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Analysis Of The Hedgehog Pathway In Pancreatic Adenocarcinoma

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ANALYSIS OF THE HEDGEHOG PATHWAY IN PANCREATIC ADENOCARCINOMA

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A DISSERTATION

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ABSTRACT

Pancreatic adenocarcinoma (PAC) is the fourth leading cause of cancer mortality in the United States. Despite the use of highly aggressive treatment regimens (surgery, chemotherapy and radiation), almost all patients succumb to metastatic disease within 6-10 months of diagnosis. The hedgehog (HH) signaling pathway was originally discovered to play a critical role in mammalian embryological development. Interestingly, recent studies have suggested that aberrant expression of this pathway is involved in the initiation and continued growth of PAC. Small molecules that antagonize the transmembrane protein Smoothened (Smo), a critical signaling component of the HH pathway, have proven effective in decreasing PAC growth both *in vitro* and *in vivo* and show promise as a new therapeutic strategy. Data from our laboratory indicate the HH pathway is overexpressed in pancreatic cancers and Smo antagonism decreases pancreatic cancer cell growth *in vitro*. The major accomplishments of this dissertation research include (1) the validation of a recently developed technique known as Taqman low-density array (TLDA) which can be used to analyze the expression of multiple genes in a single RNA sample, (2) the identification of novel, tumor-associated genes through an extensive characterization of the HH pathway and its transcriptional targets in PAC clinical specimens (both surgically resected tissues and fine-needle biopsies) and (3) the identification of genes associated with *in vitro* response to cyclopamine, a selective HH pathway inhibitor, in human pancreatic cancer cell lines. These findings contribute to the growing char-

acterization of the HH pathway in pancreatic cancer etiology and may provide a basis for future clinical applications in which PAC patients most likely to respond to HH pathway antagonists could be identified based upon gene expression profiling, thereby maximizing the efficacy of this type of therapy.

DEDICATION

To my dad, Brian Steg, whose never-ending determination to improve the lives of those around him showed me that real "superheroes" do exist. To my mom, Lynne Steg, for her unconditional love and support that ultimately made me the person I am today. To my brother, Jason Steg, my kindred spirit, who can understand and relate to me when no one else can. And finally, to my future wife, Hope Amm, whose love, devotion and patience has helped me through the trials and tribulations of graduate school. She has truly made me a better person and I would not be where I am without her.

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LIST OF ABBREVIATIONS

A_{260}	absorbance at 260 nm
cDNA	complimentary deoxyribonucleic acid
C_T	cycle threshold
CV	coefficient of variance
DMEM	Dulbecco's Modified Eagle's Medium
EN1	Engrailed-1
EUS-FNA	endoscopic ultrasound-guided fine-needle aspirate
FBS	fetal bovine serum
FPE	formalin-fixed, paraffin-embedded
GLI	Glioma-associated oncogene
GSK3B	Glycogen synthase kinase 3-beta
HH	hedgehog
M	molar
mM	millimolar
mRNA	messenger ribonucleic acid
ng	nanogram
OEA	ovarian endometrioid adenocarcinoma
OSE	ovarian surface epithelium
PAC	pancreatic adenocarcinoma
PanIN	pancreatic intraepithelial neoplasia

PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PTCH	Patched
RNA	ribonucleic acid
RPLP0	ribosomal protein large subunit, P0
rpm	rotations per minute
RTQ	real-time quantitative polymerase chain reaction
SD	standard deviation
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SMO	Smoothened
TLDA	Taqman low-density array
Tris	Tris (hydroxymethyl) aminomethane
Wnt	wingless-type
µl	microliter
µm	micrometer
µM	micromolar

INTRODUCTION

Lack of Effective Treatment for Pancreatic Adenocarcinoma (PAC)

PAC is the fourth leading cause of cancer mortality in the United States and is characterized by an unusual resistance to radiation or chemotherapy. The death incidence ratio for pancreatic cancer is approximately 0.99 making it one of the deadliest malignancies (Christiansen, 2003). Even more alarming is the realization that diagnoses of PAC have continued to increase in frequency in the last decade (Shi, Friess, Kleeff, Ozawa, & Buchler, 2001). Surgical resection remains the most effective treatment for this malignancy. However, because early stage PAC remains difficult to detect, it often presents symptoms only when it is already in an advanced state and curative resection is no longer possible. Currently, only 10% of patients diagnosed with PAC are candidates for curative resection (Lockhart, Rothenberg, & Berlin, 2005). For patients with locally advanced (unresectable) PAC, chemotherapy agents, such as the pyrimidine analogs 5-fluorouracil (5-FU) and gemcitabine, administered concurrently with radiation (50-60 Gy) remain the current standard of care (Berlin & Rothenberg, 2003; Hoffman et al., 1998; Moertel et al., 1981; Neoptolemos et al., 2003). However, despite these highly aggressive therapeutic approaches, almost all these patients succumb to local recurrence or distant metastases (Crane et al., 2001). Ultimately, this unremitting behavior that is characteristic of PAC has prevented the median survival time (6-10 months) for patients diagnosed with the disease from appreciably changing in the last 80 years (Berlin & Rothenberg, 2003).

Genetic Events Involved in PAC Formation and Progression

Arguably the best characterized of all genetic alterations associated with PAC is the mutant *KRAS* gene. Gain-of-function mutations in this gene, which codes for a GTP-binding protein (RAS) involved in growth factor-mediated signal transduction pathways, have frequently been observed in cases of PAC (more than 90%) (Almoguera et al., 1988). Interestingly, *KRAS* mutations are found in pancreatic intraepithelial neoplasia (PanIN), the precursor lesions that often lead to PAC, suggesting that constitutive activation of the RAS protein plays a key role in pancreatic tumorigenesis. RAS activation alone, however, does not appear to be sufficient for the development of PAC. In a study where mutant *Kras* was specifically expressed in the pancreas of genetically altered mice, the development of PanIN-like lesions was frequently observed; however these lesions rarely progressed to PAC (Hingorani et al., 2003). The results of this study and others suggest that additional genetic events, in addition to *KRAS* mutation, are involved in pancreatic tumor formation.

Another molecular alteration that occurs early in the development of PAC is loss-of-function mutations in the *CDKN2A*/*INK4A*/*p16* gene, which codes for a cyclin-dependent kinase inhibitor that is capable of inducing cell cycle arrest at the G1 phase (Caldas et al., 1994). Loss of CDKN2A coupled with the activation of RAS appears to be essential for pancreatic carcinogenesis and eventually leads to downstream mutations that advance the transformation of PanIN into invasive ductal carcinoma. These later modifications include loss-of-function mutations in the *TP53*/*p53* gene, which codes for a DNA binding protein that is involved in a variety of cellular functions including cell cycle arrest and apoptosis, and in the *SMAD4* gene, which codes for a signal mediator that

plays an important role in the control of cell proliferation (Casey et al., 1993; Hahn et al., 1996; Miyazono, Suzuki, & Imamura, 2003; Nakamura, 2004; Wilentz et al., 2000). While studies on *KRAS*, *CDKN2A*, *TP53* and *SMAD4* genetic mutations in PAC provide a basis for understanding this complex disease, there is still much to be learned about the exact molecular mechanisms that define the initiation and progression of PAC. Recent evidence points toward other genes and cellular pathways that may also be key mediators of pancreatic cancer formation (see below). The identification of new tumor-associated genes and/or pathways could provide potentially novel therapeutic targets and lead to the development of more effective treatment strategies.

The Hedgehog (HH) Signaling Pathway

The HH pathway is comprised of a family of secreted morphogens that were first described in *Drosophila* in early 1980s and later, in the 1990s, in mammals as playing a crucial role in embryonic development particularly organ and limb patterning (Hammerschmidt, Brook, & McMahon, 1997; Mohler, 1988; Nusslein-Volhard & Wieschaus, 1980; Weed, Mundlos, & Olsen, 1997). Nusslein-Volhard and Wieschaus were the first to name the *hedgehog* gene after noticing that when this gene is mutated, denticles (spiked cuticle that normally decorates only the anterior portion of fly body segments) were prevalent throughout the entire body (both anterior and posterior portions) of newly hatched larva. To the researchers, this continuous lawn of denticles suggested the spines of a hedgehog. The discovery of *hh* as well as other so-called segment-polarity genes including *wingless* (*wg*; ortholog of the vertebrate *WNT1* gene) and *engrailed* (*en*)

would provide the basis for defining the molecular mechanisms behind not only *Drosophila* embryonic development but also vertebrate limb and organ patterning.

Vertebrate HH genes were first reported in 1993 when a collaborative effort involving three groups (Echelard et al., 1993; Krauss, Concordet, & Ingham, 1993; Riddle, Johnson, Laufer, & Tabin, 1993) discovered that, unlike the fly, which has a single *hh* gene, there are three related mammalian HH genes – Desert HH, Indian HH and Sonic HH. Sonic HH, named after the title character in the popular video game series by Riddle et al., is arguably the best characterized of the three HH ligands as it appears to play a substantial role in the formation of several tissues and organs (Ingham & McMahon, 2001). In a process that is conserved between *Drosophila* and mammals, the HH ligand begins as a 45-kDa precursor protein that undergoes autocatalytic cleavage, which is mediated by the C-terminal portion of the precursor to yield a 25-kDa C-terminal fragment that has no other known function and a 19-kDa N-terminal fragment that is considered the mature signaling peptide (Bumcrot, Takada, & McMahon, 1995; Lee et al., 1994; Porter et al., 1995). This autoproteolysis proceeds via a thioester intermediate that undergoes a nucleophilic attack by cholesterol resulting in the covalent coupling of cholesterol to the C-terminus of the N-terminal signaling moiety (Porter, Young, & Beachy, 1996). The HH ligand is further lipid-modified by the addition of palmitic acid to its most N-terminal cysteine (Pepinsky et al., 1998). The protein responsible for this palmitoylation was later identified as Skinny HH also known as HH acyl transferase (Chamoun et al., 2001).

Dispatched, a 12-span transmembrane protein, and the Exostoses family of glycosaminoglycan transferases control the release and transport, respectively, of lipid-

modified HH ligand from HH-secreting cells to HH-target cells (Bellaiche, The, & Perrimon, 1998; Burke et al., 1999). Recently, the heparin sulfate proteoglycans (HSPGs) Dally and Dally-like protein (Dlp) have been discovered in *Drosophila* to play a role in HH ligand secretion and transport as well (Lin, 2004). These HSPGs not only transport HH ligand between cells, but they also provide protection from extracellular proteases; however, vertebrate homologues of these proteins have yet to be identified. In a study performed by Yao, Lum and Beachy (2006), it was found that Dlp directs HH ligand to a transmembrane immunoglobulin-like protein called interference hedgehog (ihog) and its relative brother of ihog (boi). The mammalian counterparts of ihog and boi were soon identified by Tenzen et al. (2006) as Cdo and Boc, respectively. It has been theorized that ihog/Cdo and boi/Boc along with HH ligand interact with the 12-span transmembrane receptor Patched (Ptch1 or Ptch2) thereby relieving Ptch-mediated inhibition of Smoothened (Smo), a 7-span transmembrane protein (Carpenter et al., 1998; Stone et al., 1996; Wilson & Chuang, 2006). The mechanism by which Ptch antagonizes Smo remains elusive; however, a recent study suggests Ptch introduces a small molecule that binds to and effectively inhibits Smo activity (Bijlsma et al., 2006). Of note, Ptch is not the only receptor for HH ligands. It competes with other proteins including HH interacting protein (Hip) and Megalin, both of which appear to act as negative regulators of HH signaling (Chuang & McMahon, 1999; McCarthy, Barth, Chintalapudi, Knaak, & Argraves, 2002).

In the absence of HH ligand (see Figure 1), Ptch inhibits signal transduction through Smo resulting in the cytoplasmic sequestration of the Glioma-associated oncogene (Gli) family of transcription factors (Gli-1, 2, 3) (Lee, Platt, Censullo, & Ruiz i

Altaba, 1997; Mo et al., 1997). Gli is retained in the cytoplasm and is believed to be anchored to the cytoskeleton by a multiprotein complex consisting of the Fused (Fu) kinase, Supressor of fused (Sufu) and Costal 2 (Cos2/Kif7/Kif27) (Monnier, Ho, Sanial, Scott, & Plessis, 2002; Murone et al., 2000). While sequestered, Gli is phosphorylated at multiple sites (serine and threonine residues) by cAMP-dependent protein kinase (Pka), Casein kinase 1 (Ck1) and Glycogen synthase kinase 3-beta (Gsk3β) and ultimately marked for proteasomal degradation by the E3 ubiquitin ligase known as β-transducin-repeat-containing protein (β-TrCP) (Pan, Bai, Joyner, & Wang, 2006; Price & Kalderon, 2002; Wang & Li, 2006). At this point, the ultimate fates of the three Gli transcription factors diverge somewhat. Proteolysis of Gli-1 by the proteasome is a complete process whereas degradation of Gli-2 and Gli-3 is only partial. Gli-2 and Gli-3, unlike Gli-1 have activator and repressor domains. Upon proteolysis, Gli-2 and Gli-3 are cleaved into smaller transcriptional repressor fragments (Gli-2R and Gli-3R) with Gli-2R being a much weaker repressor than Gli-3R, which localizes to the cell nucleus where it binds Gli-response elements to prevent HH signal transduction (Pan et al., 2006; Wang, Fallon, & Beachy, 2000).

The conversion of Gli-2 and Gli-3 to transcriptional repressors is inhibited in the presence of HH ligand (see Figure 2). When HH binds to Ptch, Smo becomes activated resulting in a signaling cascade that ultimately leads to the translocation of the full-length, activator forms of Gli into the nucleus where they transcribe HH target genes including *PTCH* and *GLI* itself thereby providing a mechanism whereby HH signaling is tightly regulated (Alexandre, Jacinto, & Ingham, 1996). Of note, recent evidence has emerged suggesting that components of HH signaling (including Smo and Gli) localize to

cilia, microtubule-based structures that protrude from the body of the cell, during mammalian development (Corbit et al., 2005; Haycraft et al., 2005). Cilia formation appears to play an important role in HH pathway activation since genetic mutations affecting cilia structure can lead to inhibition of proper HH signaling (Huangfu et al., 2003). Research on this particular aspect of HH signaling is limited but may provide novel insights on the mechanisms driving this pathway in future studies.

The mechanism by which Smo activation leads to Gli transclocation remains poorly understood. Only recently has a model emerged that helps elucidate this complex signaling cascade. Unlike its *Drosophila* counterpart, vertebrate Smo has been shown to couple with heterotrimeric (αβγ) G-proteins (Riobo, Saucy, Dilizio, & Manning, 2006). Specifically, Smo appears to couple with G-proteins of the G_i family, the members of which have been linked with reductions in cAMP through inhibition of adenylyl cyclase. This finding led Riobo et al. to speculate that HH-induced Smo activation leads to reduced activity of Pka, a negative regulator of Gli, as a consequence of reduced intracellular cAMP levels. In addition, Riobo et al. hypothesized the interaction between Smo and G_i proteins could lead to the activation of phosphoinositide 3-kinase (PI3K) which in turn activates Akt, a kinase that phosphorylates and inhibits Gsk3β, another negative regulator of Gli function. Indeed, this group of investigators found that PI3K and Akt activity are essential for HH signaling in a recent publication (Riobo, Lu, Ai, Haines, & Emerson, 2006). The concept of Smo coupling with G-proteins was further suggested by the finding that the C-terminal tail of Smo is phosphorylated by G-protein coupled receptor kinase 2 (GRK2) upon binding of HH ligand (Meloni et al., 2006). This phosphorylation leads to the recruitment of β-arrestin 2 which binds to Smo and promotes its internaliza-

tion leading to either lysosomal degradation of Smo or, conversely, intracellular signaling possibly through the MEK/ERK pathway.

The downstream effects of HH signaling and Gli activation are many. The HH pathway appears to interact with and regulate several other cellular pathways including the cell cycle (Barnes, Kong, Ollendorff, & Donoghue, 2001; Fan & Khavari, 1999), apoptosis (Regl et al., 2004), epidermal growth factor signaling (Amin, Li, & Finkelstein, 1999), platelet-derived growth factor signaling (Dahmane et al., 2001), the Wnt pathway (Nusse, 2003), the Notch pathway (Benson, Lowrey, Lamb, & Howie, 2004) and the Snail pathway (Li et al., 2006; Louro et al., 2002). The complexity of the HH signaling pathway continues to grow as more of its cellular components and functions are characterized. The relatively recent discovery of this molecular pathway indicates that there is still much to be elucidated about its exact cellular roles, although it is clear that HH signaling plays an important role in determining cell fate, proliferation and survival in different target organs and tissues. Therefore, it should not be surprising that misregulation of the HH pathway can contribute to the formation of a variety of cancers including PAC.

Increasing Evidence the HH Pathway is Important in the Initiation and Progression of PAC

Mutations in members of the HH pathway are found in several distinct cancers of the skin, brain, muscle and lung (Pasca di Magliano & Hebrok, 2003). For example, loss-of-function mutations in *PTCH* and activating mutations in *SMO* impair the ability of Ptch to restrain Smo-mediated activation of the Gli transcription factors resulting in con-

stitutively active HH pathway. *GLI1* expression alone is transformative in immortalized primary rat kidney cells (RK3E) *in vitro* indicating its oncogenic activity (Ruppert, Vogelstein, & Kinzler, 1991). In addition, there are studies that demonstrate activation of the HH pathway and co-expression of *Sonic HH* and/or *Indian HH* with *PTCH1* in cell lines derived from carcinomas of several organ systems (Berman et al., 2002; Berman et al., 2003; Nishimaki et al., 2004; Qualtrough, Buda, Gaffield, Williams, & Paraskeva, 2004; Sanchez et al., 2004; Thayer et al., 2003; Watkins et al., 2003). Within these same studies it was reported that stimulation of HH signaling results in increased proliferation and, conversely, inhibition of HH signaling results in decreased proliferation and/or apoptosis *in vitro* (Berman et al., 2002; Berman et al., 2003; Qualtrough et al., 2004; Sanchez et al., 2004).

Recently, two reports in *Nature* suggest that activation of the HH signaling pathway alters the expression of growth factors as well as genes important for cell proliferation and appears to be involved in pancreatic cancer initiation and sustained tumor growth and survival (Berman et al., 2003; Thayer et al., 2003). Studies performed in Pdx-Sonic HH mice (in which Sonic HH is misexpressed in the pancreatic endoderm) indicate that induction of the HH pathway results in abnormal tubular structures similar to those observed in human PanIN lesions (Thayer et al., 2003). Moreover, these PanIN-like lesions contain mutations in codon 12 of the *KRAS* gene and overexpressed the HER-2/*neu* protein, which are genetic alterations found early in the progression of human pancreatic cancer (Hruban, Goggins, Parsons, & Kern, 2000). Other investigators have expanded upon these initial studies by providing explanations as to how the HH pathway becomes misregulated and why this event leads to PAC formation. It is interesting to

note that HH signaling occurs primarily during embryogenesis and, for the most part, remains inactive in adult tissues. However, studies have shown that this pathway contributes to the maintenance and function of adult stem cells, particularly those that give rise to epithelial progenitor cells found in the skin, lungs and pancreas (Hebrok, Kim, St Jacques, McMahon, & Melton, 2000; Oro & Higgins, 2003; Watkins et al., 2003). The study performed by Watkins et al., in particular, demonstrated that the HH pathway becomes transiently activated in progenitor cells during airway epithelial regeneration following tissue injury. Constitutive activation of HH signaling in these progenitor cells, however, was believed to be the basis for small-cell lung cancer formation. These findings led other investigators to hypothesize that such an event might also occur in the pancreas during prolonged inflammatory processes such as chronic pancreatitis (Kayed, Kleeff, Keleg, Buchler, & Friess, 2003; Nakashima et al., 2006) Kayed et al. showed that Indian HH, Ptch and Smo mRNA and protein levels are significantly overexpressed in chronic pancreatitis tissue specimens in comparison to normal pancreas. Nakashima et al. took this analysis one step further and proposed a mechanism whereby inflammatory stimuli, in particular nuclear factor-κB (NF-κB), that result from chronic pancreatitis eventually contribute to the overexpression of Sonic HH and its receptors and ultimately PAC formation. Dennler et al. (2007) demonstrated transforming growth factor-β (TGF-β), another mediator of inflammation and tissue repair, can induce the mRNA and protein expression of Gli-1 and Gli-2 *in vitro*.

Other reports have suggested mechanisms other than inflammatory stimuli by which HH signaling is activated in PAC. Interestingly, in a study by Pasca di Magliano et al.(2006), it was found that overexpression of GLI2 in the pancreatic epithelium of

mice, which resulted in HH pathway activation, did not lead to the formation of early PanIN lesions or PAC. This suggests that, as with *KRAS* mutations, aberrant HH signaling alone is not sufficient to cause to PAC. However, in the same study by Pasca di Magliano et al., simultaneous activation of HH and Ras signaling in the pancreas of transgenic mice resulted in the formation of extensive PanIN lesions. In addition, tumor formation occurred more rapidly and the life span of these mice was significantly shortened in comparison to mice with either HH signaling or *KRAS* mutation alone. This suggests that HH and Ras signaling work in conjunction to promote the formation of pancreatic cancer. Ras signaling, in particular, appears to be responsible for the induction of HH pathway activation, and not the converse, by means of the downstream effector RAF/MEK/MAPK signaling pathway (Ji, Mei, Xie, & Cheng, 2007). Through this signaling pathway, Ras can increase GLI transcriptional activity independent of HH ligands and receptors. Taken collectively, these studies suggest that misregulation of HH pathway activity is among the key genetic events that lead to pancreatic neoplasia (Furukawa, Sunamura, & Horii, 2006). Further, inhibition of the HH pathway may be an effective, novel approach to treating patients with this devastating disease.

Antagonism of the HH Pathway as a Potential Therapeutic Strategy for PAC

Because aberrant HH signaling appears to be limited to tumor cells, antagonists of this pathway could serve as attractive anti-cancer agents. Antibodies directed against Sonic HH have been reported and Pka agonists such as forskolin, which could theoretically target Gli, have been suggested as HH inhibitors; however neither of these approaches has been tested in a cancer model (Marti, Bumcrot, Takada, & McMahon,

1995; Pasca di Magliano & Hebrok, 2003). A considerable amount of research, however, has been devoted to identifying and testing inhibitors of Smo. Cyclopamine (see chemical structure Figure 3), a naturally occurring steroidal alkaloid derived from the plant *Veratrum Californicum*, represents the first member of a class of small molecule compounds that selectively target the HH pathway (Cooper, Porter, Young, & Beachy, 1998; Incardona, Gaffield, Kapur, & Roelink, 1998). This compound inactivates HH transcriptional activity by directly binding to Smo's heptahelical bundle and inducing a conformational change similar to that induced by Ptch (Chen, Taipale, Cooper, & Beachy, 2002; Taipale et al., 2000). Cyclopamine was first used as an anti-cancer agent to inhibit the proliferation of brain cancer cells *in vitro* (Dahmane et al., 2001). Of particular interest, Thayer et al. (2003) showed that exposure to cyclopamine suppresses PAC cell proliferation *in vitro* and causes durable regression of pancreatic cancer xenografts. This same study demonstrated that tomatidine (see chemical structure Figure 3), a structural analog of cyclopamine that does not inhibit HH signaling (Cooper et al., 1998), had no effect on pancreatic cancer cells. With the success of cyclopamine effecitively targeting cancer cells, other Smo antagonists, both naturally-occuring (e.g. vitamin D3) and synthetic (e.g. SANT, CUR61414) have been discovered and/or developed that are structurally distinct from cyclopamine and may be more selective for Smo (Bijlsma et al., 2006; Chen, Taipale, Young, Maiti, & Beachy, 2002; Williams et al., 2003). The concentrations of these compounds that were required to inhibit cancer cell proliferation, however, were similar to those observed with cyclopamine (i.e. in the micromolar range). Interestingly, no adverse systemic effects of cyclopamine administration in adult animals (up to 1.25 mg/day for 24 days in mice) have been described (Berman et al., 2002; Berman et al., 2003;

Thayer et al., 2003). This observation is likely due to the inactivity of HH signaling in most adult tissues, which suggests this pathway could provide a tumor-associated target much like, for example, *bcr-abl* and HER-2/*neu* are for Imatinib mesylate and Trastuzumab, respectively (Buchdunger et al., 1996; Pegram et al., 1998). However, Smo antagonists can interfere with normal embryonic development in vertebrates resulting in congenital abnormalities such as holoprosencephaly also known as cyclopia; hence the name cyclopamine (Binns, James, Shupe, & Everett, 1963; Incardona et al., 1998). As more is learned about HH signaling and Smo function, it may be possible to develop less teratogenic Smo antagonists.

Potential for Pharmacogenomic Analysis of PAC

Several non-genetic factors including age, organ function and drug interactions can influence the effects of medications. However, genetic variation can account for as much as 95% of variability in drug response and side effects (Kalow, Tang, & Endrenyi, 1998). There are numerous examples of inter-individual difference in drug response caused by common genetic variations (called polymorphisms) in genes encoding drug-metabolizing enzymes, drug transporters or drug targets (Evans & Johnson, 2001; Evans & McLeod, 2003; Evans & Relling, 1999). Genetic determinants of drug response can be used in conjunction with other, non-genetic predictors in addition to having the advantage of remaining unchanged throughout a person's lifetime. These characteristics, therefore, are potentially useful for designing rational therapeutic strategies. The relevancy of being able to identify genetic determinants of drug response becomes apparent when con-

sidering that, currently, there are several medications available for a given condition but no single best treatment (Lanfear & McLeod, 2007).

The field of pharmacogenetics focuses on the interaction of drug therapies and genetic variation. Because most drug effects are determined by the interaction of multiple gene products throughout the entire drug pathway, pharmcogenetics has now extended to all aspects of drug response (i.e. absorption, distribution and excretion) and drug targets as well as mediators of downstream cellular effects (Meyer, 2000). Pharmacogenetics has given way to the more recent field of pharmacogenomics, which focuses on genome-wide approaches, rather than just one or two genes of interest, to identify genetic variations that govern drug response. Successful application of pharmacogenomic studies has led to the identification of human genes involved in response to, for example, the anti-cancer agent mercaptopurine (thiopurine methyltransferase (*TPMT*)) (Relling et al., 1999; Yates et al., 1997), the anticoagulant warfarin (cytochrome P450 2C9 (*CYP2C9*)) (Voora et al., 2005) and the analgesic codeine (*CYP2D6*) (Gasche et al., 2004; Lotsch, Skarke, Liefhold, & Geisslinger, 2004). Genetic variations identified within these genes are in various stages of becoming molecular diagnostics in medicine.

