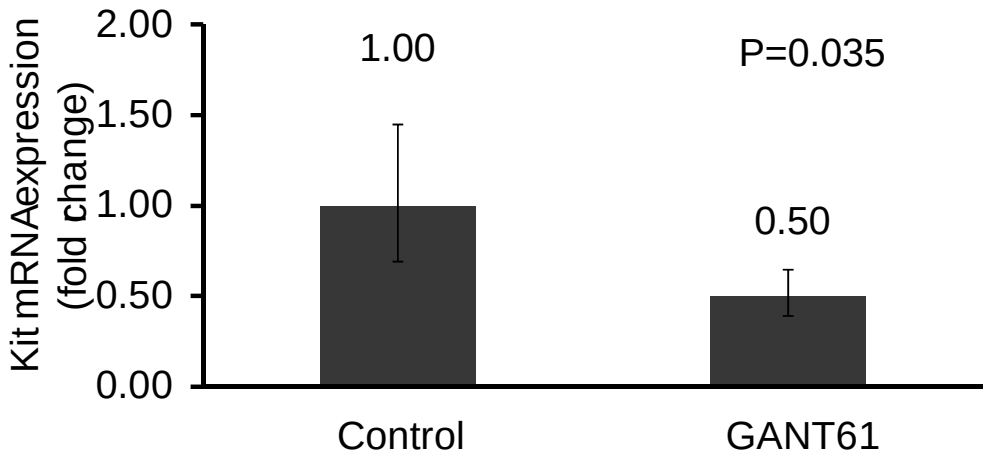

Figure 17. Pharmacologic inhibition of Gli1/2 reduces KIT expression. GIST882 cells were treated with GANT61, a Gli1/2 inhibitor, compared to GIST882 cells treated with 0.1% DMSO. It was followed by real-time RT-PCR amplifying KIT mRNA.

Discussion

Through preliminary data gathered in the Sicklick lab, we now provide evidence that the Gli transcription factors (Gli1/2/3) within the Hh pathway regulate KIT expression in human GISTs. We found that Gli1, Gli2 and Gli3 transcriptionally regulate mRNA expression of the KIT oncogenes via genetic and pharmacologic modulation. Gli1 and Gli2 activate KIT mRNA expression while Gli3 represses KIT mRNA and protein expression. As we decrease the expression of the Gli transcription factors, we see a decrease in KIT with the Gli activating transcription factors (Gli1/2) and an increase in the Gli repressing transcription factor (Gli3). Since Gli3 created the most significant increase in KIT mRNA expression, the expectation was to see a significant decrease in KIT mRNA expression with overexpression of Gli3. Using the GIST882 line, a $Gli3^{hi}$ line, it created a better test for observing the effect of Gli3 on KIT. We had used the GIST882 line because electroporation with the GIST-T1 line, a $Gli3^{hi}$ line, was unsuccessful in our laboratory, as well in other laboratories that study GISTs. In addition, we wanted to confirm those result with observations on the protein level. Our experiments show a successful decrease in KIT protein expression with the overexpression of a Gli3 plasmid.

In addition, Gli3 overexpression decreases KIT and leads to an increase in apoptosis, whereas Gli3 knockdown increases KIT and slightly increases cell viability. Since constitutively active KIT has been repeatedly shown to play a role in activating and maintaining GISTs, this data confirms these findings. It also shows that by modulating Gli3, we can target KIT expression without binding to the ATP binding pocket on KIT as IM does, the current treatment for non-resectable GISTs.

Likewise, inhibition of Gli2 via genetic means leads to a reduction in GIST viability compared to the viability effects of the scrambled siRNA and Gli3 siRNA. The Gli2 inhibition had a greater effect on viability than Gli3 inhibition. However, Gli3 is low in the GIST882 cells that were used and can contribute to Gli2 siRNA having a greater effect on viability than Gli3 siRNA. In addition, inhibition of Gli2 via pharmacologic means leads to a reduction in KIT expression and GIST viability comparable to the current drug for the treatment of GISTs, TKI. Pharmacological inhibition of Gli2 could be a novel target for GISTs because it is able to reduce KIT expression to 50%. Using pharmacologic inhibition of Gli2 could be accomplished with both GIST882 and GIST-T1 cell lines because the drug, GANT61, would be able to enter the cell.