Recent advancements in technology and genomic knowledge have created opportunities to expand and refine the understanding of pharmacogenomics. In particular, the capabilities of quantifying gene expression levels using real-time quantitative PCR (RTQ) have recently been expanded by the development of Taqman low-density arrays (TLDAs). These arrays are capable of simultaneously quantifying the expression of up to 384 user-defined genes in a single RNA sample. This is an improvement upon RTQ's limitation of being able to only examine one gene at a time. Large clusters of genes and

even whole families of genes could potentially be analyzed quickly and efficiently using TLDA technology. In the current dissertation research, TLDA was used in a focused, molecular analysis of genes directly associated with the HH pathway or influenced by HH signaling (see Table 1 for a full list and description of these genes). The total 46 genes examined were chosen based upon extensive literature reviews of the HH molecular pathway (Ingham & McMahon, 2001; Nusse, 2003; Pasca di Magliano & Hebrok, 2003; Roy & Ingham, 2002). Because of the relatively recent discovery of this pathway, its true molecular portrait remains to be fully elucidated and genes involved in HH signaling continue to be discovered. Therefore, the 46 genes examined in this dissertation should not be taken as a definitive analysis of the entirety of the HH pathway. Validation of TLDA methodology was required for the analysis of clinical specimens including snap-frozen and paraffin-embedded tissues. These studies were carried out in ovarian and not pancreatic carcinomas because of the availability of matched snap-frozen and archival tissues. The development and validation of TLDA in ovarian cancer ultimately allowed subsequent examination of HH gene expression in matched surgically resected pancreatic cancer (both snap-frozen and paraffin-embedded) and fine-needle aspirates.

One of the primary goals in the development of rational treatment paradigms is the identification of tumor associated pathways that could be selectively targeted in cancer cells. Understanding the molecular basis of response and identifying markers capable of stratifying patients toward these more effective treatments also remains a central effort in current pharmacogenomic studies. Genetic analysis has been successfully employed in other cancers to enable the identification of specific targets (Evans et al., 2006; Halvorsen et al., 2007), individualized treatments (Torres-Arzayus et al., 2006; Zondor & Medina,

2004) and prognostic indicators (Ishikawa et al., 2006; Larsson, Holck, & Christensen, 2007) as well as to elucidate molecular mechanisms involved in tumor etiology (Cristiano et al., 2006; Herszenyi et al., 2007). These pharmacogenomic principles could potentially be applied to the HH pathway and its relationship to PAC. However, certain obstacles must be overcome in order to obtain the benefits of such an approach. One obstacle is the heterogeneity of pancreatic tumors due to the host desmoplastic stromal reaction. Neoplastic cells often represent only a small percentage of the cellular content (<40%) in many parts of the tumor mass which can necessitate difficult and time-intensive procurement of malignant epithelia from within heterogeneous tissues (Crnogorac-Jurcevic et al., 2002). In addition, tumor heterogeneity can vary between patients and the type of biopsy collected (snap-frozen, paraffin-embedded or fine-needle aspirates) further complicating genetic analyses of this tumor type. Overcoming the difficulty this heterogeneity represents could potentially lead to the identification of novel tumor-specific targets, a prerequisite to the future development of prognostic, diagnostic or targeted anti-HH therapies. Another obstacle is that molecular indicators of response and/or resistance to HH pathway inhibition remain unknown. Knowledge of these molecules could ultimately be used to develop a molecular basis for the rational selection of newly diagnosed pancreatic cancer patients who may be candidates for HH inhibitor therapy. Lastly, the molecular basis of response to HH pathway inhibition in pancreatic cancer cells remains largely undefined. Elucidation of the molecular changes that result in a physiological response to HH pathway inhibition could, ultimately, provide a mechanistic basis for combining HH antagonists with other therapeutic agents to improve clinical outcome (e.g. synergistic effects). Since activation of the HH pathway appears to be an early event in the develop-

ment of pancreatic cancer, these analyses may also be useful in understanding tumor etiology.

Specific Aims

The primary objectives of this dissertation research were to characterize differential expression of the HH pathway in clinical PAC specimens (snap-frozen, paraffin-embedded and fine-needle aspirates) and examine the molecular basis of *in vitro* response to cyclopamine in human pancreatic cancer cell lines. The following specific aims for this dissertation were proposed:

1. To examine differential expression (mRNA) of 46 genes directly associated with the HH pathway or influenced by HH signaling in PAC clinical specimens and uninvolved pancreas. We hypothesized that HH gene expression levels will vary between neoplastic and uninvolved tissue. In addition, we hypothesized that these analyses may be affected by (1) different methodologies used for tissue collection (surgical resection versus fine-needle aspiration), (2) storage (snap-frozen versus archival paraffin-embedded) and (3) tissue heterogeneity.
2. To identify genes associated with response and/or resistance to HH pathway inhibition. (a) To determine, *in vitro*, the relationship between HH pathway gene expression levels (prior to treatment) and response to cyclopamine (a HH pathway inhibitor) in human pancreatic cancer cell lines. (b) To modulate the expression of these genes and determine how altered expression affects *in vitro* response to cyclopamine. We hypothesized that response to cyclopamine is associated with differential expression of genes in the HH pathway.

3. To examine the physiological and molecular changes that result from HH pathway inhibition in pancreatic cancer cells. We hypothesized that inhibition of the HH pathway in human pancreatic cancer cells results in reduced cell proliferation, the induction of apoptosis or a combination of both. Further, we hypothesized that these physiological responses result from molecular changes that occur following HH pathway inhibition.

Significance of the Study

Recent studies have shown that HH signaling appears to be required for PAC initiation, growth and continued survival and that selective inhibitors of this pathway could provide novel therapeutic approaches for the treatment of tumors which remain resistant to conventional trimodal treatment paradigms (surgery, chemotherapeutics, radiation). However, the molecular basis of response and/or resistance to HH pathway inhibition remains to be elucidated. This dissertation research was designed to characterize the HH pathway in clinical PAC specimens as well as identify genes that are associated with efficacy to HH antagonism. The data from Specific Aims 1 and 2 offer the exciting possibility of not only providing a basis for future targeted therapies but also stratifying newly diagnosed PAC patients toward more effective treatment with HH pathway inhibitors. In addition, these analyses will provide a better understanding of the HH signaling pathway in pancreatic cancer and, ultimately, provide insight into the molecular basis of antineoplastic activity observed following inhibition of the HH pathway (Specific Aim 3).

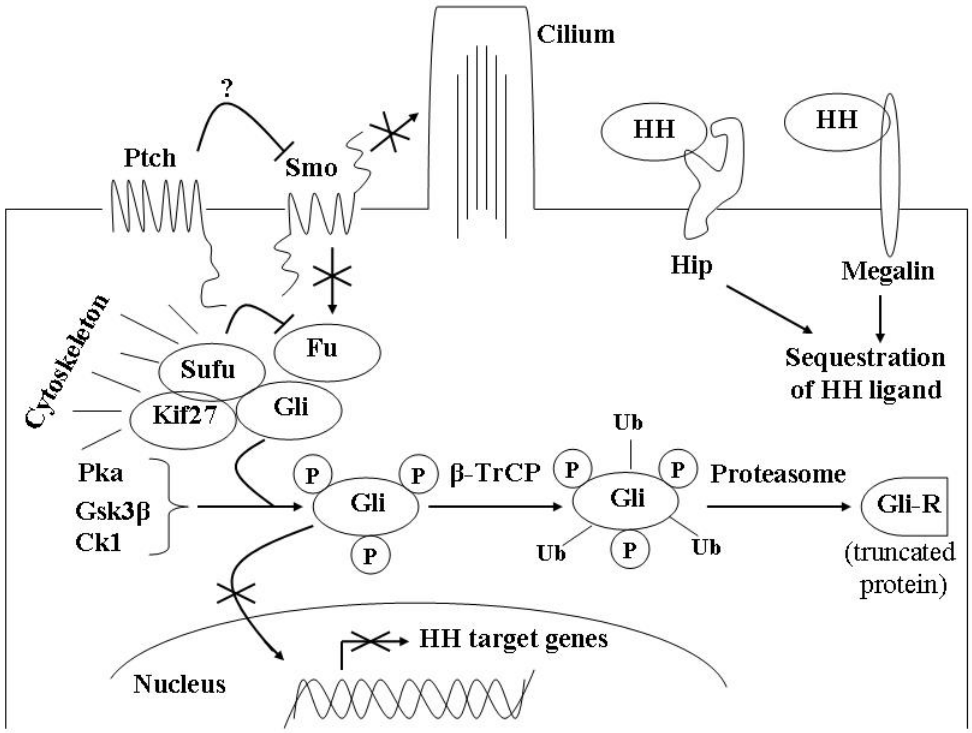


Figure 1. Inactive hedgehog signaling pathway. In the absence of HH ligand, the transmembrane receptor Patched (Ptch) inhibits the activity of Smoothened (Smo), a seven-span transmembrane protein. The mechanism by which this occurs remains unclear; however, it has been suggested that Ptch introduces a small molecule that binds to and effectively inhibits Smo activity. Smo cannot localize to cilia and the transcription factor Gli is prevented from translocating into the nucleus through interactions with cytoplasmic proteins including Fused (Fu), Suppressor of fused (Sufu) and Kinesin family member 27 (Kif27). cAMP-dependent protein kinase (Pka), Glycogen synthase kinase 3-β (Gsk3β) and Casein kinase 1 (Ck1) phosphorylate Gli at multiple sites priming it for the E3 ubiquitin (Ub) ligase β-transducin-repeat-containing protein (β-TrCP). Phosphorylated and polyubiquitinated Gli is then degraded by the proteasome. This proteolysis is only partial for Gli-2 and Gli-3, which can act as transcriptional repressors in their truncated forms. The receptors hedgehog interacting protein (Hip) and Megalin negatively regulate HH signaling by attenuating the binding of HH ligand to Ptch.

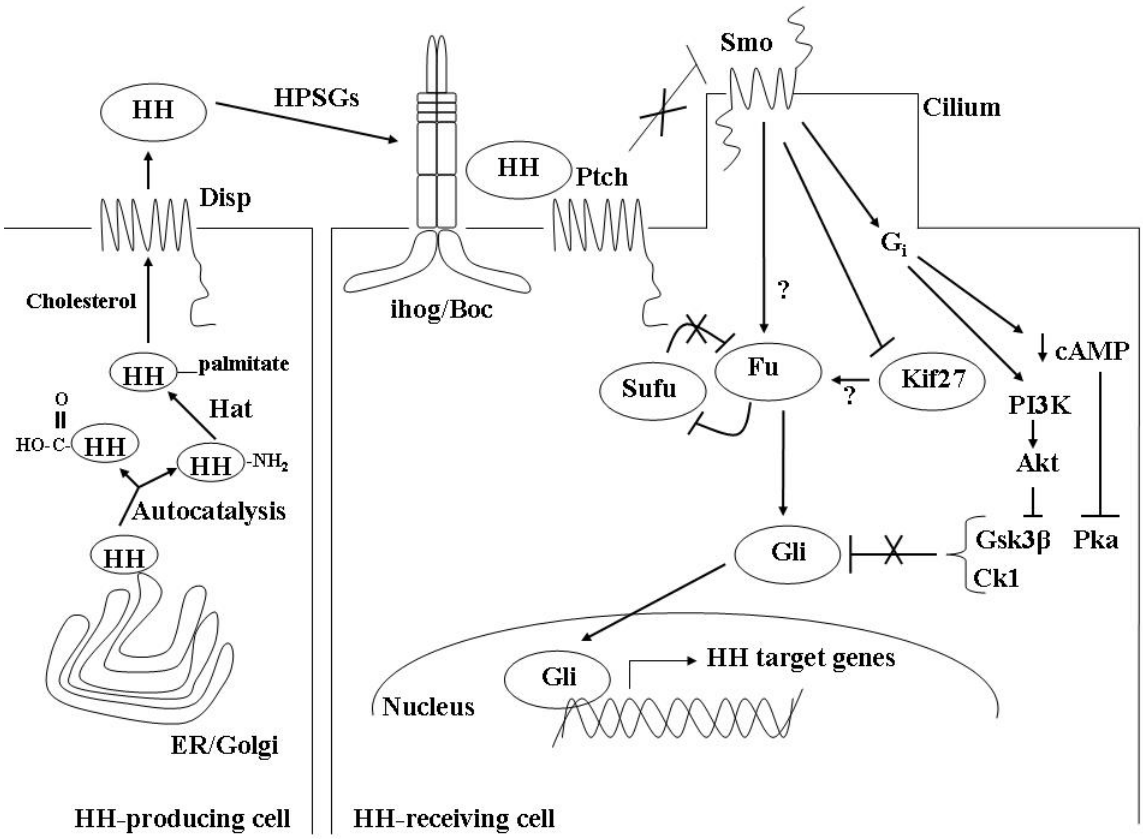
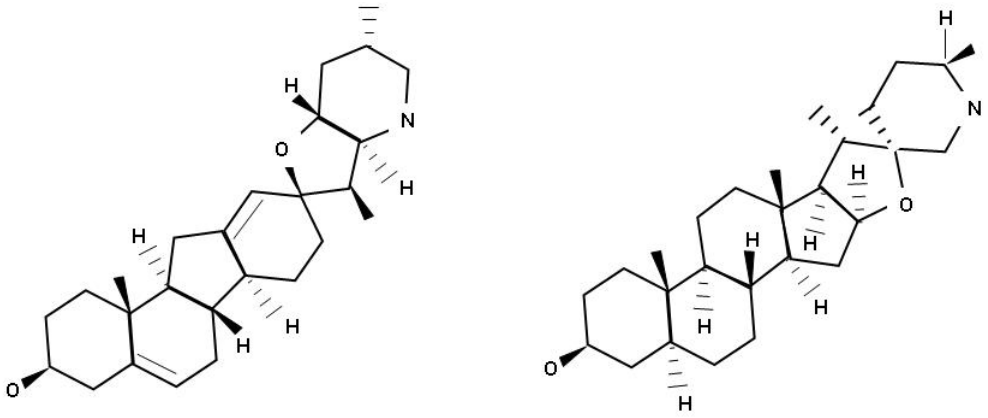


Figure 2. Active hedgehog signaling. In a HH-producing cell, the HH ligand undergoes autocatalytic cleavage to yield a C-terminal peptide, which has no known biological function, and an N-terminal peptide, which goes on to perform cell signaling. This truncated peptide undergoes the addition of palmitic acid (in a reaction catalyzed by hedgehog acyl transferase (Hat)), and cholesterol to become a mature signaling peptide. Lipid-modified HH ligand is released from HH-producing cells through the transmembrane protein Dispatched (Disp) and is transported to HH-receiving cells by interactions with heparin sulfate proteoglycans (HPSGs). Binding of HH ligand to Patched (Ptch) is mediated by the immunoglobulin-like receptor interference hedgehog (ihog or Boc) and relieves the inhibition of Smoothened (Smo) activity. Activated Smo is believed to localize to the cilia where it can activate Gli. The mechanism by which Smo signals to Gli remains unclear. It has been suggested that by coupling with members of the G_i family of G-proteins, Smo promotes the decrease of intracellular cAMP levels and activation of phosphoinositide 3-kinase (PI3K). Thjs leads to the inhibition cAMP-dependent protein kinase (Pka) and Glycogen synthase kinase 3- β (Gsk3β) thereby promoting the activity of the Gli transcription factors. Activator forms of Gli are allowed to translocate into the nucleus where they transcribe HH target genes including *PTCH* and *GLI* itself.



Cyclopamine Tomatidine

Figure 3. Chemical structures of cyclopamine and tomatidine.

Table 1

List of Genes Examined Using TLDA

Gene Name (Symbol)	Description	Connection to the HH Pathway	References
Hedgehog Pathway Genes			
Sonic Hedgehog (SHH)	Signaling peptide	HH ligand; binds to Ptch receptors	Riddle et al., 1993
Indian Hedgehog (IHH)	Signaling peptide	HH ligand; binds to Ptch receptors	Echelard et al., 1993
Desert Hedgehog (DHH)	Signaling peptide	HH ligand; binds to Ptch receptors	Echelard et al., 1993
Hedgehog acyl transferase (HHAT)	Acyl transferase	Adds palmitic acid to the HH ligand	Chamoun et al., 2001
Dispatched homolog 1 (DISP1)	12-span transmembrane receptor	Controls release of HH ligand from HH-producing cells	Burke et al., 1999
Dispatched homolog 2 (DISP2)	12-span transmembrane receptor	Controls release of HH ligand from HH-producing cells	Burke et al., 1999
Tout velu homolog 1 (EXT1)	Glycosaminoglycan transferase	Controls transport of HH ligand to HH-receiving cells	Bellaiche et al., 1998
Tout velu homolog 2 (EXT2)	Glycosaminoglycan transferase	Controls transport of HH ligand to HH-receiving cells	Bellaiche et al., 1998
Hedgehog interacting protein (HHIP)	Transmembrane protein	Attenuates HH ligand diffusion by competing with the Ptch receptors	Chuang et al., 1999
Megalin (LRP2)	Low-density lipoprotein receptor	Binds HH ligands and promotes their endocytosis; inhibits HH signaling	McCarthy et al., 2002
Open brain homolog (RAB23)	GTPase vesicular transport protein	Inhibits HH signaling through an unknown mechanism	Evans et al., 2003
Patched 1 (PTCH1)	12-span transmembrane receptor	Binds HH ligands and inhibits Smo	Stone et al., 1996
Patched 2 (PTCH2)	12-span transmembrane receptor	Binds HH ligands and inhibits Smo	Carpenter et al., 1998
Smoothened (SMO)	G-protein coupled receptor	Disinhibited in the presence of HH ligand; activates Gli transcription factors	Stone et al., 1996
Fused homolog (STK36)	Serine-threonine kinase	Complexes with Sufu and Kif27 to sequester Gli in the cytoplasm	Murone et al., 2000
Suppressor of fused homolog (SUFU)	Cytoplasmic protein	Complexes with Stk36 and Kif27 to sequester Gli in the cytoplasm	Murone et al., 2000
Costal-2 homolog (KIF27)	Cytoplasmic protein	Complexes with Sufu and Stk36 to sequester Gli in the cytoplasm	Monnier et al., 2002
Glycogen synthase kinase-3 beta (GSK3B)	Cytoplasmic protein kinase	Inhibits HH signaling by phosphorylating Gli	Price et al., 2002
Glioma-associated oncogene 1 (GLI1)	Zinc-finger transcription factor	Transcribes HH pathway target genes	Lee et al., 1997
Glioma-associated oncogene 2 (GLI2)	Zinc-finger transcription factor	Transcribes HH pathway target genes; can repress and activate transcription	Mo et al., 1997
Glioma-associated oncogene 3 (GLI3)	Zinc-finger transcription factor	Transcribes HH pathway target genes; can repress and activate transcription	Mo et al., 1997

Table 1 (Continued)

List of Genes Examined Using TLDA

Gene Name (Symbol)	Description	Connection to the HH Pathway	References
Genes Influenced By Hedgehog Signaling			
Cyclin B1 (CCNB1)	Protein involved in G2-M transition	Activity is regulated by interaction with Ptch	Barnes et al., 2001
Cyclin D1 (CCND1)	Protein involved in G1-S transition	Transcriptional target of Gli transcription factors	Roy et al., 2002
Cyclin D2 (CCND2)	Protein involved in G1-S transition	Transcriptional target of Gli transcription factors	Roy et al., 2002
Cyclin D3 (CCND3)	Protein involved in G1-S transition	Transcriptional target of Gli transcription factors	Roy et al., 2002
Cyclin E1 (CCNE1)	Protein involved in G1-S transition	Transcriptional target of Gli transcription factors	Roy et al., 2002
Cyclin E2 (CCNE2)	Protein involved in G1-S transition	Transcriptional target of Gli transcription factors	Roy et al., 2002
p21, Cip1 (CDKN1A)	Inhibitor of cyclin-dependent kinases	Activity is blocked by HH signaling	Fan et al., 1999
E2F1 transcription factor (E2F1)	Protein involved in G1-S transition	Transcriptional target of Gli transcription factors	Roy et al., 2002
Retinoblastoma 1 (RB1)	Protein involved in cell proliferation	Activity is blocked by HH signaling	Roy et al., 2002
c-Myc oncogene (MYC)	Protein involved in cell proliferation	Transcriptional target of Gli transcription factors	Louro et al., 2002
Epidermal growth factor ligand (EGF)	Mitogenic factor	Transcriptional target of Gli transcription factors	Amin et al., 1999
Epidermal growth factor receptor (EGFR)	Mitogenic factor	Transcriptional target of Gli transcription factors	Amin et al., 1999
Platelet-derived growth factor receptor alpha (PDGFRA)	Mitogenic factor	Transcriptional target of Gli transcription factors	Dahmane et al., 2001
Transforming growth factor beta-1 (TGFB1)	Mitogenic factor	Transcriptional target of Gli transcription factors	Dahmane et al., 2001
Transforming growth factor alpha (TGFA)	Mitogenic factor	Transcriptional target of Gli transcription factors	Dahmane et al., 2001
Engrailed homolog 1 (EN1)	Transcription factor	Transcriptional target of Gli transcription factors	Tabata et al., 1992
Engrailed homolog 2 (EN2)	Transcription factor	Transcriptional target of Gli transcription factors	Tabata et al., 1992
Notch homolog 1 (NOTCH1)	Developmental protein	Interacts with HH pathway during mammalian development	Benson et al., 2004
Notch homolog 2 (NOTCH2)	Developmental protein	Interacts with HH pathway during mammalian development	Benson et al., 2004
Snail homolog 1 (SNAI1)	Zinc-finger transcription factor	Transcriptional target of Gli transcription factors	Louro et al., 2002
Snail homolog 2 (SNAI2)	Zinc-finger transcription factor	Transcriptional target of Gli transcription factors	Louro et al., 2002
K-ras oncogene (KRAS2)	GTPase	Induces Gli-1 expression and HH activity in pancreatic cancer	Pasca di Magliano et al., 2006
Bcl-2 oncogene (BCL2)	Anti-apoptosis protein	Transcriptional target of Gli transcription factors	Regl et al., 2004
Beta-Catenin (CTNNB1)	Transcription factor	Activity can be regulated by Gli and Snail	Li et al., 2007
Archipelago homolog (FBXW7)	Ubiquitin ligase	No known connection; mistakened for Slimb homolog (β-TrCP)	Wang et al., 2006

MULTIPLE GENE EXPRESSION ANALYSES IN PARAFFIN-EMBEDDED TISSUES BY TAQMAN LOW-DENSITY ARRAY: APPLICATION TO HEDGEHOG AND WNT PATHWAY ANALYSIS IN OVARIAN ENDOMETRIOID ADENOCARCINOMA

by

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Format adapted and errata corrected for dissertation

Abstract

Recent studies have shown the hedgehog and Wnt families of signaling proteins to be associated with tumor initiation, growth, and survival. However, these pathways remain unexplored in ovarian endometrioid adenocarcinoma (OEA). Here, we describe a novel Taqman low-density array to examine the expression of 26 and 20 genes in the hedeghog and Wnt pathways, respectively, in six matched snap-frozen and formalin-fixed, paraffin-embedded (FPE) OEA specimens. Expression values were normalized to uninvolved ovarian epithelium. Gene expression in matched frozen and FPE tissues demonstrated significant concordance ($r = 0.92$, $P < 0.0001$). However, comparison of amplified and unamplified RNA from frozen OEA tissues revealed an altered molecular profile in amplified RNA. Amplification of RNA from FPE tissues was not successful. The expression of *Desert hedgehog* (*DHH*), *Indian hedgehog* (*IHH*), *Hedgehog interacting protein* (*HHIP*), *Wnt10B*, *Wnt9B* and *Wnt inhibitory factor* (*WIF1*) were tumor-specific with no detectable expression in normal ovarian epithelium. In addition, several genes were significantly ($P < 0.025$) down-regulated in OEA, including *cyclin E2*, *Porcupine*, *c-Myc*, and *Axin 2* (4.8, 3.6, 2.9 and 1.9-fold respectively). Taqman low-density array provides an effective multivariate technique for examining gene expression in RNA isolated from either snap-frozen or archival FPE tissues and can identify tumor-specific genes, possibly leading to novel treatments.

Introduction

Ovarian cancer is distinguished by particularly aggressive local invasiveness potential and in the United States, remains the fourth highest cause of cancer death in

women.[1, 2] Epithelial ovarian cancer is the major ovarian malignancy consisting of four histological subtypes including serous, mucinous, endometrioid, and clear cell with ovarian endometrioid adenocarcinoma (OEA) being the second most common.[3] Unfortunately, ~80% of patients diagnosed with advanced OEA die within 5 years.[4] In addition, the limited knowledge of the molecular mechanisms involved in the development and clinical progression of OEA have hampered attempts to develop novel rationally designed treatment paradigms.

Activation of the hedgehog signaling pathway, normally involved in embryogenesis, can lead to tumor formation and is necessary for tumor survival in several types of cancer (including medulloblastoma, basal cell carcinoma, small-cell lung cancer and breast cancer).[5-10] Recent studies have also implicated hedgehog signaling as an early mediator of tumorigenesis in cancers of the digestive tract, particularly pancreatic adenocarcinoma.[11, 12] Collectively, these studies suggest that abrogation of the hedgehog pathway may provide a novel, targeted therapeutic approach. Interestingly, the hedgehog pathway has not been examined in OEA. The Wnt pathway, also involved in embryogenesis, has been found to possess similarities to the hedgehog pathway with respect to post-translational modification, secretion, signaling mechanisms and tumorigenesis.[13, 14] Recent studies have shown that increased expression of components in the Wnt pathway have been implicated in ovarian tumorigenesis, although the exact molecular mechanisms remain to be elucidated.[15, 16]

Despite recent advances in gene quantitation technology, there is limited analysis of large clusters of genes, such as the hedgehog and Wnt families, in ovarian carcinomas in part because of the limited availability of fresh frozen tissue. Conversely, formalin-

fixed, paraffin-embedded (FPE) tissues, derived from institutional archives, offer a more readily available alternative to frozen tissue in most cancer treatment/research facilities. Several studies have shown that real-time quantitative (RTQ) polymerase chain reaction (PCR) can be used to quantify gene expression from RNA isolated from FPE tissue.[17-22] However, these studies have been limited by RTQ's ability to quantify only one gene at a time in a single RNA sample. In addition, validation of gene expression profiles obtained from frozen versus FPE tissue has been problematic due to the difficulty in obtaining matched frozen and paraffin-embedded samples. The capabilities of RTQ have recently been expanded with the development of Taqman low-density arrays (TLDAs) (Applied Biosystems, Foster City, CA), which are able to determine the expression of multiple, user-defined gene clusters simultaneously.

In the current study, we examine whether TLDA could be used for the analysis of archival, paraffin-embedded tissues by correlating the expression of 26 and 20 genes in the hedgehog and Wnt pathways, respectively, in six matched snap-frozen and FPE OEA specimens. In addition, expression profiles of amplified versus unamplified RNA were compared to determine whether the small amounts of RNA available from needle biopsies or laser capture-microdissected samples could be increased and quantified while conserving the expression profile. These analyses represent the first multivariate examination of the hedgehog and Wnt pathways in OEA.

Materials and Methods

Tissue Collection and Processing

Six ovarian endometrioid tumors were obtained from the Department of Pathology at the University of Alabama at Birmingham using an institutional review board-approved protocol. The samples were collected in the operating room and sectioned into three pieces. The central piece was immediately snap-frozen in liquid nitrogen and stored at -80°C before RNA isolation. The other two end pieces were immediately fixed in neutral-buffered formalin for 6 to 18 hours before paraffin embedding. Paraffin-embedded tissues were cut into 20-μm sections and stored at room temperature before RNA isolation. Normal ovarian surface epithelium (OSE) that harbored no neoplasms was scraped from the ovaries of two unrelated patients and immediately snap-frozen in liquid nitrogen and stored at -80°C before RNA isolation.

RNA Extraction

Total RNA was isolated from frozen tissues using Trizol reagent (Invitrogen, Carlsbad, CA) as per manufacturer's instructions. RNA was then DNase treated and purified using the RNeasy Mini Kit (QIAGEN, Hilden, Germany) as per manufacturer's instructions. RNA was eluted in 30 μl of RNase-free water and stored at -80°C.

Paraffin tissue sections were deparaffinized by incubation with 800 μl of xylene and 400 μl of 100% ethanol. The samples were then centrifuged and the supernatant was removed. Tissue pellets were washed with 1 ml of 100% ethanol and dried for 10 minutes at 55°C. The RNA isolation that followed was performed using the Roche High

Pure RNA Paraffin Kit (Roche Diagnostics, Manheim, Germany) as per manufacturer's instructions. RNA was eluted in 30 µl of RNase-free water and stored at -80°C.

Housekeeping Gene Variability

Assessment of housekeeping gene variability between normal and neoplastic ovarian tissues was performed as previously described.[23] Briefly, the RNA concentrations of both the OEA and OSE samples were determined spectrophotometrically by A_{260} measurement and adjusted to 20 ng/µl to ensure that differences in housekeeping gene expression were not due to variability in RNA concentrations. Because of the low amounts of tissue obtained from the 2 scraped OSE specimens, their RNA was combined to obtain a sufficient concentration for further analysis. The concentration of each sample was confirmed by densitometry and RNA integrity (degradation) was verified by electrophoresis and ethidium bromide staining on a 1% agarose gel. Primers and probe for the *ribosomal protein*, *large*, *P0* (*RPLP0*) (NM_001002) gene were obtained from Applied Biosystems (Foster City, CA) and used according to the manufacturer's instructions. The concentration of all RNA samples (OSE and OEA) was then determined using *RPLP0* and linear regression analysis of a standard curve derived from known concentrations of normal ovary total RNA (Ambion, Austin, TX) as previously described by our laboratory.[23, 24]

RNA Amplification

A fixed amount of 20 ng of total RNA isolated from either frozen or paraffin-embedded OEA was amplified using the Ovation Nanosample RNA amplification system

(NUGEN Technologies, Inc., San Carlos, CA), Full Spectrum Global amplification kit (System Biosciences, Mountain View, CA), MessageAmp aRNA kit (Ambion) and RiboAmp RNA amplification kit (Arcturus, Mountain View, CA) as per manufacturer's instructions. The yield obtained from each amplification procedure was assessed by the *RPLP0* housekeeping gene.

Reverse Transcription

Before cDNA synthesis, all RNA samples, amplified and unamplified, were diluted to 4 ng/µl using RNase-free water containing 12.5 ng/µl of total yeast RNA (Ambion) as a carrier. cDNA was prepared using the High Capacity cDNA archive kit (Applied Biosystems) as per manufacturer's instructions. The resulting cDNA samples were used immediately for TLDA analysis.

PCR Amplification Efficiency

For RNA isolated from frozen and FPE tissues, a standard curve was prepared using 20, 10, 5, 2.5, 1.25 ng RNA. RTQ was performed for the *RPLP0* housekeeping gene. The slope to each standard curve was then calculated and the efficiency of PCR amplification was determined using the equation $e = 10^{(-1/\text{slope})}$. In a PCR reaction that is 100% efficient, the amount of amplicon doubles at each cycle such that an *e* of 2 represents 100% PCR amplification efficiency.

TLDA

TLDA design. For each card of the low-density array, there are eight separate loading ports that feed into 48 separate wells for a total of 384 wells per card (Figure 1). Each 2-µl well contains specific, user-defined primers and probes, capable of detecting a single gene. In this study, the TLDA card was configured into eight identical 48-gene sets (Figure 1). Genes were chosen based on literature reviews of the hedgehog, Wnt and cell cycle molecular pathways and their involvement in tumorigenesis.[5, 6, 13, 14, 25] Each set of 48 genes (Table 1) also contains two housekeeping genes, *RPLP0* and *18S* (a mandatory control designed into each card by the manufacturer). In this study, however, *RPLP0* was used exclusively as the housekeeping gene.

TLDA preparation. Each cDNA sample (100 µl) was added to an equal volume of 2X Taqman Universal PCR Master Mix (Applied Biosystems). After gentle mixing and centrifugation, the mixture was then transferred into a loading port on a TLDA card (Applied Biosystems). Each of the six matched OEA samples were run in quadruplicate with one matching pair (frozen and FPE) on each card. Amplified RNA samples were also run in quadruplicate on separate cards. To distinguish tumor-specific activation of the hedgehog and Wnt pathways, RNA from two combined snap-frozen OSE samples were also analyzed. The array was centrifuged twice for one minute each at 1200 rpm (306 x *g*) to distribute the samples from the loading port into each well. The card was then sealed and PCR amplification was performed using an Applied Biosystems Prism 7900HT *sequence* detection system. Thermal cycler conditions were as follows: 2 minutes at 50°C, 10 minutes at 94.5°C, 30 seconds at 97°C, 1 minute at 59.7°C for 40 cycles.

TLDA Analysis

Expression values were calculated using the comparative C_T method as previously described (User Bulletin No. 2, Applied Biosystems). Briefly, this technique uses the formula $2^{-\Delta\Delta CT}$ to calculate the expression of target genes normalized to a calibrator. The threshold cycle (C_T) indicates the cycle number at which the amount of amplified target reaches a fixed threshold. C_T values range from 0 to 40 (the latter representing the default upper limit PCR cycle number that defines failure to detect a signal). ΔC_T values ($\Delta C_T = C_T$ (target gene) – C_T (RPLP0)) were calculated for the combined frozen OSE sample and subsequently used as the calibrator, for which all gene expression values were assigned a relative value of 1.00, to determine comparative gene expression such that $\Delta\Delta C_T = \Delta C_T$ (OEA sample) - ΔC_T (OSE sample). A range for each expression value was calculated based on the SD (s) of the $\Delta\Delta C_T$ value where $2^{-(\Delta\Delta CT+s)}$ is the lower limit and $2^{-(\Delta\Delta CT-s)}$ is the upper limit.

Validation of TLDA

Primers and probes for *Desert Hedgehog* (*DHH*) (NM_021044), *Indian Hedgehog* (*IHH*) (NM_002181), *Sonic Hedgehog* (*SHH*) (NM_000193), *Patched* (*PTCH*) (NM_000264), *Patched 2* (*PTCH2*) (NM_003738), *Smoothened* (*SMO*) (NM_005631), *Glioma-associated oncogene homolog 1* (*GLI*) (NM_005269) and *Glioma-associated oncogene homolog 3* (*GLI3*) (NM_000168) were obtained from Applied Biosystems and used according to manufacturer's instructions. RTQ was then performed for these eight genes on the six matched frozen and FPE OEA samples as well as the combined OSE

sample using an ABI Prism 7900HT sequence detection system. Gene expression was calculated using the comparative C_T method.

Statistical Analysis

All statistical analyses were conducted with SAS Ver. 9.1 (SAS Institute, Cary, NC). Because of the small sample size, a more stringent $P < 0.025$ was used to establish statistically significant differences rather than 0.05, which is typically used. To check the reproducibility of TLDA, the coefficient of variance (CV) was calculated for each gene in each sample. The C_T values of all 48 genes examined in both snap-frozen and FPE tissue were measured four times (four replicates) for each of the six matched OEA samples. The average and range of CVs for the four replicates of each gene were calculated for ΔC_T values, which were used instead of C_T values so that different amounts of RNA added to each of the four replicates would not be reflected in the average CV.

To examine the correlation between matched frozen and FPE tissue, average ΔC_T values for the four replicates of each gene were calculated in each of the six matched OEA samples. A two-dimensional plot was then created depicting ΔC_T values from the six frozen samples as the explanatory variable (x) and ΔC_T values from the six paraffin-embedded samples as the dependent variable (y). Linear regression analysis and Pearson correlation was then performed to determine the agreement in gene expression between frozen and FPE tissue. A similar comparison of amplified and unamplifed RNA was performed, however C_T values were used instead of ΔC_T values to illustrate the significant number of genes that could not be detected following amplification.

To distinguish significant differences between expression levels in OEA relative to normal tissue, a one-sample, two-sided *t*-test was applied to compare the average expression level of the six samples to the normalized ovarian epithelium (which was assigned an expression level of 1.00 for each of the genes examined). The significance level for this test is 0.025.

Results

Quantitation of Housekeeping Gene Expression in Human Tissues Using RTQ

Comparative analysis demonstrated no significant ($P < 0.01$) difference in *RPLP0* expression between normal (OSE) and neoplastic (OEA) tissues.

Reliability of TLDA

For either frozen or FPE samples, the average coefficient of variance (CV) for all four replicates, using ΔC_T values, is ~5%, with a SD of 5%, over the 48 genes examined. In calculating the average CV, genes with a C_T value of 40, the default upper limit PCR cycle number that defines no signal, as well as *RPLP0* and *18S*, were excluded. Those genes that failed to express ($C_T = 40$) are designated as "NE" in Table 1 and *include insulin promoter factor 1* (*IPF1*), *Wnt16*, *Wnt8B*, and *Wnt9A*.

PCR Amplification Efficiency

Analysis of the standard curves for *RPLP0* amplification in both frozen and FPE OEA RNA yielded slopes of -3.00 and -3.11, respectively. The PCR amplification efficiency was calculated as 108% for frozen tissue and 105% for FPE tissue.

Correlation of Gene Expression in Matched Frozen and FPE Tissue

Analysis of the standard curves for *RPLP0* amplification in both frozen and FPE OEA RNA yielded slopes of -3.00 and -3.11, respectively. The PCR amplification efficiency was calculated as 108% for frozen tissue and 105% for FPE tissue.

Correlation of gene expression in matched frozen and FPE tissue

To determine whether archival FPE tissue is suitable for use with TLDA, we examined the correlation of 48 genes in frozen versus FPE OEA. As shown in Figure 2, a two-dimensional plot depicting average ΔC_T values from the six frozen (x) and matching six FPE samples (y) demonstrates a significant ($P < 0.0001$) linear correlation ($r^2 = 0.85$) with a Pearson correlation coefficient (r) of 0.92.

Correlation of Gene Expression in Amplified and Unamplified RNA

To examine whether commercially available kits could be used to amplify small amounts of RNA without altering the expression profile, we examined the correlation of 48 genes in matched amplified versus unamplified frozen and FPE OEA samples. Total RNA from frozen OEA successfully amplified using the Ovation, MessageAmp RiboAmp and Full Spectrum amplification protocols and resulted in increases of 448-, 110-, 2757- and 333-fold respectively. Repeated attempts to amplify total RNA isolated from FPE tissue were unsuccessful using the four protocols examined.

The conservation of gene expression in matched amplified versus unamplified (template) RNA from frozen OEA samples was examined using TLDA. As shown in Figure 3A, a two-dimensional plot depicting average C_T values from the amplified (x) and unamplified (y) frozen OEA sample using the Ovation amplification system demonstrates

a weak correlation with $r = 0.55$ ($P < 0.0001$). Twenty-four out of a total forty-two genes (57%) that expressed in unamplified RNA were undetectable after amplification (as shown by the boxed data points). Similar results were obtained for RNA amplified using the MessageAmp and RiboAmp kits (data not shown). Figure 3B depicts a comparison of the same frozen OEA sample using the Full Spectrum amplification system and demonstrates a stronger correlation with $r = 0.75$ ($P < 0.0001$) and only 12 out of 42 genes (29%) becoming undetectable following amplification. Although C_T values were plotted instead of ΔC_T values to emphasize undetectable genes in amplified RNA (boxed), identical r^2 and r values were obtained when ΔC_T values were used (data not shown).

Expression of Hedgehog and Wnt Genes in OEA

The expression of 46 independent genes in the hedgehog and Wnt pathways across six pairs of matched frozen and FPE OEA samples are summarized in Table 1. For both the frozen and FPE categories, each gene expression value represents an average of the six samples ± SD. Thus, the standard deviations in Table 1 reflect the range in expression over the six samples examined. Gene expression and statistical analysis show that 11 genes from frozen and 10 genes from FPE (Table 1, bolded) were differentially expressed in OEA compared to OSE (91% agreement).

Three members of the hedgehog pathway (*DHH*, *IHH*, *HHIP*) and three members of the Wnt pathway (*Wnt9B*, *Wnt10B*, *WIF1*) were found to be tumor-specific in both frozen and FPE tissues. The average expression, SD and *P* value for each of these genes could not be calculated since none of them express in the OSE calibrator. These six genes are designated as X in Table 1. Several other hedgehog and Wnt-related genes

were significantly lower in OEA (both frozen and FPE) compared to normal ovarian epithelium including *cyclin E2* (*CCNE2*), *Porcupine* (*PPN*), *c-Myc* (*MYC*), and *Axin 2* (*AXIN2*). The proapoptotic *Gli-pathogenesis-related protein* (*GLIPR1*) was significantly lower in frozen but not FPE OEA compared to normal ovarian epithelium. The difference in expression of the remaining 19 and 12 genes in the hedgehog and Wnt pathways, respectively, were not found to be significantly different due to their range in expression over the samples examined (Table 1). *Insulin promoter factor 1* (*IPF1*), *Wnt16*, *Wnt8B* and *Wnt9A* did not express in either OSE or OEA (designated as NE in Table 1).

Validation of TLDA

To validate the gene expression results obtained with TLDA, 8 genes (*DHH*, *IHH*, *SHH*, *PTCH*, *PTCH2*, *SMO*, *GLI*, *GLI3*) out of the original 46 were individually analyzed in both frozen and FPE samples using Taqman RTQ. A similar gene expression correlation ($r = 0.91$, $P < 0.01$) (Figure 4) between matched frozen and FPE tissues was obtained. When compared separately, gene expression values for these eight genes were not significantly different from the values obtained with TLDA in either frozen or FPE OEA (data not shown).

Discussion

Initial studies examined the reliability and amplification efficiency of the TLDA method and demonstrated an intra-assay CV of ~5% with almost equal PCR amplification efficiency in RNA isolated from matched frozen and FPE tissues (108% and 105%, respectively). Pearson correlation (r) between the six matched fresh frozen and FPE

OEA tissues for all tested genes was 92% (Figure 2). This agreement was further validated when similar gene expression correlation (r = 0.91, Figure 4) between the matched OEA samples was obtained by individually analyzing 8 genes out of the original 46 using RTQ. This multi-variate analysis is unique in that both snap-frozen and matched archival FPE specimens, routinely processed through the University of Alabama at Birmingham's Department of Pathology, were examined simultaneously. Although encouraging, future studies involving the analysis of expression profiles in FPE tissues should consider the variability in the stability of any particular gene being examined and must be independently validated.

Based on these promising preliminary data, we examined whether RNA amplification could be used in combination with TLDA. RNA amplification is a method whereby nanogram amounts of total RNA (usually obtained from needle biopsies or laser-capture microdissected clinical samples) undergo a multistep process for linear amplification of the mRNA fraction. Pearson correlation (r) between matched amplified versus unamplified RNA isolated from frozen OEA varied among the four amplification protocols tested from a low of 55% to a high of 75% (Figure 3). The three amplification protocols (Ovation, MessageAmp, RiboAmp) using polydT oligomer priming (which requires the 3' poly-A tail in template mRNA to bind with a modified oligo-dT primer) gave the poorest correlations of 55% (Figure 3A), 44%, and 53% (data not shown), respectively. The Full Spectrum amplification protocol, which utilizes a random hexamer (N_6) priming method that does not require an intact 3' poly-A tail, demonstrated the best correlation of 75% between amplified and unamplified RNA (Figure 3B). Unfortunately, RNA isolated from FPE samples failed to amplify using any of the four procedures and

correlative studies could not be conducted. Collectively, these data suggest random hexamer priming may have an advantage over oligo-dT priming when comparing expression profiles in snap-frozen tissues but that sheared or degraded RNA (such as that obtained from FPE tissues) cannot be amplified using these protocols.

To identify potential tumor-specific therapeutic targets, we used TLDA to quantify the expression of 26 and 20 genes in the hedgehog and Wnt pathways, respectively, in six matched frozen and FPE OEA specimens (Table 1). These pathways, normally involved in embryogenesis, have both been implicated in cancer initiation and are similar in terms of post-translational modification, secretion and some signaling mechanisms and may have evolved from a common pathway.[13] Recent studies have shown activation of the hedgehog signaling pathway in adult tissues can initiate and sustain tumor growth; however, this pathway has never been examined in OEA.[5-10] Inhibition of the hedgehog pathway by small molecule inhibitors such as cyclopamine has been shown to be effective in decreasing tumor growth and is a promising new therapeutic strategy.[26, 27] Similarly, Wnt signaling is involved in normal follicular development and ovarian function.[28] Because OSE is believed to be the origin of ovarian adenocarcinomas and Wnts have been implicated in oncogenic transformation of epithelial cells, it is thought that aberrant expression of this pathway could lead to ovarian carcinogenesis.[16] Quantitation of hedgehog- and Wnt-associated genes in OEA revealed several tumor-specific genes including the two hedgehog ligands, *DHH* and *IHH*, and hedgehog pathway regulator, *HHIP*, as well as the two Wnt ligands, *Wnt10B* and *Wnt9B*, and Wnt pathway regulator, *WIF1* (Table 1). Interestingly, these genes did not consistently express in all of the OEA samples. Other genes directly involved in the hedgehog and Wnt pathways including

Smoothened (*SMO*), *Glioma-associated oncogene* (*GLI*), *GLI2*, *GLI3* and *Wnt7A*, *Frizzled homolog* (*FRZD1*), *low-density lipoprotein receptor-related protein 6* (*LRP6*), *Frizzled related protein* (*FRZB*) were all over-expressed in both frozen and FPE OEA in comparison to normal OSE, but did not reach statistical significance. Similar gene expression results were obtained when eight differentially expressed genes (*DHH*, *IHH*, *SHH*, *PTCH*, *PTCH2*, *GLI*, *GLI3*, *SMO*) were examined individually using RTQ in both frozen and FPE samples (data not shown). In relation to statistically significant and tumor-specific genes, the differing genetic profiles among the OEA samples suggest variable activity of the hedgehog and Wnt pathways in this cancer. Thus, future studies involving individual analysis of a larger population of both normal and cancer patients to distinguish tumor-specific differences from interindividual variation are warranted. These studies could then offer the potential of identifying patients with advanced OEA who would benefit from anti-hedgehog or anti-Wnt therapy such as cyclopamine and non-steroidal anti-inflammatory drugs, respectively.[27, 29, 30]

The TLDA methodology presented in this study represents a robust and reproducible technique for quantifying gene expression in tens to hundreds of independent genes concurrently in RNA samples isolated from either frozen or FPE tissues. This approach represents a significant advance in multivariate gene analysis that is less time and labor intensive than individually analyzing single genes by RTQ. The correlation of gene expression profiles between matched frozen and FPE tissues offers the exciting possibility that archival, paraffin-embedded tissues (a more abundant alternative to frozen tissue available from most cooperative groups) may be examined to identify specific therapeutic targets and/or prognostic indicators. The importance of these multivariate analyses have

been emphasized by recent studies that have shown that examination of genes acting collectively in a specific pathway, such as the hedgehog or Wnt pathway, offers more information about clinical outcome than examination of individual genes.[31-34] In the current study, TLDA analysis was used to quantify the expression of hedgehog and Wnt-related genes in OEA and determined that elements of both these pathways are expressed in a subset of the patient samples examined. These analyses could potentially be used to identify patients with advanced OEA who would benefit from specifically targeted anti-hedgehog and/or anti-Wnt therapy.

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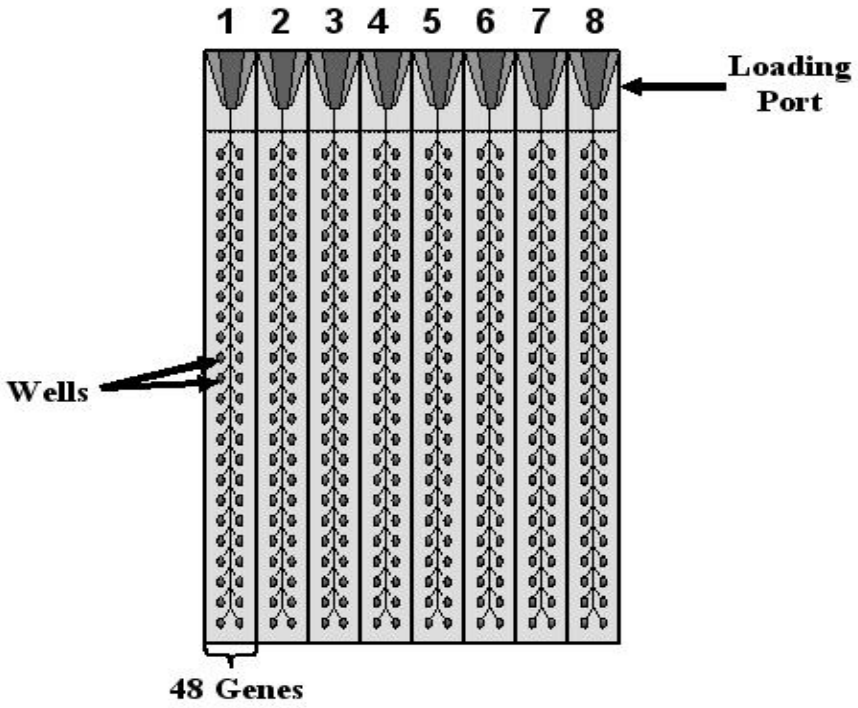

Figure 1. TLDA design using a 48-gene template.