There are several potential implications of this data for the study and management of GIST. Hedgehog pathway dysregulation may occur early in GIST formation because Hh overexpression in mesenchymal GI SCs results in a GIST phenotype. Gli3 itself could serve as a novel target for treating KIT independent or dependent GISTs that could possibly have drug resistant KIT mutations. Secondary drug resistant KIT mutations occur in 50% of patients in the ATP binding pocket and kinase activation loop, where IM binds to.[19] Thus, IM cannot bind to KIT in the GIST cells and the GISTs become resistant to pan-tyrosine kinase therapies. This leads to disease recurrence, progression, metastasis and death. By targeting the Hh pathway, specifically the Gli family transcription factors, we can bypass the mechanisms that cause resistance, such as primary drug resistance due to mutations in other genes beside KIT or secondary drug resistance due to acquired KIT mutations after initial treatment with IM. In addition, the third and most important

possibility exists and that is of GSCs that are KIT^{low}.[12] Thus, drugs that target KIT cannot target the GSC population. IM and other drugs that target KIT can only kill most of the immature and the mature population of GIST cells because they are KIT^{hi}, but miss the GSC population completely. Given that Gli2 overexpression drives KIT and Gli3 knockdown drive KIT, directly targeting the Gli family transcription factor would regulate KIT. The Hh pathway could be a potential druggable target. Giving drugs against Gli1/2 or drugs that can overexpress Gli3 (i.e. viral vectors) would decrease KIT and increase apoptosis. Targeting Hh signaling in GSC may serve as a novel approach for treating GIST by depleting self-renewing cancer cells. Taken together, it is important to have drugs that can avoid the need to only solely targeting tyrosine kinases such as KIT, given concurrently with a tyrosine kinase inhibitor (TKI) or given sequentially. We have identified a novel putative target for GSC, the Hh embryonic signaling pathway, which is critical for supporting CSC in other cancers.

In addition to discovering novel drug targets for GISTs, there are also other cancers with mutations in various Hh pathway components and would also benefit from these novel drug targets. Gli1 has been shown to be amplified in rhabdomyosarcomas by 30-fold.[29,56] Because Gli1 is a transcriptional activator like Gli2, Gli1 could be target with GANT61 or a drug similar to GANT61. In colon carcinoma, there is a Gli3 missense mutation[57] and Gli3 upregulation in colon cancer tissue compared to normal tissue.[58] Understanding the regulation of KIT via Gli3, in addition to understanding the relationship between Gli1/2 and Gli3, drugs can be made similar to GANT61 to treat GISTs and colon cancers as well.

Furthermore, there are some cancers that don't have known mutation in Hh pathway components, but do upregulate the Hh ligands. PDAC has increase of Shh ligand, which activates the Hh pathway in the neighboring stromal cells. These stromal cells cause a paracrine mitogen feedback loop to the tumor cells, which prevent angiogenesis in the stroma. The dense, avascular stroma is characteristic of PDAC. With the drugs inhibiting the Hh pathway, the stromal vasculature can return to normal and allow other chemotherapeutic drugs to be used on the tumor.[8] Melanoma arises from melanocytes in the skin or from neural crest-derived precursor cells. There is no genetic change in the Hh pathway components, but increases in Gli1 and Gli2 have been shown to activate a more aggressive form of the cancer in which there is bone metastasis.[29]

Additionally, there are many cancers that have KIT mutations or have become KIT independent. Mastocytosis (systemic and cutaneous) have been shown to have KIT activating mutations in various portions of the KIT biomarker, the most common is in the activating loop of the receptor. This creates constitutively active KIT, which causes increased proliferation and reduced apoptosis. Thus, the tumors become ligand independent. The only therapeutic agent approved for patients with systemic mastocytosis is IM. However, IM only can target wild-type KIT and the V560G KIT mutant. It is less effective in targeting the most common mutation in mastocytosis, D816V in the activating loop of the receptor.[59] Likewise, seminomas also have a KIT mutation mostly in the kinase activation loop (exon 17), but also seen in the juxtamembrane region (exon 11).[60] Similarly, IM is also used to treat Dermatofibrosarcoma protuberans, which has a translocation/fused gene of PDGFB

(encodes for a part of the ligand PDGF which binds to PDGFRA and PDGFRB) and COL1A1. This fusion event is a pathogenic event that causes constitutive activation of the PDGFR through binding of the PDGF ligand to the receptor.[61,62] Taken together, our findings could have great implications for the treatment of the previously mentioned cancers due to the fact that their mutations are normally targeted by IM therapy. In addition, our findings could possibly be used in the treatment of other sarcomas and even other carcinomas.