Table 1

Hedgehog and Wnt Gene Expression in Ovarian Endometrioid Adenocarcinoma

Gene	Average frozen*	P *value* †	*Ratio* ‡	Average paraffin*	P *value* †	*Ratio* ‡
Desert Hedgehog (DHH)	**X**		**6/6**	**X**		**3/6**
Hedgehog Interacting Protein (HHIP)	**X**		**2/6**	**X**		**2/6**
Indian Hedgehog (IHH)	**X**		**4/6**	**X**		**3/6**
Wnt Inhibitory Factor 1 (WIF1)	**X**		**5/6**	**X**		**4/6**
Wingless-type MMTV integration site family, member 10B (WNT10B)	**X**		**5/6**	**X**		**4/6**
Wingless-type MMTV integration site family, member 9B (WNT9B)	**X**		**1/6**	**X**		**1/6**
Porcupine (PPN)	**0.28 ± 0.11**	**<0.0001**	**6/6**	**0.48 ± 0.39**	**0.021**	**6/6**
Cyclin E2 (CCNE2)	**0.21 ± 0.20**	**<0.0001**	**6/6**	**0.38 ± 0.45**	**0.020**	**6/6**
c-Myc oncogene (MYC)	**0.35 ± 0.39**	**0.010**	**6/6**	**0.12 ± 0.08**	**0.000**	**6/6**
Axin 2 (AXIN2)	**0.54 ± 0.29**	**0.011**	**6/6**	**0.54 ± 0.23**	**0.005**	**6/6**
GLI-pathogenesis related 1 (GLIPR1)	**0.46 ± 0.29**	**0.006**	**6/6**	2.16 ± 2.53	0.312	6/6
HER-2/Neu (ERBB2)	1.39 ± 2.03	0.654	6/6	0.36 ± 0.51	0.027	6/6
Patched (PTCH)	0.83 ± 0.63	0.536	6/6	1.64 ± 0.55	0.035	6/6
Epidermal Growth Factor Receptor (EGFR)	1.09 ± 0.86	0.802	6/6	2.90 ± 2.07	0.074	6/6
Wingless-type MMTV integration site family, member 7A (WNT7A)	7.01 ± 11.00	0.238	4/6	16.49 ± 18.06	0.090	4/6
Smoothened (SMO)	1.61 ± 1.44	0.345	6/6	2.96 ± 2.29	0.091	6/6
Frizzled homolog (FRZD1)	5.56 ± 10.29	0.328	6/6	9.34 ± 10.46	0.108	6/6
Patched 2 (PTCH2)	0.66 ± 0.56	0.194	6/6	5.60 ± 5.80	0.110	6/6
Glioma-associated oncogene (GLI)	3.84 ± 6.20	0.312	6/6	12.99 ± 15.39	0.114	5/6
Kringle containing transmembrane protein 1 (KREMEN1)	1.22 ± 1.29	0.692	6/6	0.68 ± 0.43	0.126	6/6
Platelet-derived growth factor receptor alpha (PDGFRA)	10.49 ± 7.80	0.031	6/6	12.79 ± 15.84	0.128	6/6
Glioma-associated oncogene 3 (GLI3)	1.22 ± 1.09	0.645	6/6	6.16 ± 7.06	0.134	6/6
Glioma-associated oncogene 2 (GLI2)	3.17 ± 4.47	0.289	6/6	5.41 ± 6.47	0.156	6/6
Low density lipoprotein receptor-related protein 6 (LRP6)	1.85 ± 1.57	0.241	6/6	4.35 ± 4.99	0.161	6/6
Cyclin B1 (CCNB1)	0.46 ± 0.42	0.026	6/6	0.61 ± 0.58	0.163	6/6
p21, Cip1 (CDKNA1)	1.08 ± 1.30	0.881	6/6	2.93 ± 2.97	0.172	6/6
Frizzled related protein (FRZB)	1.60 ± 2.37	0.563	6/6	2.38 ± 2.16	0.178	6/6
K-ras oncogene 2 (KRAS2)	1.00 ± 0.85	0.992	6/6	0.75 ± 0.41	0.185	6/6
Receptor-like Tyrosine Kinase (RYK)	1.05 ± 0.93	0.896	6/6	2.39 ± 2.37	0.209	6/6
Cyclin D1 (CCND1)	0.67 ± 0.53	0.182	6/6	1.71 ± 1.24	0.218	6/6
Sonic Hedgehog (SHH)	0.88 ± 1.41	0.840	3/6	5.26 ± 7.76	0.237	3/6
Cyclin D3 (CCND3)	2.97 ± 3.33	0.208	6/6	4.23 ± 6.18	0.257	6/6
Wingless-type MMTV integration site family, member 8A (WNT8A)	0.83 ± 1.95	0.837	2/6	0.45 ± 1.10	0.278	1/6
Suppressor of Fused (SUFU)	0.54 ± 0.45	0.052	6/6	1.56 ± 1.13	0.279	6/6
Cyclin D2 (CCND2)	1.10 ± 1.29	0.860	6/6	2.51 ± 3.21	0.302	6/6
Low density lipoprotein receptor-related protein 5 (LRP5)	0.61 ± 0.32	0.029	6/6	1.34 ± 0.84	0.368	6/6
E2F1 Transcription Factor (E2F1)	1.01 ± 0.73	0.987	6/6	2.14 ± 3.16	0.416	6/6
Retinoblastoma 1 (RB1)	1.20 ± 0.68	0.511	6/6	1.24 ± 0.87	0.537	6/6
Wingless-type MMTV integration site family, member 1 (WNT1)	0.25 ± 0.60	0.028	1/6	2.66 ± 6.51	0.560	1/6
Wingless-type MMTV integration site family, member 7B (WNT7B)	0.60 ± 0.92	0.338	5/6	0.68 ± 1.44	0.610	4/6
Axin 1 (AXIN1)	0.73 ± 0.54	0.278	6/6	1.18 ± 0.82	0.621	6/6
Cyclin E1 (CCNE1)	0.92 ± 0.87	0.836	6/6	1.10 ± 1.56	0.881	6/6
Insulin Promotor Factor 1 (IPF1)	NE		0/6	NE		0/6
Wingless-type MMTV integration site family, member 16 (WNT16)	NE		0/6	NE		0/6
Wingless-type MMTV integration site family, member 8B (WNT8B)	NE		0/6	NE		0/6
Wingless-type MMTV integration site family, member 9A (WNT9A)	NE		0/6	NE		0/6

*Values are expressed as the mean of six samples ± SD. Genes designated by X did not express in normal ovarian epithelium. Genes designated by NE (no expression) did not expressin either normal or OEA.

†$P < 0.025$ was considered statistically significant.

‡Ratio indicates OEA samples expressing gene/total samples examined.

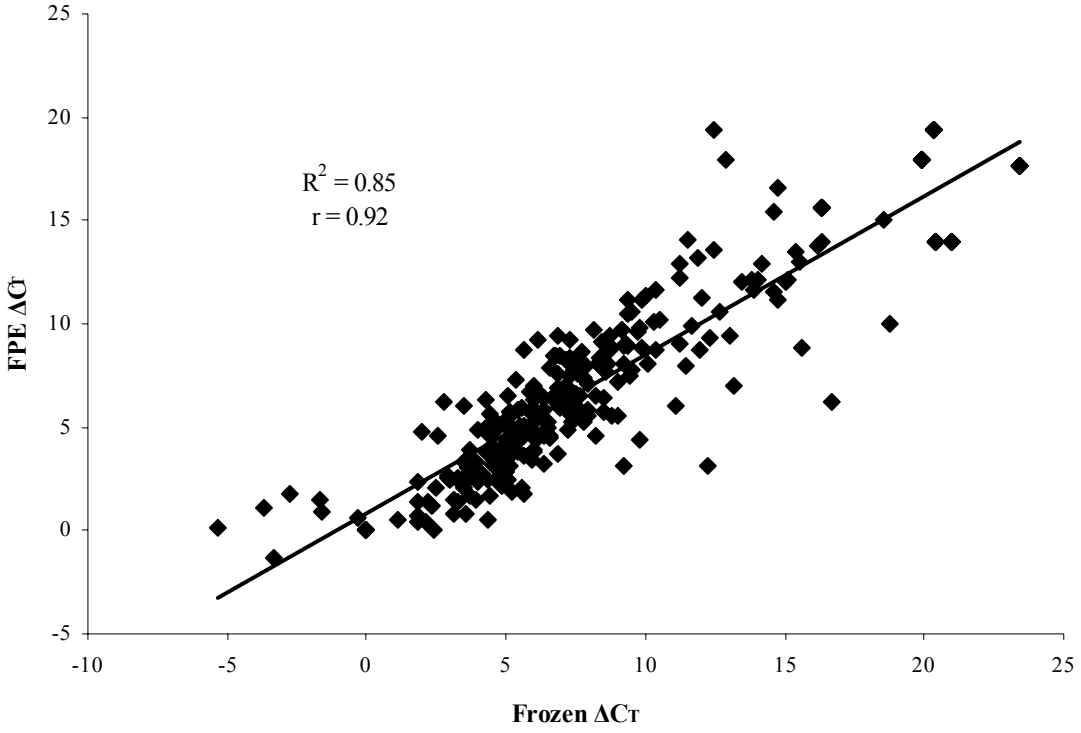

Figure 2. Correlative plot of mean ΔC_T values obtained by TLDA analysis of 48 genes in six frozen (x) compared to six matching paraffin-embedded OEA samples (y). Regression analysis demonstrated a coefficient of determination (r^2) of 0.85 and a Pearson correlation coefficient (r) of 0.92 ($P < 0.0001$).

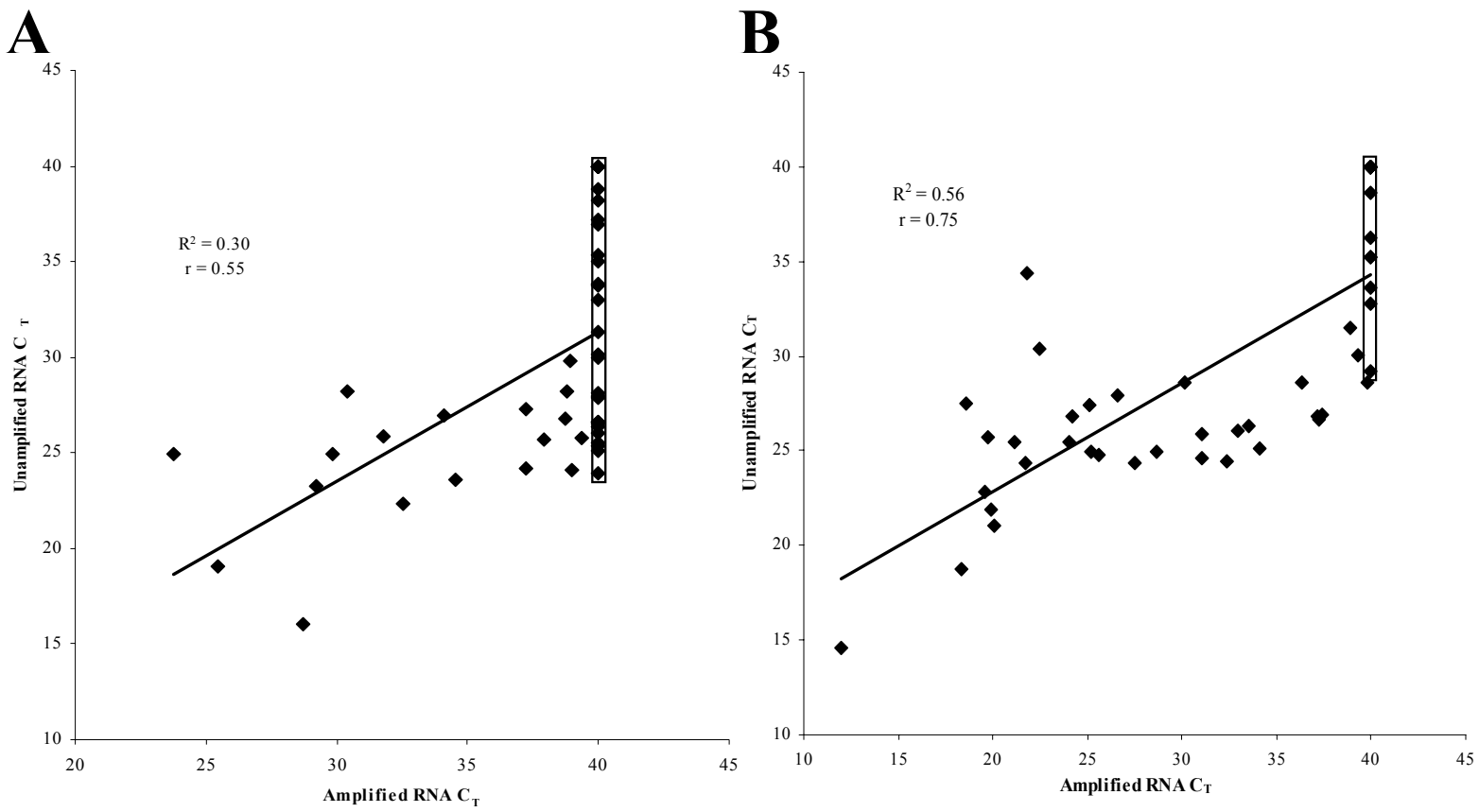

Figure 3. Correlative plot of CT values obtained by TLDA analysis of 48 genes in amplified (*x*) compared to unamplified (template) (*y*) using the Ovation Nanosample RNA amplification system with a coefficient of determination (r^2) of 0.30 and a Pearson correlation coefficient (*r*) of 0.53 ($P < 0.0001$) (A); and the Full Spectrum Global RNA amplification kit with a coefficient of determination (r^2) of 0.56 and a Pearson correlation coefficient (*r*) of 0.75 ($P < 0.0001$) (B).

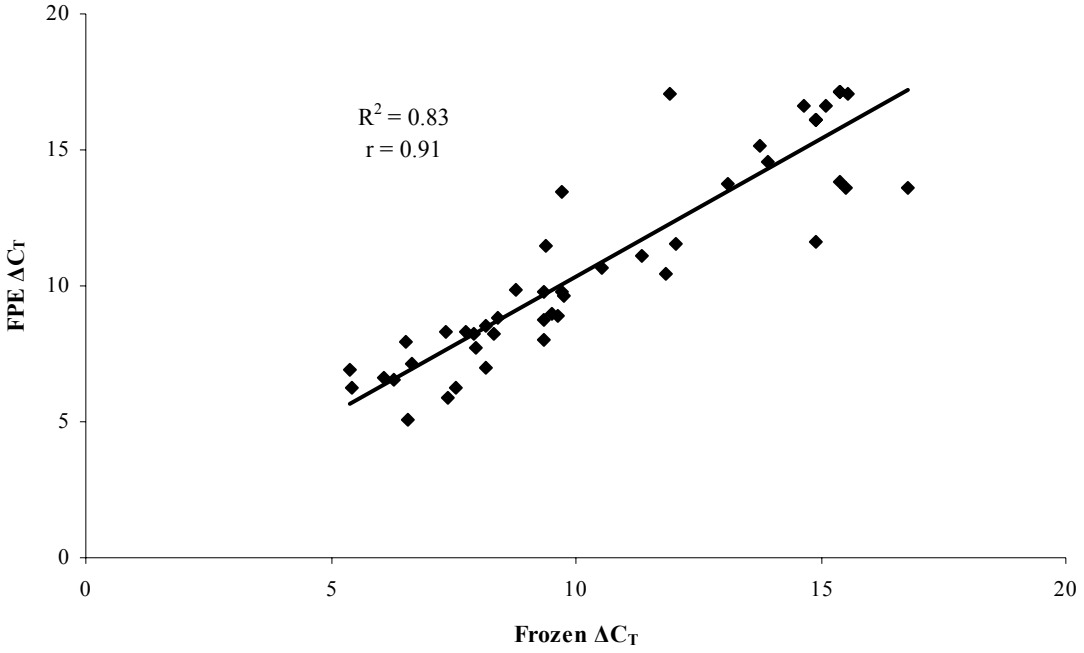

Figure 4. Correlative plot of ΔC_T values obtained by RTQ analysis of eight genes (*DHH*, *IHH*, *SHH*, *PTCH*, *PTCH2*, *SMO*, *GLI*, *GLI3*) in six frozen (*x*) compared to six matching paraffin-embedded OEA samples (*y*). Regression analysis demonstrated a coefficient of determination (r^2) of 0.83 and a Pearson correlation coefficient (*r*) of 0.91 ($P < 0.0001$).

HEDGEHOG PATHWAY EXPRESSION IN HETEROGENEOUS PANCREATIC ADENOCARCINOMA: IMPLICATIONS FOR THE MOLECULAR ANALYSIS OF CLINICALLY AVAILABLE BIOPSIES

by

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Format adapted and errata corrected for dissertation

Abstract

Recent studies suggest that hedgehog (HH)-pathway signaling is required for the initiation and continued growth of pancreatic adenocarcinoma (PAC). Definitive gene expression analysis of PAC remains difficult, owing to the host desmoplastic stromal interaction and subsequent tumor heterogeneity. The primary goal of this study was to evaluate the effect of heterogeneity within a series (n = 5) of matched clinical PAC biopsies [snap-frozen, formalin-fixed paraffin-embedded (FPE), endoscopic ultrasound-guided fine-needle aspirate (EUS-FNA)]. Differential expressions, specific to tumor cells, were evaluated by comparisons of uninvolved pancreas (n = 9), EUS-FNA (n = 14), and macrodissected (tumor-cell-enriched) biopsies (n = 16). To determine whether treatment modulates gene expression, a unique (independent) set of synchronous EUS-FNA samples (n = 4) was obtained before, and 2 weeks after, chemoradiation. mRNA levels were evaluated using real-time quantitative polymerase chain reaction formatted in a Taqman low-density array, which was capable of simultaneously quantifying 46 independent genes in the HH pathway. Protein levels for Patched, Smoothened, and glioma-associated oncogene (Gli-1) in FPE tissues were determined using immunohistochemistry. A significant concordance ($p<0.0001$) was observed in HH-pathway mRNA levels between matched surgically resected (both snap-frozen and FPE) and EUS-FNA biopsies. HH-pathway mRNA levels changed (increased) only after macrodissection, suggesting localization to tumor cells. Immunohistochemical staining for Patched, Smoothened and Gli-1 confirmed the increased ($p<0.001$) levels of protein in the PAC cells, compared with cells from uninvolved pancreas. EUS-FNA biopsies that were obtained before and during chemoradiation demonstrated no significant changes in HH-pathway gene expres-

sion. Collectively, these studies demonstrate presence of HH-pathway expression in all clinical PAC biopsies examined, suggesting that this is a significant tumor-associated target and offering the possibility that specific molecular profiling might be attempted from these heterogeneous tissues.

Introduction

Pancreatic adenocarcinoma (PAC) is the fourth leading cause of cancer mortality in the United States and is characterized by an unusual resistance to radiation or chemotherapy. A death incidence ratio of approximately 0.99 makes PAC one of the deadliest malignancies.[1] Surgical resection remains the most effective treatment for PAC. However, at the time of diagnosis, the majority of patients present with advanced (unresectable) disease, which requires aggressive chemoradiotherapy (primarily 5-fluorouracial or gemcitabine, administered concurrently with 50 to 60 Gy radiation).[2-5] Despite the recent introduction of gemcitabine (which increased the median survival time by 4 mo), almost all these patients succumb to disease that is secondary to the local one, or to a distant recurrence.[6] Genetic analysis has been successfully employed in other cancers to enable the identification of specific targets[7, 8], individualized treatments[9, 10] and prognostic indicators.[11, 12] It can also elucidate the molecular mechanisms involved in tumor etiology.[13, 14] However, a similar analysis of PAC remains difficult, owing to the host desmoplastic stromal reaction and subsequent tumor heterogeneity.

The hedgehog (HH) pathway, originally identified as a potential target for treatment in basal cell carcinoma, has recently been implicated as a potential mediator of pancreatic carcinogenesis. This pathway is dominated by a family of highly regulated pro-

teins that direct development and cell proliferation. Binding of the secreted ligand (Sonic, Indian or Desert HH) to the Patched (Ptch) receptor reverses the inhibition of Ptch on Smoothened (Smo), a transmembrane protein. This results in a cascade, leading to the translocation of the active forms of Gli transcription factors [glioma-associated oncogenes (Gli-1, 2, 3)] to the nucleus, and the subsequent transcriptions of target genes such as *PTCH*, *epidermal-derived*, *platelet-derived*, and *vascular-endothelial-growth factors*, *cyclins B*, *D*, and *E*, and *GLI* itself.[15] In a recent study, ectopic expression of *Sonic HH* in the pancreatic endoderm of transgenic mice has been shown to result in the formation of pancreatic intraepithelial neoplasia (PanIN)-like lesions.[16] The histological progression of pancreatic neoplasia in these transgenic mice is accompanied by the induction of *HER-2/neu* expression and mutations of the proto-oncogene K-*ras* – genetic alterations that are frequently characterized as early events in PAC tumor etiology.[17-19] Immunohistochemical analysis of Sonic HH, Ptch, and Smo in paraffin-embedded PAC tissues suggests that expression of these HH-signaling components progressively increases from PanIN lesions to adenocarcinoma.[16] In addition, an examination of a panel of 26 human PAC cell lines revealed that all the lines expressed 2 or more components of HH signaling.[16, 20] In vitro and in vivo studies examining treatment with the HH-pathway antagonist, cyclopamine, have reported reduced cell proliferation, apoptosis. and cell cycle arrest.[16, 21, 22] Taken collectively, these studies suggest that the HH pathway is aberrantly expressed in PAC, that it might be involved in tumor development or progression, and that targeting this pathway may offer a unique therapeutic strategy.

In the current study, we examined the expression of 46 independent genes associated with the HH pathway, within a series of matched heterogeneous PAC biopsies that

included snap-frozen, formalin-fixed paraffin-embedded (FPE), and endoscopic ultrasound-guided fine-needle aspirate (EUS-FNA), using real-time quantitative polymerase chain reaction (PCR) formatted in a Taqman low-density array (TLDA). In addition, differential expression in tumor cells was evaluated by comparative analysis with uninvolved pancreas and macrodissected (tumor cell enriched) biopsies. Immunohistochemistry was used to evaluate the protein levels of Ptch, Smo, and Gli-1 to determine concordance with mRNA and in the distribution of HH signaling within PAC. In addition, an independent set of EUS-FNA samples obtained before, and 2 weeks after, chemoradiation was examined, to determine whether treatment alters gene expression. Collectively, these analyses suggest that the HH pathway is a significant tumor-associated target, that treatment with chemoradiation does not alter pathway expression, and that molecular profiling might be possible from these heterogeneous tissue biopsies.

Materials and Methods

Tissue Specimens

All studies using human tissues were approved by the Institutional Review Board at the University of Alabama at Birmingham (UAB, Birmingham, AL) and conducted in accordance with its policies. Before analysis, all tissues were histologically examined and classified according to TNM staging guidelines as uninvolved pancreas, stage IIA, IIB, or III PAC by a cytopathologist (W.E.G. and A.R.F.). This study was conducted using uninvolved pancreata (n = 9) and snap-frozen (n = 5), FPE (n = 16), and EUS-FNA (n = 14) PAC specimens obtained from the UAB Pancreatic Tumor SPORE Tissue Core Facility. The uninvolved pancreata are a unique population of samples that are unrelated

to any of the patients from whom PAC specimens were obtained and examined. Concordance of HH-pathway expression among the different types of clinical PAC biopsies (snap-frozen, FPE, and EUS-FNA) was evaluated using a series of matched samples that were obtained from 5 individual patients. In this study, snap-frozen, FPE, and EUS-FNA are singular descriptions; a specimen is never referred to, for example, as being both snap-frozen and EUS-FNA; the sample is either the one or the other. The effect of chemoradiation on HH-pathway gene expression was evaluated in a unique (independent) set of synchronous EUS-FNA samples (n = 4) that were collected from patients before, and 2 weeks after, the start of treatment, as part of a phase 1 clinical protocol.[23] For macrodissected samples, FPE blocks (n = 16) were cut into 10-μm sections and placed onto glass microscope slides. Tumor tissue was subsequently macrodissected away from the surrounding paraffin-embedded section and placed into a microcentrifuge tube; in which it was stored at room temperature before RNA isolation. These macrodissected FPE specimens are a unique population and were obtained from patients different from those who supplied the matched samples. Ten of these FPE samples were also processed for immunohistochemistry. Throughout the course of this study, a large portion of each of the FPE samples was used for macrodissection and subsequent RNA isolation. In 6 of these samples, the amount of paraffin-embedded tissue was diminished so much that immunohistochemistry could not be performed; hence only 10 samples were used.

Immunohistochemistry

Formalin-fixed, paraffin-embedded, 5-μm thick tissue sections were deparaffinized in xylene and rehydrated in progressively decreasing concentrations of ethanol.

Slides were placed in washing buffer (50 mM Tris-HCl, 150 mM NaCl, 0.01% Triton-X-100, pH 7.6) and subjected to immunostaining. After antigens were retrieved by boiling the tissue sections in 0.1 M Tris-HCl, pH 9.0; 10% Tween-20 (for Ptch, Smo), and 0.01 M sodium citrate, pH 6.0 (for Gli-1) for 10 minutes in a pressure cooker, sections were incubated in 3% hydrogen peroxide to quench endogenous peroxidases. This was followed by incubation in 1% goat serum for 1 hour at room temperature, to block nonspecific binding sites. Sections were incubated with a rabbit anti- Ptch polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:25, a rabbit anti-Smo polyclonal antibody (LifeSpan Biosciences, Seattle, WA) diluted 1:100, and a rabbit anti-Gli-1 polyclonal antibody (Abcam Inc., Cambridge, MA) diluted 1:100 or rabbit IgG Universal Negative Control (Dako Cytomation, Carpintera, CA) for 1 hour at room temperature. Secondary detection was accomplished with a streptavidin/horseradish peroxidase secondary-detection system (Signet Laboratories, Dedham, MA) and diaminobenzidine (BioGenex, San Ramon, CA). Harris hematoxylin was used as a counterstain.

The immunohistochemical stains were examined and scored by 2 of the authors (A.R.F. and A.S.). Stained pancreatic islets were used as positive internal control, owing to their intense staining for Ptch, Smo, and Gli-1. The intensity of immunostaining of individual cells was scored on a scale of 0 (no staining) to 4+ (strongest intensity), and the percentage of stained cells in each of these categories was estimated. The proportion of cells in each stain-intensity category was multiplied by the corresponding intensity value, and these products were added to obtain an immunostaining score.[24]

RNA Extraction

Total RNA was isolated from surgically resected specimens (snap-frozen uninvolved pancreas and PAC) and EUS-FNA biopsies, using Trizol reagent (Invitrogen, Carlsbad, CA) as per manufacturer's instructions. RNA was then DNase treated and purified, using the RNeasy Mini Kit (Qiagen, Hilden, Germany) as per manufacturer's instructions. Total RNA was eluted in 50 μL of RNase-free water and stored at -80°C. Paraffin tissue sections were deparaffinized by incubation with 800 μL of xylene and 400 μL of 100% ethanol. The samples were then centrifuged, and the supernatant was removed. Tissue pellets were washed with 1 mL of 100% ethanol, and then dried for 10 minutes at 55°C. Total RNA isolation was performed using the Roche High Pure RNA Paraffin Kit (Roche Diagnostics, Manheim, Germany) as per manufacturer's instructions. Total RNA was eluted in 30 μL of RNase-free water, and stored at -80°C. The concentration of all RNA samples was quantitated using the *ribosomal protein, large, P0* (*RPLP0*) housekeeping gene, and linear regression analysis of a standard curve derived from known concentrations of normal pancreas RNA, as previously described by our laboratory.[25, 26]

Reverse Transcription

Before cDNA synthesis, all RNA samples were diluted to 4 ng/μL using RNase-free water containing 12.5 ng/μL of total yeast RNA (Ambion, Austin, TX) as a carrier. cDNA was prepared using the High Capacity cDNA Archive Kit (Applied Biosystems,

Foster City, CA) as per manufacturer's instructions. The resulting cDNA samples were used immediately for TLDA analysis.

TLDA

For each card of the low-density array (Applied Biosystems), there are 8 separate loading ports that feed into 48 separate wells for a total of 384 wells per card. Each 2-µL well contains specific, user-defined primers and probes that are capable of detecting a single gene. In this study, the TLDA card was configured into 8 identical 48-gene sets. Genes were chosen on the basis of literature reviews of the HH-molecular pathway.[15, 27-29] Each set of 48 genes also contains 2 housekeeping genes, *RPLP0* and *18S*. All samples were loaded onto a TLDA card and PCR amplification was performed as previously described.[30]

TLDA Analysis

Expression values were calculated using the comparative C_T method as previously described.[26, 30] Briefly, this technique uses the formula $2^{-\Delta\Delta CT}$ to calculate the expression of target genes normalized to a calibrator. The threshold cycle (C_T) indicates the cycle number at which the amount of amplified target reaches a fixed threshold. C_T values range from 0 to 40 (the latter representing the default upper limit PCR cycle number that defines failure to detect a signal). Normal pancreas RNA (Ambion) was used as the calibrator [$\Delta\Delta C_T = \Delta C_T$ (tumor) - ΔC_T (normal)]. A range for each expression value was calculated, which was based on the standard deviation (s) of the $\Delta\Delta C_T$ value where $2^{-(\Delta\Delta C_T+s)}$ is the lower limit and $2^{-(\Delta\Delta C_T-s)}$ is the upper limit.

Statistical Analysis

All statistical analyses were conducted with SAS Ver. 9.1. To examine the correlation between matched snap-frozen, EUS-FNA, and FPE tissues and between EUS-FNA biopsies obtained before, and 2 weeks after, the start of chemoradiotherapy, ΔC_T values [$\Delta C_T = C_T$ (gene of interest) - C_T (*RPLP0*)] for each of the 46 genes examined were calculated in 5 matched snap-frozen, EUS-FNA, and FPE (heterogeneous and macrodissected) pancreatic-cancer tissue samples, and 4 matched prechemoradiation and postchemoradiation EUS-FNA PAC biopsies. These ΔC_T values were then compared using linear regression and Pearson correlation analysis to determine the agreement in gene expression. The Pearson correlation coefficient (*r*), in statistical terms, is a measure of the correlation of 2 variables, *x* and *y*, or a measure of the tendency of these variables to increase or decrease together. In this study, *x* and *y* represent ΔC_T values, which in turn are representative of gene expression. The linear equation that best describes the relationship or correlation between *x* and *y* can be found by linear regression. This equation, of the form $y = mx + b$, can then be used to predict the value of one variable, on the basis of the other known variable, where "m" is the slope and "b" is the *y*-intercept of the line derived from the linear regression analysis. The Pearson correlation coefficient only applies to a correlation of *x* and *y* if the relationship is significantly ($p<0.05$) linear [i.e., the slope (m) is significantly non-zero]. Significant differences in gene expression between uninvolved tissues and tumor tissue, immunostaining of pancreatic ducts and PAC, and prechemora-

diation and postchemoradiation were determined by student *t* test. Significance was defined as $p < 0.05$. The small sample size in this study affects the type II error rate and reduces power in such manner that not all the genes that are truly differentially expressed can be detected. A gene might be differentially expressed when using a significance value of 0.05 (the type I error rate); however, the statistical significance has been established and should not change with sample size.

Results

Correlation of HH Expression in Matched Snap-Frozen, FPE, EUS-FNA, and Macrodissected FPE Tissues

Initial studies examined HH-pathway expression in matched synchronous clinical PAC biopsies (snap-frozen, FPE, and EUS-FNA) obtained from 5 individual patients. FPE tissues were subsequently macrodissected and compared with matched samples to evaluate the effects of tumor heterogeneity. Linear regression and Pearson correlation analyses of the ΔC_T values for 46 genes involved in the HH pathway were carried out; they demonstrated a significant ($p < 0.0001$) correlation for matched snap-frozen and EUS-FNA tissues, and the Pearson correlation coefficients (r) were 0.93, 0.93, 0.93, 0.95, and 0.96, respectively (Fig. 1A). Similar results were obtained with significant correlations observed between matched snap-frozen and FPE samples ($r = 0.86, 0.93, 0.93, 0.94, 0.96$; $p < 0.0001$), and matched FPE and EUS-FNA samples ($r = 0.81, 0.86, 0.87, 0.89, 0.90$; $p < 0.0001$) (Figs. 1B, C). However, enrichment of tumor cells by macrodissection of FPE tissue significantly altered the expression profile and reduced the correlation ($r = 0.63, 0.67, 0.67, 0.70, 0.70$) (Fig. 1D). Collectively, analysis of snap-frozen, FPE and

EUS-FNA heterogeneous de-novo PAC tissues demonstrated a significant correlation in HH-pathway gene expression (shown in Figs. 1A-C in a single patient); similar results obtained for all matched patient samples. Macrodissection of FPE tissues (with enrichment of tumor cell population) significantly altered HH expression, compared with matched heterogeneous FPE biopsies (shown in Fig. 1D). Similar results were obtained by comparative analysis of macrodissected FPE tissues with all the matched heterogeneous snap-frozen and EUS-FNA biopsies (data not shown).

Increased Expression of the HH Pathway in Cancer Cell Enriched PAC

To determine differential expression specific to tumor cells, 46 genes in the HH pathway were evaluated by comparison with those from 9 uninvolved pancreatic tissues, 14 heterogeneous EUS-FNA biopsies, and 16 macrodissected (cancer cell enriched) FPE tissues (Table 1). Differential expression analysis of tissues from uninvolved pancreas and heterogeneous EUS-FNA biopsies demonstrated a significant increase in HH ligands (*Sonic* and *Indian*) and downstream targets [*E2F1* and cyclin B1 *CCNB1*)]. A significant decrease was observed in HH-signaling components (*PTCH*, *SMO*, *GLI3*) and downstream targets [epidermal growth factor (*EGF*), platelet-derived growth factor receptor alpha (*PDGFRA*), cyclin D2 (*CCND2*)]. Enrichment of cancer-cell population by macrodissection altered and increased (2.9-fold) the number of differentially expressed genes (compared with uninvolved pancreas) from 10 to 29. Nine of the 10 genes (*Sonic HH*, *Indian HH*, *PTCH*, *SMO*, *GLI3*, *E2F1*, *EGF*, *CCNB1*, and *PDGFRA*) identified in EUS-FNA biopsies were also significantly different in macro-dissected FPE tissues. Five of these 9 genes showed the same statistical trends (increased *Sonic HH*, *Indian HH*, *E2F1*,

CCNB1, and decreased *EGF*) in tumor compared to uninvolved pancreas. The other 4 genes (*PTCH*, *SMO*, *GLI3*, and *PDGFRA*) showed decreased expression in EUS-FNA biopsies and increased expression in macrodissected FPE tissues, demonstrating the significant impact of tissue heterogeneity on gene-expression analysis.

Immunohistochemical Analysis of FPE Tissues

A total of 10 FPE samples were randomly selected from the 16 samples shown in Table 1 for immunohistochemistry. Cytopathological examination of FPE tissues used in this study included both PAC and adjacent uninvolved pancreatic tissues. Subsequent immunohistochemical analysis used these matched (same patient, tumor and uninvolved) samples to evaluate the differential protein expressions of Ptch, Smo, and Gli-1. Weak to negative staining was observed in the normal ducts of all 10 cases for Ptch, Smo, and Gli-1 (Figs. 2A-C). Conversely, in all 10 cases, adjacent PAC demonstrated higher staining for Ptch, Smo, and Gli-1 (Figs. 2D-F). Comparisons of the average immunoscores for Ptch, Smo, and Gli-1 in all 10 FPE tissues revealed a significant ($p<0.0005$) increase in all 3 proteins in PAC, compared with pancreatic ducts (Fig. 3).

Effects of Chemoradiation on HH Gene Expression

EUS-FNA PAC biopsies were collected from patients before, and 2 weeks after, the start of chemoradiotherapy (concurrent administration of capecitabine and radiation), as part of a phase 1 clinical trial.[23] The effect of chemoradiation on HH pathway expression was evaluated by a comparison of the genetic profiles in matched patient samples (n = 4), before and after treatment. Linear regression and Pearson correlation analysis of

ΔC_T values for 46 genes associated with the HH pathway, in prechemoradiotherapy and postchemoradiotherapy EUS-FNA biopsies, revealed a significant concordance ($p<0.0001$) in their gene expressions, as evidenced by the Pearson correlation coefficients (r) of 0.89, 0.91, 0.93, and 0.94, respectively (Fig. 4 representative of a single patient). Statistical analysis of each individual gene demonstrated no difference ($p>0.05$) before and after chemoradiation. In addition, no correlation was identified between clinical response to the fluoro-pyrimidine anti-metabolite capecitabine and HH-pathway expression. These data suggest that, within the first 2 weeks, none of the 46 genes associated with the HH pathway is significantly altered as a result of chemoradiotherapy in PAC.

Discussion

The recent introduction of technologies capable of quantifying the expression profiles of specific pathways (TLDA) in available clinical samples (including snap-frozen, archival paraffin-embedded, or EUS-FNA) has provided researchers with an opportunity to examine the effects of tissue heterogeneity on gene expression and to determine the changes in expression secondary to treatment. Initial studies demonstrated a concordance in the molecular profiles of matched (same patient) snap-frozen, archival FPE, and diagnostic EUS-FNA PAC biopsies (Figs. 1A-C). The ability to quantitatively perform multivariate gene-expression analysis in FPE PAC tissue (the most widely available source of clinical samples at most treatment research facilities) represents a significant advance in the characterization of archival tissue samples. Ultimately, the analysis of FPE tissues may be most useful in determining dynamic gene-expression ranges (within a population) and/or for identifying correlations with clinical parameters (as available from most coop-

erative groups). Although needle biopsies are increasingly being used for the diagnosis of PAC,[31, 32] this study represents the first molecular-pathway analysis that includes matched EUS-FNA biopsies. The similarity or concordance in HH gene expression profiles between EUS-FNA biopsies and surgically resected tissues suggests that, although needle biopsies could be used as a reliable tissue source for molecular analyses, using one type of tissue collection over the other for genetic analyses seems to have no benefit. However, the ability to examine EUS-FNA biopsies will ultimately increase the number of PAC specimens for molecular analysis, because only 10 to 15% of the patients diagnosed with PAC are candidates for surgical resection.[2] Examination of molecular profiles in EUS-FNA biopsies will enable future studies to include patients presenting with more advanced, unresectable disease. In addition, as demonstrated in this study, EUS-FNA biopsies can be used for multiple sampling from a single patient to determine the modulation of gene expression with treatment.

Tissue heterogeneity represents a major obstacle in the molecular analysis of clinical PAC specimens and has been observed during the histological examination of the FPE tissues used in this study. Differential expression in tumor cells was evaluated by comparative analysis of matched heterogeneous and macrodissected (tumor cell enriched) FPE samples (Fig. 1D). Results demonstrated differential genetic profiles after macrodissection; this led to extensive studies evaluating HH-pathway expression in tissues from uninvolved pancreas, from heterogeneous samples represented by EUS-FNA biopsies, and from macrodissected FPE tissues (Table 1). Uninvolved pancreatic tissues were included to discriminate between tumor-specific genes and those that are expressed in uninvolved tissue, a prerequisite to the future development of prognostic, diagnostic or

targeted anti-HH therapies. Comparisons of uninvolved pancreas and EUS-FNA PAC biopsies led to the identification of 10 significantly ($p<0.05$) different genes (Table 1, bolded). The increased expression of the *Sonic* and *Indian HH* ligands in EUS-FNA biopsies is in concordance with previous studies that demonstrated that the HH pathway is active in PAC.[16, 20, 22] However, other markers associated with HH signaling (e.g., *PTCH*, *SMO*, *GLI1*) were decreased. In contrast to heterogeneous EUS-FNA biopsies, macrodissected FPE samples demonstrated a 2.9-fold increase in the total number of genes (from 10 to 29) that were differentially expressed ($p<0.05$) over those from uninvolved pancreas (Table 1, bolded). The mean expression of *Sonic* and *Indian HH* ligands as well as the HH-signaling components *PTCH*, *SMO*, and *GLI1* became significantly higher in the cancer cell-enriched samples, suggesting that HH signaling may be localized within cancer cells.

Protein levels of Ptch, Smo, and Gli-1 were evaluated by immunohistochemistry in FPE tissues to determine the distribution of HH signaling in PAC. Higher staining of the HH-signaling components, Ptch, Smo, and downstream transcription factor Gli-1, was observed in the malignant ductal epithelial cells relative to the matching normal pancreatic ductal cells (Fig. 2). Protein expression remained localized to the ductal PAC cells and did not extend into the surrounding stroma, which is an abundant component of this heterogeneous cancer. [33] Immunoscore analysis of all 10 FPE samples revealed a statistically significant increase in the protein expression of Ptch, Smo, and Gli-1 (Fig. 3). These data support the molecular analysis obtained from macrodissected samples (Table 1), suggesting the compartmentalization of HH-pathway activity in PAC tumor cells; hence, they offer the possibility that anti-HH therapy (e.g., cyclopamine and other Smo

antagonists) can specifically target neoplastic cells that contain an active HH pathway, with low toxicity to the surrounding uninvolved tissues.

The concordance in gene expression between matched heterogeneous PAC specimens, including EUS-FNA biopsies, offers the exciting possibility that multiple sampling of fine-needle biopsies can be obtained from a single patient to determine the modulation of gene expression with treatment. To identify the changes in HH-pathway expression as a result of chemoradiation, a comparison of EUS-FNA PAC biopsies before, and 2 weeks after, the start of chemoradiotherapy (concurrent administration of capecitabine and radiation[23]) was performed. A significant concordance in gene expression was observed in all 4 matched-patient samples (representative plot shown in Fig. 4); interestingly, a statistical analysis of each of the 46 genes examined confirmed that there were no significant changes in expression between prechemoradiotherapy and 2 weeks postchemoradiotherapy. Similar results were observed by our laboratory in pancreatic cancer cell lines that had been irradiated (4 Gy); their HH-pathway expressions were analyzed 12, 24, 48, 72, and 96 hours after radiation (data not shown). These data suggest that the majority of the HH-pathway elements including ligands, signaling components, and downstream targets are not altered by chemoradiotherapy in PAC. However, the small sample size used for this analysis prevents us from making a definitive conclusion as to whether chemoradiation affects HH-pathway expression. Little has been reported on this aspect of the HH pathway; however, a recent study performed on clinical esophageal-tumor specimens attributes tumor regrowth after chemoradiotherapy to increases in activity of Sonic HH and Gli-1.[34] The changes in HH-pathway expression observed in esophageal cancer and the lack of HH-pathway alteration in PAC could be due to differ-

ences in timing, chemotherapy, and cancer type. Additional studies to elucidate the effects of chemoradiation on the HH pathway in PAC are warranted.

One of the primary goals in the development of rational treatment paradigms is the identification of tumor-associated pathways that can be selectively targeted in cancer cells. Understanding the molecular bases of response and identifying markers capable of directing patients toward these more effective treatments also remains a central effort in current pharmacogenomic studies. Although some success has been achieved (e.g., Bcr-abl tyrosine kinase inhibition by imatinib mesylae or blocking Her-2/Neu by trastuzumab), these strategies have had little impact on the treatment of patients diagnosed with PAC. Incremental progress (such as the introduction of gemcitabine) measures increases in patient survival in months instead of years. In addition, molecular analyses of PAC biopsies have been hampered by tissue heterogeneity and low tumor cellularity. In the current study, we utilize a comparative molecular and protein analysis of all available types of clinical PAC specimens, including uninvolved pancreas, snap-frozen, FPE, EUS-FNA tissue, and macrodissected FPE tissue. Taken together, these data demonstrate concordance in the molecular profiles of heterogeneous surgically resected (both snap-frozen and FPE) and EUS-FNA PAC biopsies. Both macrodissection and immunohistochemistry demonstrate localization of the HH pathway to tumor cells; this finding suggests that the enrichment of cancer-cell population might be necessary to accurately determine the expression of some components of the HH-signaling pathway (*PTCH*, *SMO*, and *GLI3*). The ability to examine EUS-FNA biopsies ultimately increases the number of available samples for molecular analysis, and enables multiple sampling from single patients to determine changes in gene expression after treatment. Collectively, these studies demon-

strate an overexpression of the HH pathway in all the clinical PAC biopsies examined, with localization of the HH pathway to tumor cells. In addition to suggesting a potential tumor-associated target, these analyses also offer the possibility of using molecular profiling to identify patients with PAC, who can, thus, benefit from specifically targeted anti-HH therapy.

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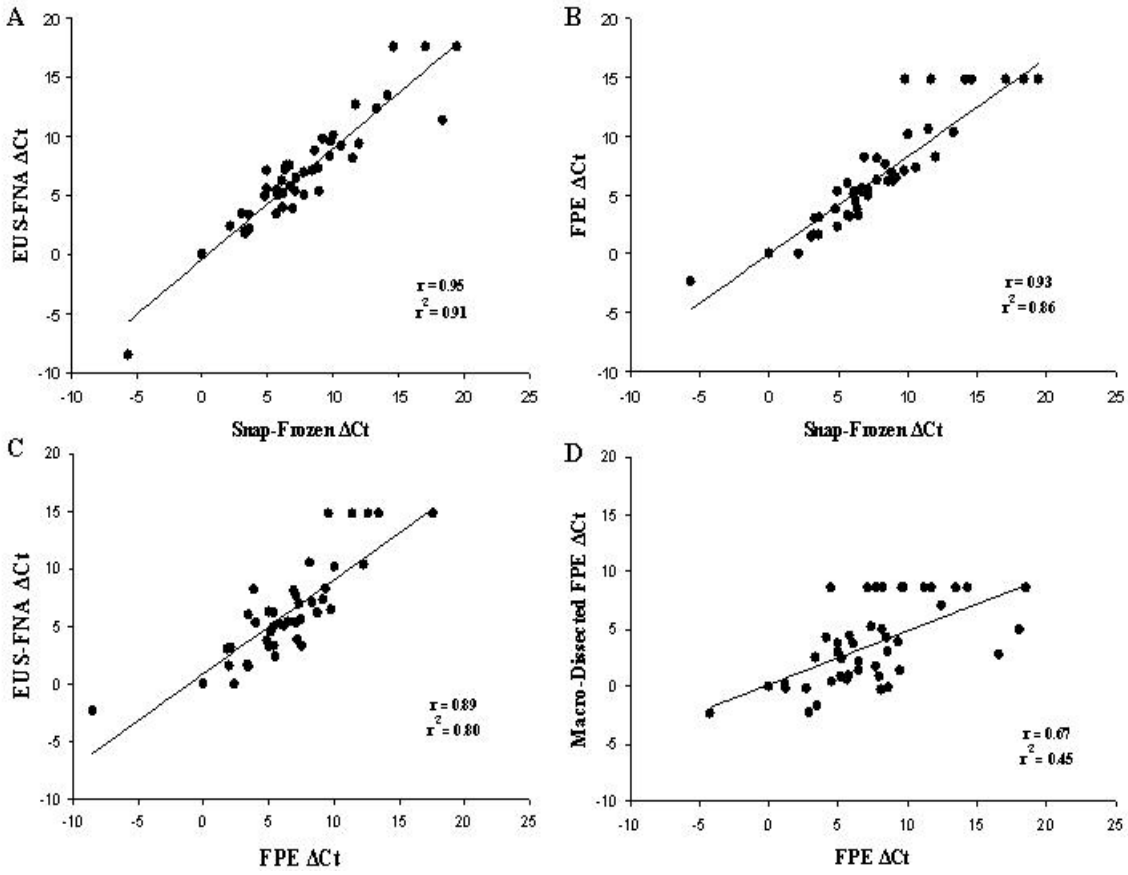

Figure 1. Comparison of 46 genes associated with the HH pathway in matched snap-frozen, EUS-FNA, and FPE PAC tissues. Correlative plots depicting ΔC_T values obtained from matching snap-frozen and EUS-FNA tissue (A), matching snap-frozen and FPE tissue (B), and matching FPE and EUS-FNA tissue (C) demonstrate statistical concordance ($p<0.0001$) in HH gene expression. Upon macrodissection of FPE tissue, the genetic profile is altered and the correlation is reduced (D). These data show representative correlative plots from a single patient; similar results were obtained for all other matched samples.

Table 1

Hedgehog Pathway Gene Expression in Uninvolved Pancreas and PAC

Gene Name (Symbol)	Uninvolved Pancreas (n = 9)*	EUS-FNA Tumor Biopsy (n = 14)*	Fold Change†	*P* value‡	Macro-Dissected FPE Tumor (n = 16)*	Fold Change†	*P* value‡
HH Ligands							
Sonic Hedgehog (SHH)	6.94 (0.61 - 79.35)	**468.38 (138.07 - 1588.95)**	**67.44 ↑**	**0.0006**	**515.67 (174.18 - 1526.70)**	**74.25 ↑**	**0.0005**
Indian Hedgehog (IHH)	151.65 (30.52 - 753.69)	**3677.89 (865.39 - 15630.90)**	**24.25 ↑**	**0.0001**	**549.04 (164.08 - 1837.14)**	**3.62 ↑**	**0.0327**
Desert Hedgehog (DHH)	4.22 (0.51 - 34.79)	3.13 (0.86 - 11.30)	1.35 ↓	0.6750	**30.62 (8.28 - 113.25)**	**7.26 ↑**	**0.0078**
HH Signaling Components							
Patched (PTCH)	6.87 (2.31 - 20.39)	**2.39 (1.02 - 5.62)**	**3.22 ↓**	**0.0137**	**44.53 (19.95 - 99.41)**	**6.48 ↑**	**0.0001**
Smoothened (SMO)	2.41 (0.61 - 9.47)	**0.47 (0.15 - 1.42)**	**5.15 ↓**	**0.0048**	**39.41 (16.05 - 96.75)**	**16.35 ↑**	**0.0000**
Glioma-associated oncogene 3 (GLI3)	42.63 (12.46 - 145.82)	**9.85 (3.90 - 24.91)**	**4.33 ↓**	**0.0038**	**303.17 (155.45 - 591.26)**	**7.11 ↑**	**0.0011**
Patched 2 (PTCH2)	38.53 (7.65 - 193.97)	12.93 (3.24 - 51.59)	2.98 ↓	0.0982	**678.39 (270.03 - 1704.29)**	**17.61 ↑**	**0.0005**
Glioma-associated oncogene 1 (GLI1)	42.27 (9.74 - 183.50)	18.76 (5.77 - 61.01)	2.25 ↓	0.1575	**787.01 (380.48 - 1627.90)**	**18.62 ↑**	**0.0002**
Glioma-associated oncogene 2 (GLI2)	7.04 (2.12 - 23.33)	7.55 (2.97 - 19.22)	1.07 ↑	0.8759	**79.91 (33.70 - 189.49)**	**11.35 ↑**	**0.0000**
Costal-2 homolog (KIF27)	20.83 (4.74 - 91.53)	16.22 (7.64 - 34.44)	1.28 ↓	0.6482	**0.53 (0.08 - 3.46)**	**39.35 ↓**	**0.0000**
Glycogen synthase kinase 3-beta (GSK3B)	14.01 (5.42 - 36.23)	21.67 (10.93 - 42.95)	1.55 ↑	0.2137	**51.75 (24.19 - 110.73)**	**3.69 ↑**	**0.0010**
Megalin (LRP2)	109.76 (11.26 - 1069.86)	394.16 (37.94 - 4094.95)	3.59 ↑	0.2105	**579.70 (108.54 - 3096.15)**	**5.28 ↑**	**0.0474**
Dispatched homolog 2 (DISP2)	3.31 (0.44 - 24.97)	1.88 (0.59 - 6.02)	1.76 ↓	0.4611	**19.78 (2.45 - 159.36)**	**5.97 ↑**	**0.0491**
Exostoses 1 (EXT1)	26.22 (7.50 - 91.61)	18.96 (11.03 - 32.59)	1.38 ↓	0.4799	51.22 (27.94 - 93.92)	1.95 ↑	0.1617
Exostoses 2 (EXT2)	7.65 (1.86 - 31.39)	6.39 (3.92 - 10.41)	1.20 ↓	0.7209	15.25 (8.30 - 28.02)	1.99 ↑	0.1941
Suppressor of Fused homolog (SUFU)	25.07 (5.72 - 109.84)	16.98 (9.38 - 30.73)	1.48↓	0.4691	45.55 (23.35 - 88.86)	1.82 ↑	0.2780
F-box and WD-40 domain protein 7 (FBXW7)	16.79 (4.23 - 66.63)	6.12 (3.55 - 10.53)	2.74 ↓	0.0638	25.43 (14.53 - 44.52)	1.52 ↑	0.4084
Dispatched homolog 1 (DISP1)	4.85 (1.63 - 14.39)	6.47 (3.46 - 12.09)	1.33 ↑	0.4841	3.18 (0.68 - 14.87)	1.52 ↓	0.4787
Hedgehog acetyl transferase (HHAT)	12.34 (4.30 - 35.36)	16.73 (7.87 - 35.60)	1.36 ↑	0.4269	9.22 (2.36 - 36.02)	1.34 ↓	0.5852
Fused homolog (STK36)	29.17 (4.06 - 209.40)	10.88 (4.33 - 27.33)	2.68 ↓	0.1892	40.52 (17.59 - 93.30)	1.39 ↑	0.6442
Hedgehog interacting protein (HHIP)	4.43 (1.04 - 18.86)	1.76 (0.70 - 4.43)	2.51 ↓	0.1149	3.52 (0.71 - 17.53)	1.26 ↓	0.7262

*Values are expressed as the mean of tissues examined (range in expression)

†Relative to uninvolved pancreas

‡$p<0.05$ was considered statistically significant; Calculated using Student's t-test

Bolded text indicates significant differences in gene expression

Table 1 (Continued)