The cell lines, GIST-T1 and GIST882, are ideal lines because they are derived from human tumors and have opposite Gli3/KIT expression patterns. In order to test the various disease presentations in patients with IM resistant or KIT-independent GISTs, a variety of lines are needed. Both lines show a similar pattern of the Hh pathway component expression. The expression pattern in these lines is comparable to the expression pattern in the primary human tumors that were previously tested. In using murine models of GIST, we chose to use mice developed to carry the same mutation as in the GIST-T1 and GIST882 cell lines. However, the mRNA expression patterns in the murine models did not correlate with the expression patterns of both primary human tumors and cell lines. Another factor to consider in these experiments is that siRNA downregulation of the Gli family transcription factors could only cause a temporary change. A future experiment would include looking at the long term manipulation of the Gli family transcription factors, including using shRNA instead of siRNA. Furthermore, additional experiments would be needed to further characterize the Gli expression pattern

in different populations of GIST cells since it already been shown that GSCs are KIT^{low} and perhaps will not respond to IM therapy.

Appendix

Table 1. RT-PCR primers for human samples.

Gene	Direction	Sequence	Amplicon size (bp)
Shh	Forward	GCTCGGTGAAAGCAGAGAAC	109
	Reverse	CTCAGGTCCTTCACCAGCTT	
Ihh	Forward	TCCGTCAAGTCCGAGCAC	109
	Reverse	GCCTCACGGCTGACAAGG	
Ptc	Forward	CCACCAGACGCTGTTTAGTCA	72
	Reverse	CGATGGAGTCCTTGCCTACAA	
Smo	Forward	CAGTTCCAAACATGGCAAACAG	200
	Reverse	TGCTATGTCAGGCCAATGTGA	
Gli1	Forward	TGCAGTAAAGCCTTCAGCAATG	132
	Reverse	TTTTCGCAGCGAGCTAGGAT	
Gli2	Forward	CGAGAAACCCTACATCTGCAAGA	88
	Reverse	GTGGACCGTTTTCACATGCTT	
Gli3	Forward	AAACCCCAATCATGGACTCAAC	98
	Reverse	TACGTGCTCCATCCATTTGGT	
c-KIT	Forward	TGTCATCAGCCACCATCCTA	156
	Reverse	GCAGAATTGGAGAAGCCTTG	
DOG-1	Forward	TGTCAGAGCCAAAGACATCG	183
	Reverse	AAGGAGGAGAGGGTGTGGTT	
*E*Actin	Forward	AATGTGGCCGAGGACTTTGATTGC	93
	Reverse	AGGATGGCAAGGGACTTCCTGTAA	

Table 2. RT-PCR primers for mouse samples.

Gene	Direction	Sequence	Amplicon size (bp)
Shh	Forward	CTGGCCAGATGTTTTCTGGT	117
	Reverse	TAAAGGGGTCAGCTTTTTGG	
Ihh	Forward	CCGAACCTTCATCTTGGTG	124
	Reverse	ACAGATGGAATGCGTGTGAA	
Ptc	Forward	ATGCTCCTTTCCTCCTGAAACC	168
	Reverse	TGAACTGGGCAGCTATGAAGTC	
Smo	Forward	GCCTGGTGCTTATTGTGG	75
	Reverse	GGTGGTTGCTCTTGATGG	
Gli1	Forward	AACTCCACAGGCACACAGG	79
	Reverse	GCTCAGGCTTCTCCTCTCTC	
Gli2	Forward	CCATTCATAAGCGGAGCAAG	105
	Reverse	CCAGGTCTTCCTTGAGATCG	
Gli3	Forward	GCTCTTCAGCAAGTGGTTCC	122
	Reverse	CTGTCGGCTTAGGATCTGTTG	
c-KIT	Forward	GGCTAGCCAGAGACATCAGG	159
	Reverse	AGGAGAAGAGCTCCCAGAGG	
E-Actin	Forward	GCTCTGGCTCCTAGCACC	74
	Reverse	CCACCGATCCACACAGAGTACTTG	

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