Hedgehog Pathway Gene Expression in Uninvolved Pancreas and PAC

Gene Name (Symbol)	Uninvolved Pancreas (n = 9)*	EUS-FNA Tumor Biopsy (n = 14)*	Fold Change†	*P* value‡	Macro-Dissected FPE Tumor (n = 16)*	Fold Change†	*P* value‡
HH Downstream targets							
E2F1 transcription factor (E2F1)	18.12 (6.88 - 47.75)	**50.94 (20.89 - 124.22)**	**2.81 ↑**	**0.0158**	**103.16 (17.22 - 618.15)**	**5.69 ↑**	**0.0045**
Epidermal growth factor ligand (EGF)	17.00 (3.91 - 73.98)	**1.07 (0.18 - 6.48)**	**15.87 ↓**	**0.0009**	**0.59 (0.08 - 4.19)**	**28.84 ↓**	**0.0002**
Cyclin B1 (CCNB1)	8.30 (2.62 - 26.27)	**48.47 (23.84 - 98.56)**	**5.84 ↑**	**0.0002**	**133.34 (55.30 - 321.49)**	**16.07 ↑**	**0.0000**
Platelet-derived growth factor receptor alpha (PDGFRA)	7.31 (3.55 - 15.07)	**2.37 (1.38 - 4.10)**	**3.08 ↓**	**0.0004**	**26.76 (14.72 - 48.67)**	**3.66 ↑**	**0.0001**
Cyclin D2 (CCND2)	31.77 (5.96 - 169.50)	**2.83 (1.21 - 6.62)**	**11.22 ↓**	**0.0022**	8.95 (4.15 - 19.32)	3.55 ↓	0.0576
K-ras 2 oncogene (KRAS2)	0.49 (0.17 - 1.36)	0.82 (0.46 - 1.46)	1.68 ↑	0.1928	**8.42 (2.70 - 26.29)**	**17.32 ↑**	**0.0000**
Transforming growth factor beta-1 (TGFB1)	10.40 (3.52 - 30.67)	12.42 (7.70 - 20.05)	1.19 ↑	0.8729	**82.76 (37.04 - 184.89)**	**7.96 ↑**	**0.0000**
Snail homolog 2 (SNAI2)	4.36 (1.42 - 13.43)	4.09 (1.40 - 11.95)	1.07 ↓	0.8930	**37.35 (14.85 - 93.96)**	**8.56 ↑**	**0.0000**
Snail homolog 1 (SNAI1)	13.67 (2.68 - 69.81)	5.42 (1.86 - 15.78)	2.52 ↓	0.1136	**288.79 (121.93 - 684.00)**	**21.13 ↑**	**0.0003**
Cyclin E2 (CCNE2)	3.07 (0.71 - 13.39)	5.41 (2.51 - 11.66)	1.76 ↑	0.3103	**23.85 (7.30 - 77.84)**	**7.76 ↑**	**0.0009**
Rab 23 homolog (RAB23)	19.32 (6.90 - 54.08)	13.79 (6.38 - 29.80)	1.40 ↓	0.3795	**54.78 (27.36 - 109.68)**	**2.84 ↑**	**0.0060**
Transforming growth factor alpha (TGFA)	10.22 (2.68 - 38.99)	18.46 (9.80 - 34.76)	1.81 ↑	0.2430	**36.66 (15.63 - 85.97)**	**3.59 ↑**	**0.0076**
p21, Cip1 (CDKNA1)	8.08 (1.92 - 33.94)	5.39 (3.17 - 9.16)	1.50 ↓	0.4366	**39.48 (17.23 - 90.44)**	**4.89 ↑**	**0.0111**
Engrailed homolog 2 (EN2)	0.71 (0.08 - 6.18)	2.30 (0.09 - 59.37)	3.25 ↑	0.3499	**10.89 (0.71 - 166.86)**	**15.44 ↑**	**0.0169**
Beta-Catenin (CTNNB1)	13.07 (5.62 - 30.41)	12.42 (7.70 - 20.05)	1.05 ↓	0.8729	**26.44 (15.10 - 46.29)**	**2.02 ↑**	**0.0194**
v-Myc oncogene (MYC)	20.28 (4.72 - 87.09)	10.53 (6.10 - 18.18)	1.93 ↓	0.2271	**6.11 (3.20 - 11.67)**	**3.32 ↓**	**0.0416**
Cyclin E1 (CCNE1)	3.43 (0.69 - 17.04)	9.72 (3.30 - 28.64)	2.83 ↑	0.0759	**15.01 (2.80 - 80.59)**	**4.37 ↑**	**0.0431**
Cyclin D3 (CCND3)	7.58 (2.06 - 27.85)	16.34 (9.38 - 28.45)	2.16 ↑	0.1251	13.17 (6.81 - 25.46)	1.74 ↑	0.2603
Engrailed homolog 1 (EN1)	15.59 (0.85 - 284.18)	5.13 (0.50 - 52.69)	3.04 ↓	0.3218	47.15 (3.76 - 591.54)	3.03 ↑	0.3292
Cyclin D1 (CCND1)	22.89 (5.88 - 89.09)	13.26 (7.14 - 24.64)	1.73 ↓	0.2836	13.99 (7.04 - 27.79)	1.64 ↓	0.3325
Bcl-2 oncogene (BCL2)	13.50 (3.37 - 54.14)	25.86 (7.42 - 90.19)	1.92 ↑	0.2566	8.17 (2.46 - 27.11)	1.65 ↓	0.3518
Notch homolog 1 (NOTCH1)	7.02 (1.55 - 31.72)	5.56 (2.38 - 13.00)	1.26 ↓	0.6816	11.12 (5.65 - 21.91)	1.58 ↑	0.4061
Notch homolog 2 (NOTCH2)	17.20 (4.99 - 59.32)	7.66 (3.99 - 14.73)	2.24 ↓	0.0989	24.63 (12.31 - 49.28)	1.43 ↑	0.4396
Retinoblastoma 1 (RB1)	7.70 (3.06 - 19.41)	12.53 (8.06 - 19.47)	1.63 ↑	0.1698	5.68 (1.02 - 31.62)	1.36 ↓	0.5692
Epidermal growth factor receptor (EGFR)	23.44 (6.46 - 85.05)	11.86 (5.97 - 23.55)	1.98 ↓	0.1726	27.28 (14.14 - 52.64)	1.16 ↑	0.7481

*Values are expressed as the mean of tissues examined (range in expression)

†Relative to uninvolved pancreas

‡$p<0.05$ was considered statistically significant; Calculated using Student's t-test

Bolded text indicates significant differences in gene expression

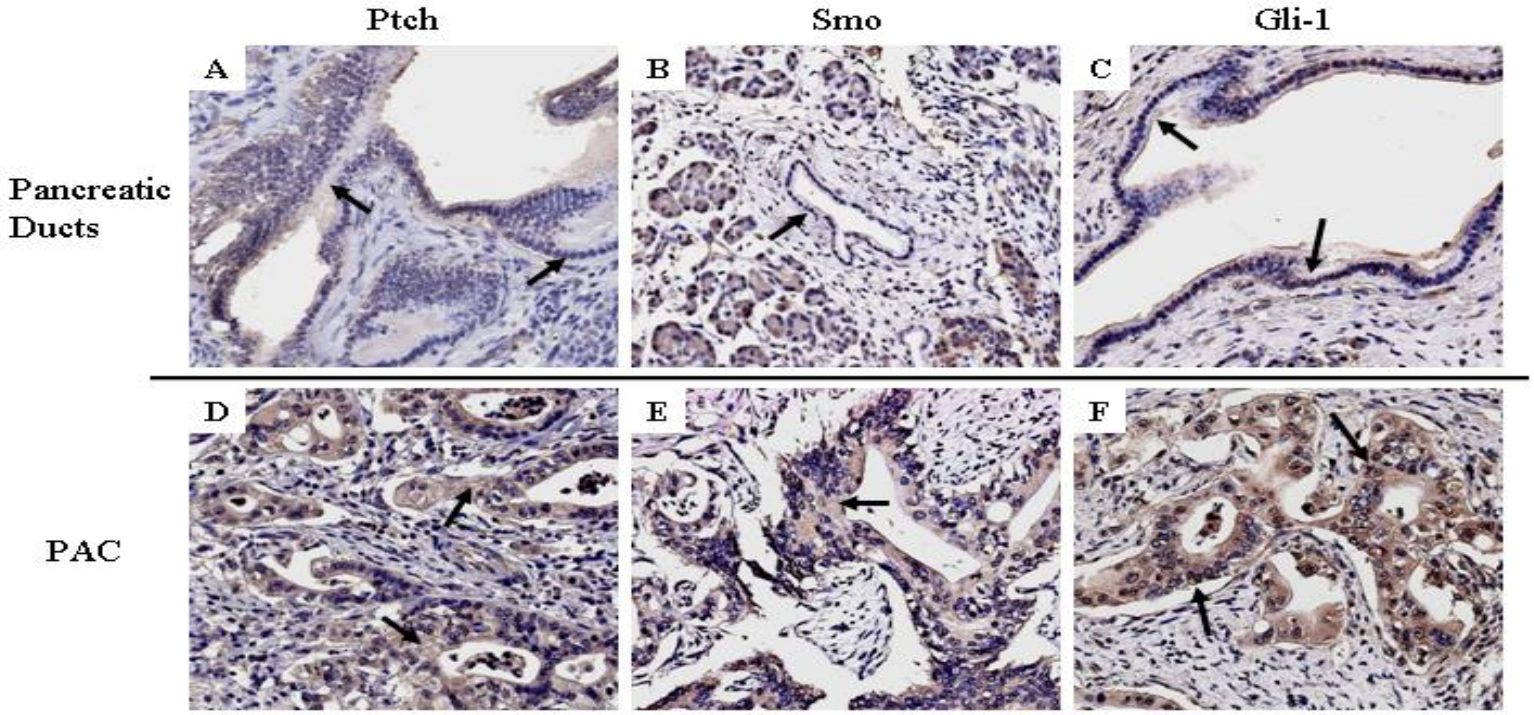

Figure 2. Immunohistochemical staining of FPE pancreatic tissues. Pancreatic ducts (A-C) and PAC (D-F) were all stained for the presence of Ptch, Smo, and Gli-1 proteins. All tissues are magnified x 200. Black arrows highlight the differences in staining between normal and cancerous ductal epithelium.

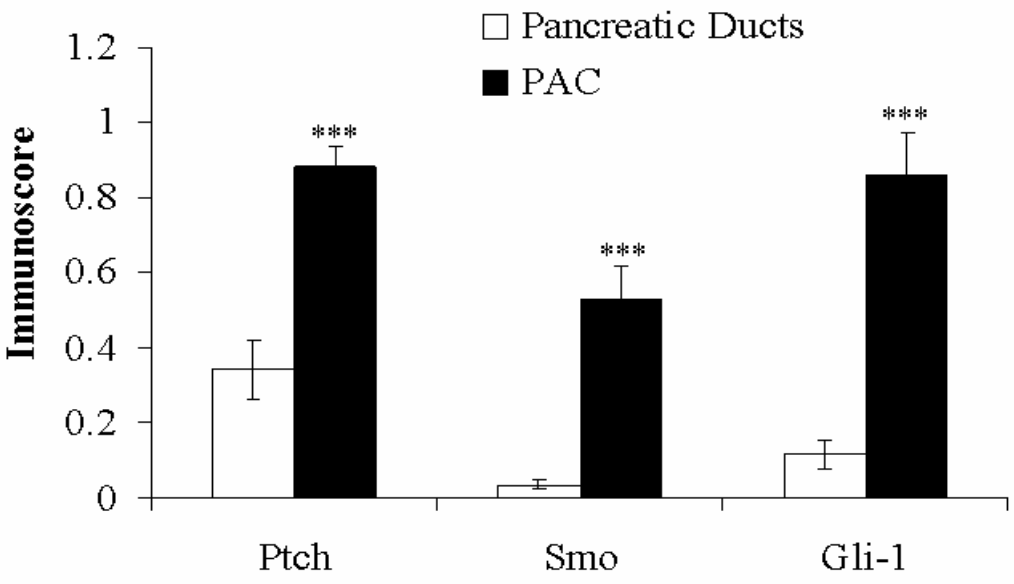

Figure 3. Average immunoscores for Ptch, Smo, and Gli-1 in 10 cases of FPE pancreatic tissues. Error bars represent standard error of the mean. Triple asterisks, $p<0.001$; calculated using paired student *t* test.

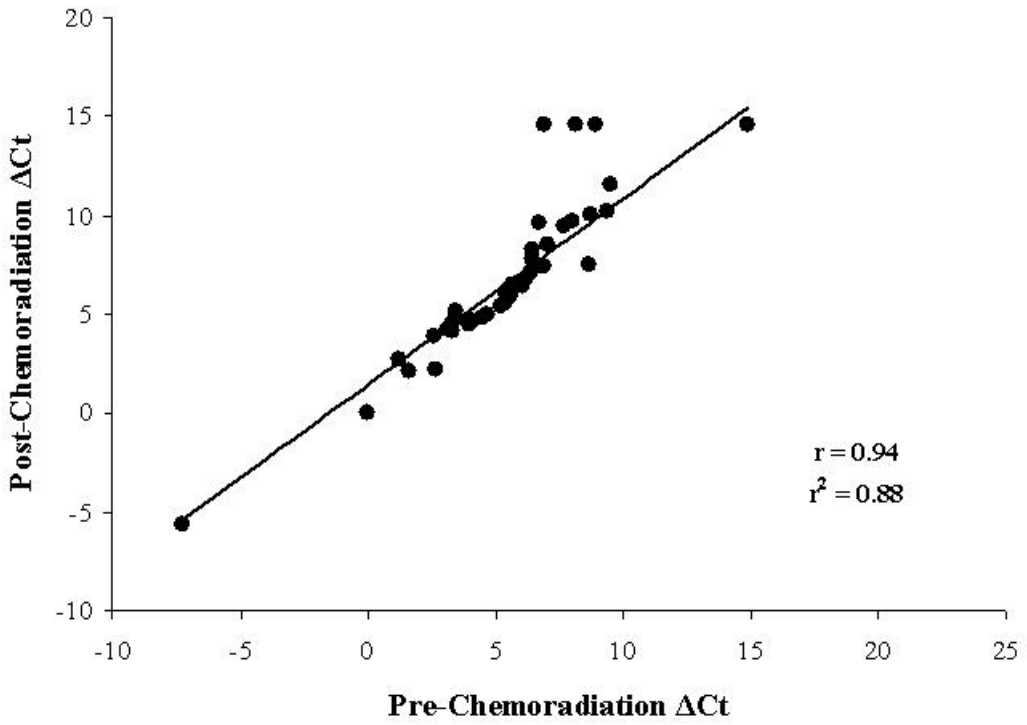

Figure 4. Comparison of 46 genes associated with the HH pathway in EUS-FNA biopsies, before and during chemoradiotherapy. A correlative plot depicting ΔC_T values obtained before, (*x*) and 2 weeks after (*y*), the start of chemoradiation demonstrated statistical concordance ($p<0.0001$) in HH gene expression. These data show a representative correlative plot from a single patient; similar results were obtained for all other patient samples.

IDENTIFICATION OF MOLECULAR DETERMINANTS OF IN VITRO RESPONSE TO CYCLOPAMINE IN PANCREATIC ADENOCARCINOMA CELL LINES

by

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Format adapted for dissertation

Abstract

Activation of the hedgehog (HH) pathway appears to play a critical role in the development and continued growth of pancreatic adenocarcinoma (PAC). Cyclopamine, a HH pathway inhibitor, has been shown to suppress cancer cell proliferation both *in vitro* and *in vivo*. However, the molecular basis of response to HH pathway inhibition in pancreatic cancer cells remains largely unexplored. The primary goals of this study were (1) to determine the relationship between HH pathway gene expression levels (prior to treatment) and response to cyclopamine in human pancreatic cancer cell lines, (2) to modulate the expression of these genes and determine how altered expression affects *in vitro* response to cyclopamine and (3) to examine the physiological and molecular changes that result from HH pathway inhibition in pancreatic cancer cells. Differential response (IC_{50} values) to cyclopamine was determined in a panel of 11 pancreatic cancer cell lines. Changes in DNA synthesis and apoptosis in cells treated with cyclopamine were examined by bromo-deoxyuridine (BrdU) incorporation and caspase activation, respectively. A statistical concordance between the expression of 46 genes associated with the HH pathway (including ligands, receptors, intracellular components, transcription factors and transcriptional targets) in each cell line (prior to treatment) and IC_{50} values was performed to identify genes associated with response. The expression of these genes was modulated *in vitro* (using siRNA) and subsequent changes in response to cyclopamine were examined. Changes in the expression of genes associated with the HH pathway after cyclopamine treatment were quantified using Taqman low-density array. Cyclopamine IC_{50} values ranged from 8.79 to >30 μM. Administered at its IC_{50} concentration, cyclopamine reduced BrdU incorporation by 83% in sensitive HPAF-2 cells (IC_{50} of 8

μM) compared to 50% in Panc-1 cells (IC_{50} of 30 μM). Treatment with low doses of cyclopamine (8 μM) resulted in cleavage of initiator and executioner caspases in sensitive HPAF-2 cells but not in resistant Panc-1 cells. *Smoothened* (*SMO*), *Glioma-associated oncogene 3* (*GLI3*), *Engrailed-1* (*EN1*) and *Glycogen synthase kinase-3 beta* (*GSK3B*) mRNA levels significantly ($p<0.05$) correlated with cyclopamine IC_{50} values. Knockdown of *SMO*, *EN1* and *GSK3B* mRNA levels did not alter *in vitro* response to cyclopamine. However, knockdown of *GLI3* mRNA levels significantly increased sensitivity. Treatment of pancreatic cancer cell lines with cyclopamine significantly altered the expression of genes involved in cell cycle progression (decreased mRNA levels of *cyclins B1*, *D1*, *D2*, *D3*, *E1* and *E2* and increased mRNA levels of *CDKN1A*/*Cip1*/*p21*). Collectively, these data suggest that Gli-3 appears to be a previously unknown indicator of *in vitro* response to cyclopamine and that the molecular basis of response to this compound could, at least in part, involve antagonism of the cell cycle and induction of apoptosis.

Introduction

Pancreatic adenocarcinoma (PAC) is the fourth leading cause of cancer mortality in the United States and is characterized by an unusual resistance to radiation or chemotherapy. Surgical resection remains the most effective treatment for PAC. However, at the time of diagnosis, up to 90% of patients present with advanced (unresectable) disease (1). In addition, the 5-year survival rate for patients who are able to undergo surgical resection is only 20% due to local secondary disease or distant metastases. Chemotherapy agents such as 5-fluorouracil (5-FU) and, more recently, gemcitabine combined with radiation (50 to 60 Gy) is the current treatment for locally advanced PAC (2-5). Despite

these highly aggressive therapeutic approaches, the median survival time (6-10 months) for patients with this disease has not appreciably changed in the last 80 years (2, 3, 6). These dismal statistics indicate a dire need for the development of novel therapeutic targets and treatment strategies. Genetic analysis has been successfully employed in other cancers to enable the identification of specific targets (7, 8), individualized treatments (9, 10), prognostic indicators (11, 12) and the molecular mechanisms involved in tumor etiology (13, 14). However, achieving these results in PAC continues to remain elusive because of the difficulty in analyzing a cancer that is often a heterogeneous mixture of several cell types (15-17).

Recent studies suggest that uncontrolled activation of the HH signaling pathway is a potential mediator of pancreatic carcinogenesis and sustained tumor growth (18, 19). This pathway, a critical component of embryogenesis, is comprised of a family of highly regulated proteins that direct cell development and proliferation. Binding of the secreted ligand (Sonic, Indian or Desert HH) to the Patched (Ptch) receptor reverses the inhibition of Ptch on Smoothened (Smo), a transmembrane protein. This results in a signaling cascade, leading to the translocation of the active forms of glioma-associated oncogene (Gli) transcription factors (Gli-1, 2 and 3) to the nucleus and the subsequent transcription of target genes such as *PTCH*, *epidermal-*, *platelet-derived-* and *vascular-endothelial-growth factors*, *cyclins B*, *D* and *E* and *GLI* itself (20). In a recent study, ectopic expression of *Sonic HH* in the pancreatic endoderm of transgenic mice has been shown to result in the formation of pancreatic intraepithelial neoplasia (PanIN)-like lesions (18). The histological progression of pancreatic neoplasia in these transgenic mice is accompanied by the induction of *HER-2/neu* expression and mutations of the proto-oncogene K-*ras* –

genetic alterations that are frequently characterized as early events in PAC tumor etiology (21-23). Immunohistochemical analysis of Sonic HH, Ptch and Smo in clinical PAC specimens suggests that expression of these HH signaling components progressively increases from PanIN lesions to adenocarcinomas (18). In addition, an examination of 26 human PAC cell lines revealed that all of them expressed 2 or more HH signaling components (18, 19). Taken collectively, these studies suggest that the HH pathway is involved in pancreatic tumor development and remains aberrantly expressed in PAC. Targeting this pathway could potentially be developed as a novel therapeutic strategy.

Cyclopamine is a naturally occurring steroidal alkaloid found in the lily plant *Veratrum Californicum* (24). This compound has been shown to inactivate hedgehog transcriptional activity by directly binding to the Smo heptahelical bundle thereby inducing a conformational change similar to that induced by Ptch (25, 26). Cyclopamine has demonstrated significant anti-cancer effects both *in vitro* and *in vivo* in models of medulloblastoma, prostate cancer and pancreatic cancer (18, 27, 28). Interestingly, cyclopamine did not adversely affect the health of treated animals. With HH signaling occurring predominately during mammalian embryogenesis, there is little to no activity of this pathway in most normal adult tissues. Synthetic derivatives of cyclopamine (e.g. KAAD-cyclopamine) as well as other small molecule inhibitors which are structurally distinct from cyclopamine (e.g. SANT1, Cur61414) show promise as more effective anti-cancer agents for future clinical application (29, 30). The molecular basis of response to cyclopamine and other HH pathway antagonists remains largely undefined and indicators of response and/or resistance, at present, remain unknown.

In the current study, we examined *in vitro* response to cyclopamine within a panel of 11 human pancreatic cancer cell lines and determined the concentration at which 50% of cell proliferation is inhibited (IC_{50}) for each cell line. Bromo-deoxyuridine (BrdU) incorporation was measured in cell lines treated at their respective IC_{50} values to determine the effect of cyclopamine on DNA synthesis. Caspase-8, -9, -3, Bid and poly (ADP-ribose) polymerase (PARP) proteins were examined in treated cells using Western blot analysis to determine whether cyclopamine influences apoptosis. The expression of 46 individual genes associated with and influenced by the HH pathway was quantified in all 11 cell lines (prior to cyclopamine treatment) using real-time quantitative polymerase chain reaction (PCR) formatted in a Taqman low-density array (TLDA). To identify genes associated with cyclopamine response, the expression of each gene was compared to IC_{50} values using linear regression analysis. Genes that significantly correlated with these values were modulated *in vitro* (using siRNA) to determine the effect on cyclopamine response. In addition, TLDA was used to quantify changes in gene expression after cyclopamine treatment. Collectively, these analyses suggest that the transcription factor, Gli-3, is a previously unknown indicator of *in vitro* cyclopamine response and that cyclopamine inhibits pancreatic cancer cell proliferation through the mechanisms of decreased DNA synthesis and induction of apoptosis.

Materials and Methods

Cell Culture

Human pancreatic adenocarcinoma cell lines were obtained from the American Tissue Culture Collection; cell lines AsPC-1, BxPC-3, Panc 2.03, Panc 6.03, Panc 8.13

and Panc 10.05 were grown in RPMI 1640 (Cellgro, Herndon, VA); HPAF-2, MiaPaCa-2, Panc-1 and S2013 were grown in DMEM (Cellgro); CFPAC1 was grown in Iscove's MEM (Cellgro). All media was supplemented with 10% FBS (Cellgro).

Cell Proliferation Assays

To test for *in vitro* response to cyclopamine, cells were cultured in triplicate (5,000 cells/well) in 96-well plates for 96 hours in control medium containing 0.5% FBS and vehicle (95% ethanol) alone or in the presence of cyclopamine or tomatidine (Toronto Research Chemicals) at concentrations of 1, 2, 3.5, 5, 7.5, 10, 12.5, 15, 17.5, 20, 22.5, 25, 27.5 and 30 μM. Cell proliferation was determined by optical density measurements at 490 nm using the CellTiter 96 (Promega, Madison, WI) MTS colorimetric assay. A dose-response curve was then created by comparing cyclopamine concentration versus cell proliferation (relative to vehicle control). Linear regression analysis was then performed to determine the concentration of cyclopamine at which 50% inhibition of cell proliferation (IC_{50}) was achieved. IC_{50} values were calculated as an average from 4 independent experiments (3 replicates per experiment). Linear regression analyses demonstrating a correlation coefficient (r^2) below 0.90 were repeated.

Flow Cytometric Analysis

Pancreatic cancer cells were seeded in 6-well plates (150,000 cells/well) and incubated for 96 hours in medium containing vehicle alone or cyclopamine at the IC_{50} concentration. To determine the effect of cyclopamine on DNA synthesis, cells were incubated in the presence of 0.2 mg/ml 5-bromo-2'deoxyuridine (BrdU) (Calbiochem, San

Diego, CA) for 1 hour. Cells were permeabilized, fixed, treated with DNase I (Roche Diagnostics, Penzberg, Germany) and stained with FITC conjugated anti-BrdU (mouse IgG1, Clone B44, BD Biosciences Immunocytometry Systems, San Jose, CA). Labeled cells were quantified by flow cytometry.

Western Blot Analysis

Pancreatic cancer cells were seeded in 60 mm cell culture dishes (500,000 cells/dish) and incubated for 96 hours in medium containing vehicle alone or cyclopamine (8 μM). Cells were subsequently washed in PBS, pH 7.4, lysed, homogenized and centrifuged at 4°C for 10 min at 14,000 rpm to remove insoluble material. The protein concentration of the supernatant was measured by spectrophotometry using the Lowry D_C protein assay method (Bio-Rad, Hercules, CA). A total of 20 μg of protein/lane was separated by SDS-polyacrylamide gel electrophoresis. After transfer to PVDF membranes, blots were incubated with mouse monoclonal antibodies to Caspase-8 and PARP (BD Pharmingen, San Diego, CA) and rabbit polyclonal antibodies to Caspase-3 (Stressgen, Ann Arbor, MI), Caspase-9 (Cell Signaling Technology, Danvers, MA), Bid (Cell Signaling Technology) and Actin (Sigma, St. Louis, MO), which was used to monitor equal sample loading. After washing, blots were incubated with goat anti-mouse (for Caspase-8 and PARP) or goat anti-rabbit (for Caspase-3, Caspase-9, Bid and Actin) secondary antibodies (Bio-Rad) conjugated with horseradish peroxidase. Visualization was performed by the enhanced chemiluminescence method (Amersham Biosciences, Buckinghamshire, U.K.).

RNA Extraction

Prior to incubation with cyclopamine, a sample of each untreated cell line was harvested for RNA extraction. Pancreatic cancer cells treated with vehicle alone or cyclopamine (at their respective IC_{50}) for 36, 48 and 96 hours were also harvested for RNA extraction. Total RNA was isolated from using Trizol reagent (Invitrogen, Carlsbad, CA) as per manufacturer's instructions. RNA was then DNase treated and purified using the RNeasy Mini Kit (QIAGEN, Hilden, Germany) as per manufacturer's instructions. RNA was eluted in 50 μL of RNase-free water and stored at -80°C. The concentration of all RNA samples was quantitated using the *ribosomal protein, large, P0* (*RPLP0*) housekeeping gene and linear regression analysis of a standard curve derived from known concentrations of normal pancreas RNA as previously described by our laboratory (31, 32).

Reverse Transcription

Prior to cDNA synthesis, all RNA samples were diluted to 4 ng/μL using RNase-free water containing 12.5 ng/μL of total yeast RNA (Ambion, Austin, TX) as a carrier. cDNA was prepared using the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) as per manufacturer's instructions. The resulting cDNA samples were analyzed using TLDA.

TLDA

For each card of the low-density array (Applied Biosystems), there are 8 separate loading ports that feed into 48 separate wells for a total of 384 wells per card. Each 2-μL

well contains specific, user-defined primers and probes, capable of detecting a single gene. In this study, the TLDA card was configured into 8 identical 48 gene sets. Genes known to be closely associated with or influenced by the HH signaling pathway were selected on the basis of literature reviews to encompass the 48 genes examined (20, 33-35). Each set of 48 genes also contains two housekeeping genes, *RPLP0* and *GAPDH*. All samples were loaded onto a TLDA card and PCR amplification was performed as previously described (36).

TLDA Analysis

Expression values were calculated using the comparative C_T method as previously described (32, 36). Briefly, this technique uses the formula $2^{-\Delta\Delta CT}$ to calculate the expression of target genes normalized to a calibrator. The threshold cycle (C_T) indicates the cycle number at which the amount of amplified target reaches a fixed threshold. C_T values range from 0 to 40 (the latter representing the default upper limit PCR cycle number that defines failure to detect a signal). Normal pancreas RNA (Ambion) was used as the calibrator ($\Delta\Delta C_T = \Delta C_T$ (Tumor) - ΔC_T (Normal)), for which all gene expression values were assigned a relative value of 1.00. A range for each expression value was calculated based on the standard deviation (s) of the $\Delta\Delta C_T$ value where $2^{-(\Delta\Delta CT+s)}$ is the lower limit and $2^{-(\Delta\Delta CT-s)}$ is the upper limit.

Transfection

SMO-, *GLI3*-, *EN1*- and *GSK3B*-specific siRNA and control siRNA were obtained from Invitrogen. Cells were cultured in triplicate (5,000 cells/well) in 96-well

plates and transfection was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Cells were transfected for 24 hours prior to treatment with vehicle alone or cyclopamine.

Taqman PCR

Primers and probes for *SMO* (Hs00170665_m1), *GLI3* (Hs00609233_m1), *EN1* (Hs00154977_m1) and *GSK3B* (Hs00275656_m1) were obtained from Applied Biosystems and used according to the manufacturer's instructions. Taqman PCR was performed using an ABI Prism 7900HT sequence detection system. Gene expression was calculated using the comparative C_T method.

Statistical Analysis

Comparisons of *in vitro* response to cyclopamine (IC_{50}) and gene expression were examined using linear regression and Pearson correlation analysis. Changes in cell proliferation, BrdU incorporation and gene expression induced by cyclopamine were determined by Student's *t*-test. Significance was defined as $p < 0.05$.

Results

Effect of Cyclopamine and Tomatidine on the Growth of PAC Cell Lines

Initial studies demonstrated that cyclopamine decreases pancreatic cancer cell proliferation in a dose-dependent manner (as determined by MTS assay) with variable sensitivity observed among the cell lines examined (Figure 1A, B). Tomatidine, a structural analog of cyclopamine that lacks the ability to inhibit HH signaling (37), had little to no effect on cell proliferation. To determine the variance in response to cyclopamine *in vitro*, IC_{50} values were calculated for 11 pancreatic cancer cell lines. As shown in Table 1, there is a broad range of response to cyclopamine observed across the cell lines examined. HPAF-2 cells ($IC_{50} = 8.79$ μM) showed the greatest reduction in cell proliferation while MiaPaCa-2 cells demonstrated no measurable response to cyclopamine treatment.

Effect of Cyclopamine on DNA Synthesis

To determine whether the reduction in cell proliferation induced by cyclopamine is due to decreased DNA synthesis, sensitive HPAF-2 cells ($IC_{50} \sim 8$ μM) and relatively resistant Panc-1 cells ($IC_{50} \sim 30$ μM) were exposed to cyclopamine for 96 hours and DNA synthesis was detected by BrdU incorporation. Cyclopamine (8 μM) significantly ($p<0.0001$) reduced BrdU labeling in HPAF-2 cells by 83% relative to vehicle control (Figure 2A). BrdU labeling of Panc-1 cells, treated at the same concentration, was reduced by 33% and treatment at their IC_{50} (30 μM) resulted in a decrease of 54% relative to vehicle control (Figure 2B).

Western Blot Analysis of PAC Cell Lines Treated With Cyclopamine

To determine whether the reduction in cell viability induced by cyclopamine is due to apoptosis, the expression of proteins known to be indicators of programmed cell death (caspase-3, -8, -9, Bid and PARP) (38, 39) were evaluated by Western blot analysis. HPAF-2 cells treated with 8 μM cyclopamine and Panc-1 cells treated with both 8 and 30 μM cyclopamine were examined. Cleavage (activation) of initiator caspases-8 and -9, executioner caspase-3 and Bid was observed in HPAF-2 cells but not Panc-1 cells treated with 8 μM cyclopamine (Figure 3). Panc-1 cells exposed to 30 μM cyclopamine, however, did display markers of apoptosis including cleavage of caspases-8 and -3. It was also observed in treated HPAF-2 cells that PARP protein expression was reduced. This result is in opposition to the expected cleavage of PARP into an 86-kDa fragment, which can occur during apoptosis (39). No reduction or cleavage of PARP protein was observed in cyclopamine-treated Panc-1 cells.

Correlation of Gene Expression With Cyclopamine Response in PAC Cell Lines

To elucidate which genes were associated with *in vitro* response to cyclopamine, we quantified the expression of 46 independent genes directly associated with or influenced by the HH pathway in 11 pancreatic cancer cell lines (prior to treatment) using TLDA. Molecular analysis was performed on cells harvested at the same time IC_{50} values were examined (see Table 1). Linear regression analysis demonstrated that resistance to cyclopamine (increasing IC_{50} values) correlated with increasing mRNA levels (positive slope; $p<0.05$) of *SMO*, *GLI3* and *EN1* (Figure 4A, B, C). Conversely, resistance to cyclopamine correlated with decreasing mRNA levels (negative slope; $p<0.05$) of *GSK3B*

(Figure 4D). MiaPaCa-2 was excluded from these analyses since IC_{50} values could not be estimated for this cell line. Individual mRNA analysis using Taqman PCR for *SMO*, *GLI3*, *EN1* and *GSK3B* also correlated with cyclopamine response (IC_{50}) validating the results obtained using TLDA (data not shown).

Effect of Gene Knockdown on In Vitro Cyclopamine Response

To further evaluate the association of *SMO*, *GLI3*, *EN1* and *GSK3B* mRNA levels with cyclopamine response, we modulated the expression of each of these genes using siRNA and observed the effect this had on *in vitro* cyclopamine response. Knockdown of *SMO* and *EN1* (in resistant Panc-1 cells) and *GSK3B* (in sensitive HPAF-2 cells) mRNA was achieved (as determined by Taqman PCR); however, this gene silencing did not significantly alter response to cyclopamine (data not shown). Alternatively, knockdown of *GLI3* mRNA levels (Figure 5A), using 2 different siRNA sequences, significantly increased the sensitivity of Panc-1 cells to cyclopamine (8 μM). As shown in Figure 5B, cyclopamine decreased Panc-1 cell proliferation by only 13-14% in mock transfection and siRNA controls. The proliferation of Panc-1 cells pre-treated with *GLI3* siRNA1 and 2, however, was reduced by 30 and 50%, respectively, after cyclopamine treatment. Interestingly, the proliferation of Panc-1 cells treated with vehicle and *GLI3* siRNA alone was reduced in comparison to mock transfection and siRNA controls. To determine if this effect could be due to apoptosis, exectutioner caspase-3 protein expression was evaluated in Panc-1 cells treated with either Lipofectamine 2000, siRNA control or *GLI3* siRNA alone or in combination with cyclopamine (8 μM) using Western blot analysis.

As indicated by Figure 5C, knockdown of *GLI3* alone or in combination with cyclopamine resulted in caspase-3 cleavage, a marker of apoptosis.

Changes in Gene Expression After Cyclopamine Treatment

To determine the molecular changes that result from cyclopamine treatment, the expression of 46 genes directly associated with or influenced by the HH pathway was quantified in HPAF-2 and Panc-1 cells treated with cyclopamine (at their respective IC_{50}) for 36, 48 and 96 hours using TLDA. As shown in Figure 6, there are significant ($p<0.05$) changes in the expression of genes that are involved in cell cycle progression including cyclins B1 (*CCNB1*), D1 (*CCND1*), D2 (*CCND2*), D3 (*CCND3*), E1 (*CCNE1*), E2 (*CCNE2*), p21, Cip1 (*CDKN1A*) and *E2F1* as a result of cyclopamine treatment in comparison to vehicle control. In particular, *CCNB1* expression was decreased in both treated HPAF-2 and Panc-1 cells with a maximum decrease achieved after 96 hours (4.40- and 6.82-fold, respectively). *CCND1* expression was decreased only in treated HPAF-2 cells (2.36-fold, 48 hours) and, interestingly, *CCND2* expression, which was undetectable in HPAF-2 cells, increased in treated Panc-1 cells (15.32-fold, 48 hours). *CCND3*, *CCNE1*, *CCNE2* and *E2F1* expression was decreased in both treated HPAF-2 and Panc-1 cells with a maximum effect achieved at 36 hours for HPAF-2 cells (2.14-, 2.18-, 8.23- and 3.13-fold, respectively) and 96 hours for Panc-1 cells (3.42-, 2.71- and 6.60-fold, respectively) with the exception of *E2F1* (4.79-fold, 48 hours). Expression of *CDKN1A*, a known inhibitor of the cell cycle, was increased in treated HPAF-2 cells (2.45-fold, 48 hours) but not in treated Panc-1 cells.

Not surprisingly, cyclopamine also affected the expression of genes directly involved in HH signaling. Gene expression of the HH ligand, Desert HH (*DHH*), which was undetectable in HPAF-2 cells, was significantly decreased in treated Panc-1 cells (22.71-fold, 36 hours). Expression of Indian HH (*IHH*), another HH ligand, was decreased in treated HPAF-2 (3.66-fold, 96 hours) and Panc-1 (3.86-fold, 96 hours; not significant) cells. Expression of the HH ligand receptors, Patched (*PTCH*) 1 and 2, was also decreased in response to cyclopamine. *PTCH1* mRNA levels were not significantly changed in treated HPAF-2 cells; however, they were significantly decreased in treated Panc-1 cells (2.62-fold, 96 hours). *PTCH2* mRNA levels were decreased in treated HPAF-2 (1.97-fold) and Panc-1 (2.19-fold) cells, both at 36 hours. Gene expression of the Gli family of transcription factors, downstream effectors of HH signaling, was also affected by cyclopamine treatment. *GLI1* mRNA levels were not significantly changed in treated HPAF-2 cells; however, there was a modest, but significant decrease (1.43-fold) in *GLI1* expression in Panc-1 cells after 96 hours of treatment. Alternatively, *GLI2* expression was decreased in both treated HPAF-2 (4.31-fold, 36 hours) and Panc-1 (4.56-fold, 96 hours) cells.

Interestingly, the expression of genes that correlated with cyclopamine response (IC_{50} values) was also altered as a result of cyclopamine treatment. *EN1*, *GLI3* and *SMO* mRNA levels, which were undetectable in HPAF-2 cells, were all significantly decreased (2.84-, 3.14- and 2.89-fold, respectively) in Panc-1 cells with a maximum effect achieved after 96 hours of treatment for all 3 genes. *GSK3B* mRNA levels were not significantly changed in either treated HPAF-2 or Panc-1 cells (data not shown).

Discussion

The HH signaling pathway consists of a family of morphogens that are essential for invertebrate and vertebrate embryogenesis (35). Recently, it has been suggested that uncontrolled activity of this pathway plays a key role in the development and continued growth of PAC (18, 19). This finding, among others, has led to several studies evaluating the usefulness of HH antagonists in the treatment of not only PAC, but also other cancers in which HH signaling is misregulated including medulloblastoma, small-cell lung, colorectal, prostate and breast cancer (18, 19, 27, 28, 40-45). In all of these studies, cyclopamine, a steroidal alkaloid that inhibits HH signaling by directly binding to Smo (25, 26), was used to target cancer cells either *in vitro* or *in vivo* or both. Interestingly, in studies evaluating the effect of cyclopamine on human PAC *in vitro*, it was found that some but not all cell lines responded to HH pathway inhibition (18, 41, 45). Similar results were obtained in the current study with the goal of providing not only a genetic explanation for this observed variability in response but also a molecular basis for the resultant physiological effects observed after treatment with cyclopamine. The identification of genes associated with *in vitro* response to cyclopamine could, ultimately, be used to develop a criteria for the rational selection of newly diagnosed pancreatic cancer patients who may be candidates for HH inhibitor therapy. Currently, specific genes in the HH pathway that could be used as molecular markers of response remain unknown. Elucidation of the molecular changes that result in a physiological response to HH pathway inhibition could, ultimately, provide a mechanistic basis for combining HH antagonists with other therapeutic agents to improve clinical outcome (e.g. synergistic effects). Since activation of

the HH pathway appears to be an early event in the development of pancreatic cancer, these analyses may also be useful in understanding tumor etiology.

Initial studies examined *in vitro* response to cyclopamine across a panel of 11 pancreatic cancer cell lines. A total of 10/11 cell lines demonstrated a dose-dependent decrease in cell proliferation following treatment with cyclopamine (1-30 μM). By comparison, tomatidine, used at the same concentrations, had little to no effect on cell proliferation in any of the cell lines examined suggesting that the decrease in cell proliferation induced by cyclopamine results from inhibition of the HH pathway (37). As shown in Table 1, IC_{50} values varied > 5-fold from 8.79 to 45.09 μM (for HPAF-2 and S2013 cells, respectively) with no measurable response observed in MiaPaCa-2 cells. This variability in response was further evident when examining the physiological effects resulting from cyclopamine treatment (i.e. decreased DNA synthesis and apoptosis). Administered at its IC_{50} concentration, cyclopamine reduced BrdU incorporation by 83% in sensitive HPAF-2 cells (IC_{50} ~ 8 μM) in comparison to only 54% in relatively resistant Panc-1 cells (IC_{50} ~ 30 μM) that were treated with almost 4 times the concentration used on HPAF-2 cells (Figure 2). Western blot analyses showed that HPAF-2 cells demonstrated markers of apoptosis in response to cyclopamine (Figure 3). Treated Panc-1 cells also demonstrated markers of apoptosis but to a lesser extent and only after exposure to 30 μM cyclopamine (IC_{50}).

To determine whether this variability in response to cyclopamine may be associated with changes in gene expression, we quantified 46 different genes directly associated with or influenced by the HH pathway in each of the 11 cell lines. Using linear regression analysis, a statistically significant correlation ($p < 0.05$) between mRNA levels and

IC_{50}'s was observed for *SMO*, *GLI3*, *EN1* and *GSK3B* (Figure 4). Interestingly, mRNA levels for *SMO*, an essential HH signaling component and the target of cyclopamine, were found to increase with increasing IC_{50}'s. Resistance to cyclopamine with increasing *SMO* expression may follow similar models observed with other targeted chemotherapeutic and non-chemotherapeutic agents where an increase in expression of drug target confers resistance (46-50). Gli-3 is a member of the Gli family of transcription factors that work downstream of Smo to regulate HH signaling. Gli-3, unlike Gli-1, is capable of both activating and repressing the HH pathway, depending upon whether it is in its full-length or truncated form, respectively (51, 52). Gsk-3β, a known inhibitor of the HH pathway, phosphorylates Gli-3 at multiple sites leading to its proteolysis and truncation (53, 54). Interestingly, inhibition of Gsk-3β activity was found to increase transcriptional activation of *EN1*, a transcription factor whose activity is positively influenced by both Wnt and HH signaling (55-57).

To determine the contribution of these candidate molecules (*SMO*, *GLI3, EN1* and *GSK3B*) to cyclopamine response and/or resistance, siRNA experiments were performed to knockdown the expression of each of these genes *in vitro* and determine what effect this has on response. Knockdown of *SMO*, *EN1* and *GSK3B* mRNA levels was achieved (as determined by Taqman PCR); however, these modifications did not have a significant impact on cyclopamine sensitivity (data not shown). It is possible that the decrease in the mRNA levels of these genes was not sufficient to reduce their corresponding protein levels to such a degree that response to cyclopamine is altered. If these proteins are relatively stable and not easily degraded, they may have a long half-life and knockdown of protein expression could potentially take longer than the amount of time used in this

study (24 hours siRNA pre-treatment + 96 hours cyclopamine treatment = 120 hours total). Evaluation of these genes and their role in cyclopamine response, therefore, warrants further investigation. As shown in Figure 5A, sustained knockdown of *GLI3* mRNA levels, using 2 different siRNA sequences, was achieved in Panc-1 cells for the entire duration of cyclopamine treatment. This resulted in a 2 to 3-fold increase in the sensitivity of Panc-1 cells to cyclopamine in comparison to mock transfection and siRNA controls (Figure 5B). It is apparent that *GLI3* siRNA2 had more of an effect on cyclopamine sensitivity than *GLI3* siRNA1 (50% versus 30% decrease). This could be due to the rise in *GLI3* gene expression in Panc-1 cells transfected with siRNA1 (after 72 hours) and the continued decrease in expression in cells transfected with siRNA2 (Figure 5A). On average, *GLI3* siRNA1 knocked down gene expression by 67% whereas siRNA2 knocked down by 75%. The results from these siRNA experiments suggest that Gli-3 does play a functional role in mediating *in vitro* response to HH pathway inhibition. The molecular mechanisms by which this occurs, however, remains unclear. It could be speculated that increased expression of Gli-3, in its full-length, transcriptional activator form, promotes increased HH signaling that cannot be overcome by cyclopamine. It has been shown that in the presence of HH ligand, full-length Gli-3 translocates into the nucleus where it binds to the promoter of Gli-1 thus leading to transcriptional activation of HH target genes (51). Alternatively, Gli-3 may influence other cellular pathways that provide protection against cell death (such as apoptosis) as suggested by the induction of caspase-3 cleavage following knockdown of *GLI3* expression (Figure 5C). There is some evidence to support this potential mechanism as studies have suggested that Gli-3 can regulate apoptosis during mammalian limb development (58, 59). To fully elucidate the

molecular basis for Gli-3's role in mediating resistance to cyclopamine, future studies, including those examining Gli-3 and its connection to programmed cell death, are warranted.

To identify genes associated with *in vitro* response to cyclopamine, mRNA levels were quantified in cell lines prior to treatment. In order to determine a molecular basis for the physiological effects observed following cyclopamine exposure (i.e. decreased cell proliferation and apoptosis), it was necessary to examine gene expression after treatment as well. As shown in Figure 6, cyclopamine altered the expression of genes involved in cell cycle maintenance that are known to be influenced by HH signaling (34). Sensitive HPAF-2 cells had significant decreases in *CCND1*, *CCND3*, *CCNE1*, *CCNE2* and *E2F1* (all of which mediate G1/S phase transition) mRNA levels that appeared to be transient after 36 hours of cyclopamine exposure. The decrease in expression of these genes (with the exception of *CCND1*), however, continued even after 96 hours of cyclopamine treatment in resistant Panc-1 cells. In addition, mRNA levels of *CDKN1A*, an inhibitor of the cell cycle, increased in treated HPAF-2 but not Panc-1 cells. Collectively, these data suggest that HPAF-2 cells could be undergoing cell cycle arrest earlier than Panc-1 cells as a result of cyclopamine exposure with apoptosis occurring later. This might explain why HPAF-2 cells appear to be farther along than Panc-1 cells in terms of apoptotic events after exposure to cyclopamine (Figure 3). Cell cycle arrest and apoptosis combined could therefore account for the decreased proliferation of both treated HPAF-2 and Panc-1 cells with apoptosis possibly playing a larger role in the physiological response of HPAF-2 cells to cyclopamine. It could also be suggested that proliferation of Panc-1 cells is not affected by cyclopamine to the extent that HPAF-2

cells are because *CCND1* and *CDKN1A* expression do not change and *CCND2* expression markedly increases (up to 15-fold) after treatment.

Previous reports have shown that cyclopamine decreases expression of HH target genes *in vitro*, in particular *PTCH1* and *GLI1* (27, 41, 44). Similar decreases were observed in this study for Panc-1 but not HPAF-2 cells (Figure 7). In addition, cyclopamine significantly decreased the expression of other HH pathway genes that have not been previously examined in treated pancreatic cancer cells. These include the HH ligands *DHH* and *IHH*, the HH receptors *PTCH2* and *SMO*, the HH transcription factors *GLI2* and *GLI3* and the HH transcriptional target *EN1*. While there was no apparent decrease in *PTCH1* and *GLI1* mRNA levels in treated HPAF-2 cells, there was a decrease in *PTCH2* and *GLI2* expression indicating that cyclopamine may suppress HH signaling differently in different cell lines (i.e. cell line-specific effects). Interestingly, cyclopamine decreased the expression of genes identified as possible indicators of response, *SMO*, *GLI3* and *EN1*, further suggesting their importance in the study of variable response to HH pathway inhibition. Of note, in studies conducted with tomatidine, the expression of these 3 genes in addition to *PTCH1* and *GLI1* did not significantly change in treated (30 μM) Panc-1 cells (data not shown). Collectively, these data suggest that cyclopamine targets members of the HH signaling pathway, which is in agreement with previous studies on this HH antagonist (26, 29, 37).

In the current study, we utilize a progressive strategy to identify genes associated with response to cyclopamine *in vitro* and provide a potential molecular basis for the physiological effects observed after cyclopamine treatment. Collectively, the data presented in this study suggest that HH gene expression profiles as well as response to

cyclopamine varied in human pancreatic cancer cell lines and these differences allowed for the identification of potential indicators of response. In addition, cyclopamine appeared to decrease pancreatic cancer cell proliferation through both decreased DNA synthesis and induction of apoptosis. Treatment with cyclopamine may decrease cell proliferation through cell cycle arrest by regulating the expression of cyclins B1, D1, D2, D3, E1 and E2, E2F1 and p21. Although correlative studies suggested 4 genes (*SMO*, *GLI3*, *EN1* and *GSK3B*) were associated with cyclopamine response, only modulation of *GLI3* expression resulted in a significant change in sensitivity suggesting that this gene is a previously unknown indicator of response to HH pathway inhibition. The identification of genes such as *GLI3* could ultimately be used to develop a molecular basis for the rational selection of newly diagnosed pancreatic cancer patients who may be candidates for HH inhibitor therapy.

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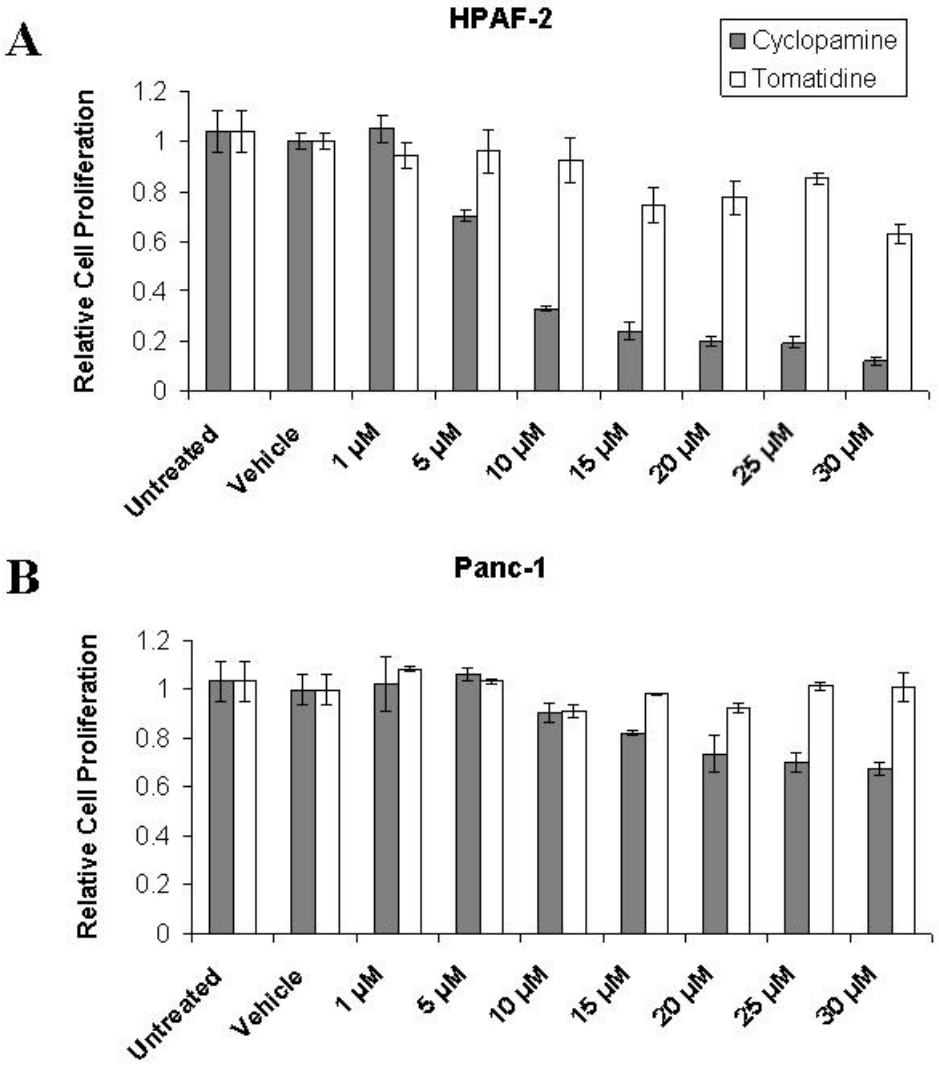

Figure 1. Effect of cyclopamine and tomatidine on the growth of pancreatic cancer cell lines. HPAF-2 (A) and Panc-1 (B) cells were exposed to increasing concentrations (1-30 μM) of cyclopamine and its structural, non-teratogenic analog, tomatidine, for 96 hours and cell proliferation was determined using MTS assay (relative to vehicle control). Error bars represent SD.

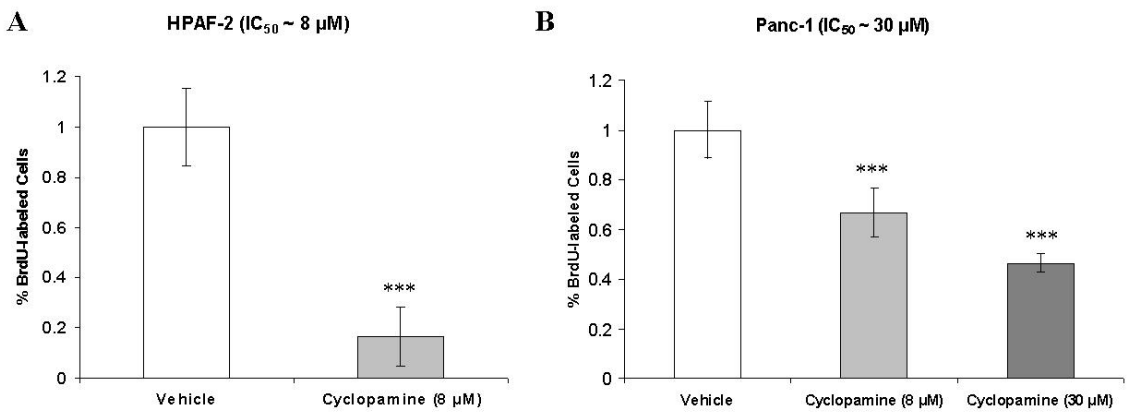

Figure 2. Effect of cyclopamine on DNA synthesis. BrdU incorporation was measured in HPAF-2 (A) and Panc-1 (B) cells treated with cyclopamine for 96 hours. Cyclopamine exposure (8 µM) led to an 83% decrease in BrdU-labeled HPAF-2 cells relative to vehicle control. BrdU-labeling of Panc-1 cells, treated at the same concentration, was reduced by only 33% relative to vehicle control. Upon exposure to 30 µM cyclopamine (IC_{50}), BrdU-labeling of Panc-1 cells was reduced by 54% relative to vehicle control. Average of 3 independent experiments performed in duplicate. Error bars represent SD; triple asterisks, $p<0.001$.

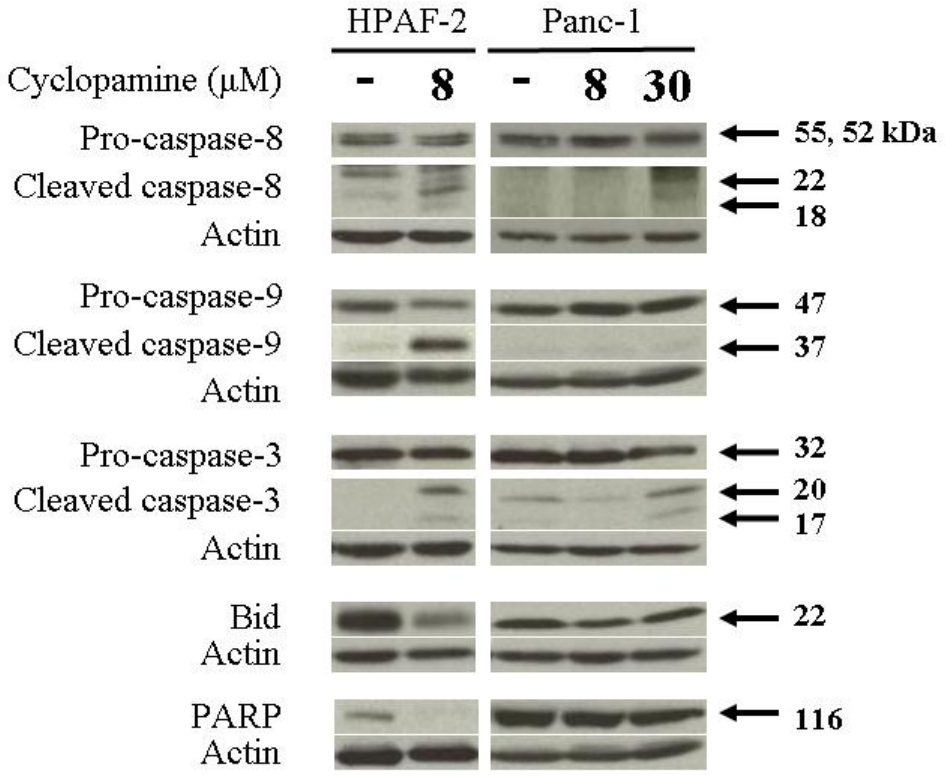

Figure 3. Effect of cyclopamine on proteins that mediate apoptosis. Western blot analysis of Caspase-3, -8, -9, Bid and PARP in HPAF-2 and Panc-1 cells treated with cyclopamine or vehicle control for 96 hours. Actin was used as a loading control.

Table 1

Response to Cyclopamine in Pancreatic Cancer Cell Lines

Cell Line	IC_{50} (μM)*†
HPAF-2	**8.79 ± 0.94**
Panc 10.05	**11.33 ± 0.41**
Panc 8.13	**14.49 ± 0.85**
Panc 2.03	**16.57 ± 0.27**
AsPC-1	**16.74 ± 1.30**
CFPAC	**19.59 ± 0.32**
Panc 6.03	**28.18 ± 1.74**
Panc-1	**31.41 ± 0.78‡**
BxPC-3	**36.17 ± 0.31‡**
S2013	**45.09 ± 1.27‡**
MiaPaCa-2	**Resistant**

*The concentration of cyclopamine that inhibits cell proliferation by 50%

†Values represent the average of 4 independent experiments ± SEM

‡IC_{50} values > 30 μM were estimated by extending linear regression analysis

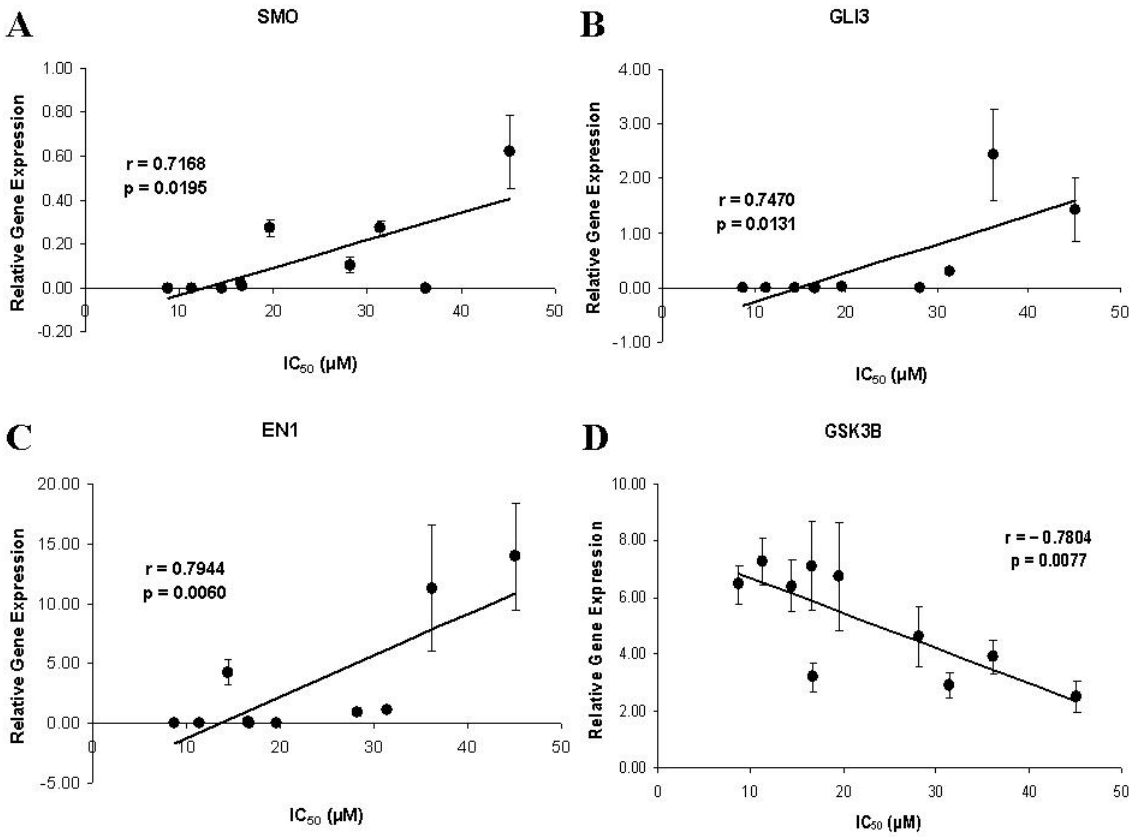

Figure 4. Genes associated with *in vitro* response to cyclopamine. Linear regression analysis of plots comparing cyclopamine IC_{50} values (*x*) and gene expression (*y*) was carried out for a total of 46 genes in 10 different pancreatic cancer cell lines prior to treatment. Only *SMO* (A), *GLI3* (B), *EN1* (C) and *GSK3B* (D) expression significantly ($p<0.05$) correlated with cyclopamine response. Gene expression was calculated relative to normal pancreas RNA. Average of 4 independent experiments. Error bars represent SEM.

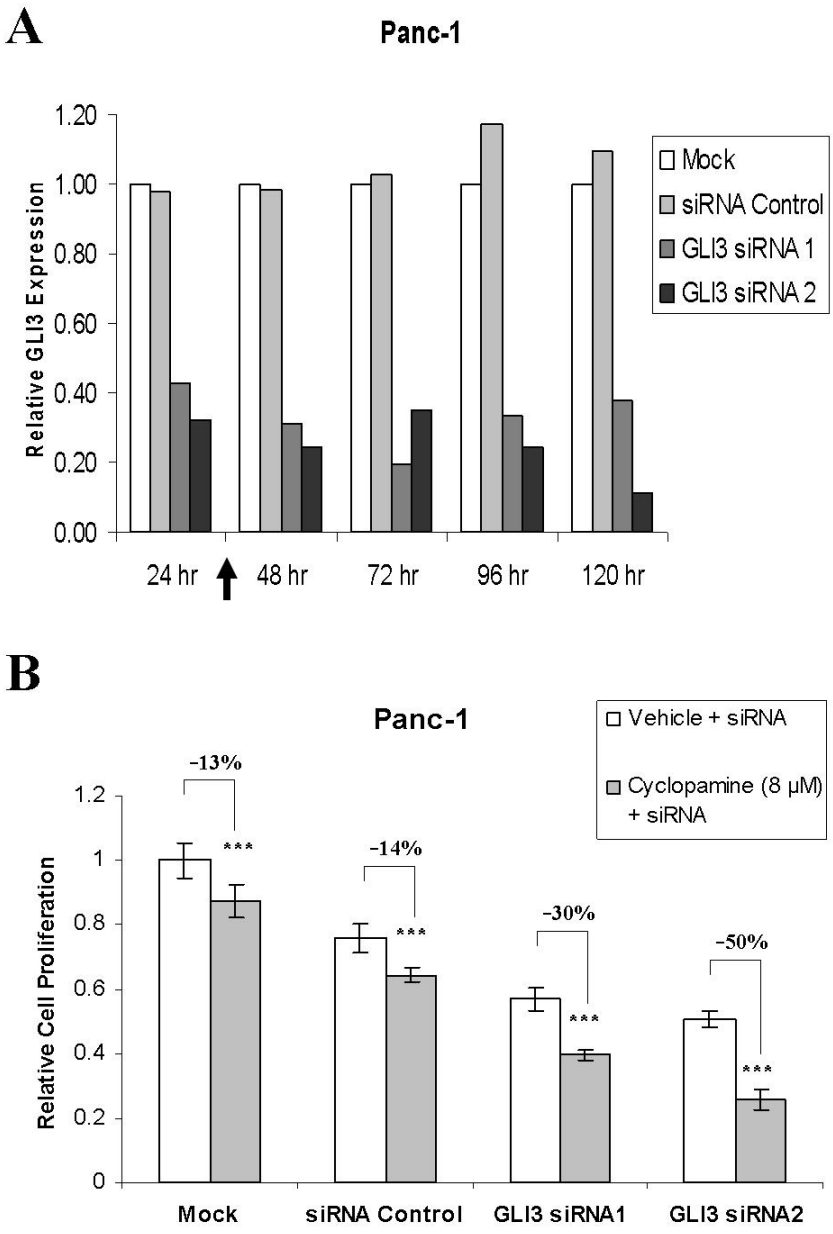

Figure 5. Effect of *GLI3* siRNA on *in vitro* sensitivity to cyclopamine. (A) *GLI3* expression was measured in Panc-1 cells treated with Lipofectamine 2000 alone (mock transfection), siRNA control or 2 different siRNA sequences directed against *GLI3* for 24, 48, 72, 96 and 120 hours to determine if expression would remain knocked down throughout cyclopamine treatment. Gene expression was calculated relative to mock transfection for each time point. Black arrow indicates when cyclopamine treatment would begin. (B) Panc-1 cells were pre-treated with Lipofectamine 2000 alone, siRNA control or *GLI3* siRNA for 24 hours and then exposed to cyclopamine (8 μM) or vehicle control for 96 hours. Cell proliferation was measured using MTS assay and calculated relative to mock transfection + vehicle control. Average of 4 independent experiments performed in triplicate. Error bars represent SD; triple asterisks, $p<0.0001$.

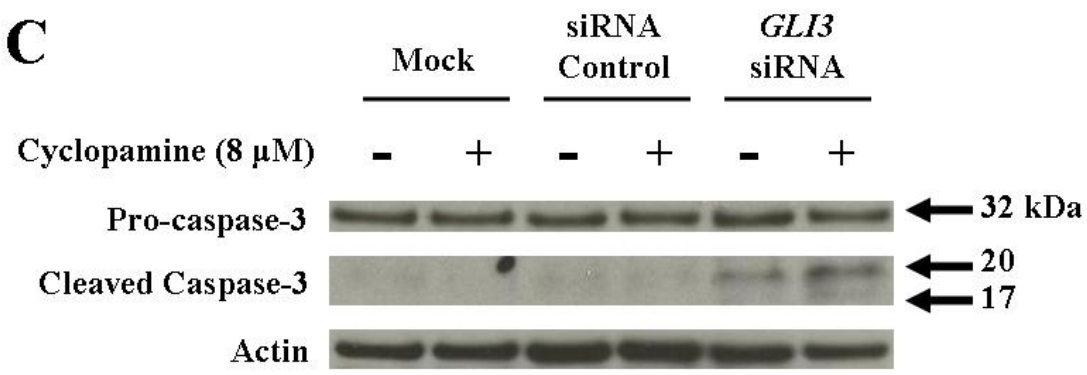

Figure 5 (Continued). Effect of *GLI3* siRNA on *in vitro* sensitivity to cyclopamine. (C) Western blot analysis of caspase-3 cleavage in Panc-1 cells pre-treated with Lipofectamine 2000 alone, siRNA control or *GLI3* siRNA for 24 hours and then exposed to cyclopamine (8 μM) or vehicle control for 96 hours. Actin was used as a loading control.

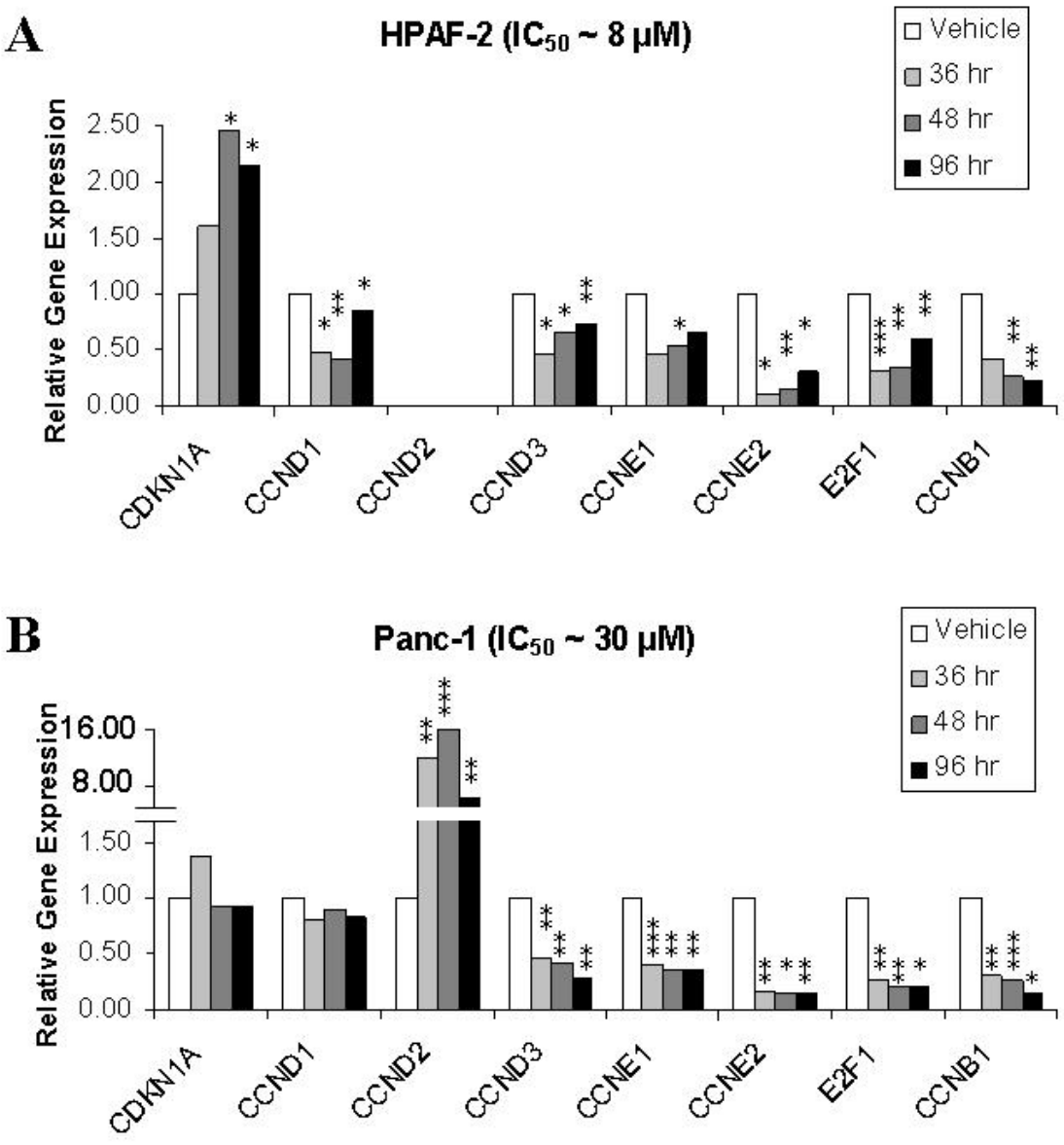

Figure 6. Effect of cyclopamine on cell cycle mediators. Gene expression of cell cycle mediators was measured in HPAF-2 (A) and Panc-1 (B) cells treated with either vehicle control or cyclopamine (IC_{50}) for 36, 48 and 96 hours using TLDA. Average of 3 independent experiments. Single asterisk, $p<0.05$; double asterisks, $p<0.01$; triple asterisks, $p<0.001$.

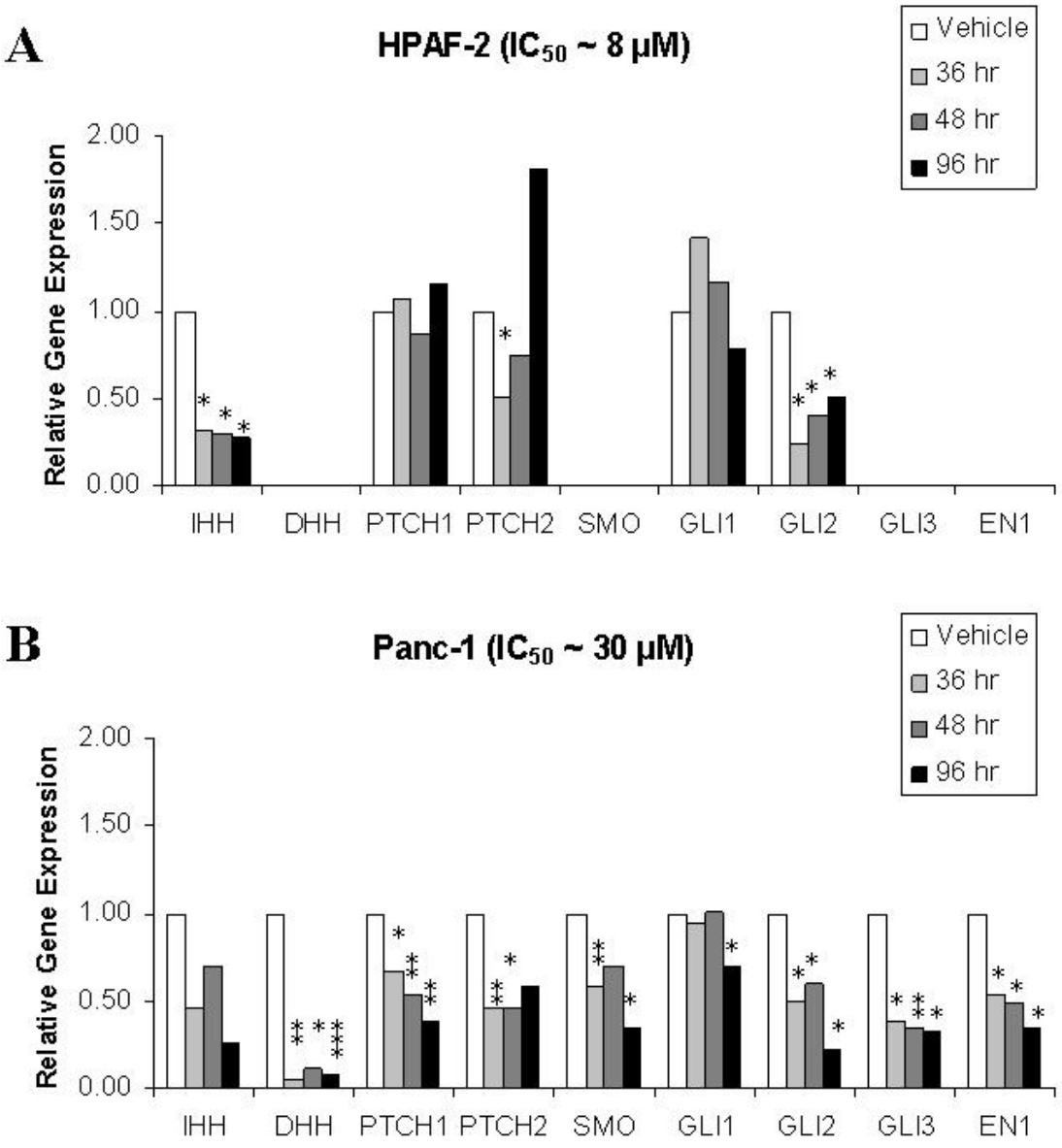

Figure 7. Effect of cyclopamine on HH pathway genes. Gene expression of HH pathway members was measured in HPAF-2 (A) and Panc-1 (B) cells treated with either vehicle control or cyclopamine (IC_{50}) for 36, 48 and 96 hours using TLDA. Average of 3 independent experiments. Single asterisk, $p<0.05$; double asterisks, $p<0.01$; triple asterisks, $p<0.001$.

SUMMARY AND CONCLUSIONS

Discussion

One of the primary goals in the development of rational treatment paradigms is the identification of tumor associated pathways that could be selectively targeted in cancer cells. Understanding the molecular basis of response and identifying markers capable of stratifying patients toward these more effective treatments also remains a central effort in current pharmacogenomic studies. While some success has been achieved (e.g. Bcr-abl tyrosine kinase inhibition by imatinib mesylae or blocking Her-2/Neu by trastuzumab) (Buchdunger et al., 1996; Pegram et al., 1998), these strategies have made little impact in the treatment of patients diagnosed with PAC. Incremental progress (such as the introduction of gemcitabine) has been made in the treatment of PAC and patient survival time is still measured in months instead of years (Crane et al., 2001). This dismal outcome indicates a dire need for the identification of novel therapeutic targets, prognostic indicators and/or molecular mechanisms of tumorigenesis in order to better understand and more effectively treat this devastating disease. Recently, it has been shown that aberrant expression of the HH signaling pathway plays a key role in the development and sustained growth of pancreatic cancers (Berman et al., 2003; Thayer et al., 2003). It was also demonstrated in these studies that cyclopamine, a known HH pathway antagonist, decreases pancreatic cancer cell viability both *in vitro* and *in vivo* suggesting the potential benefit of using HH inhibitors as therapeutic agents. These studies ultimately provided

the basis for the research encompassed within this dissertation, the overall goal of which was to characterize differential expression of HH pathway genes in clinical PAC specimens (snap-frozen, paraffin-embedded and fine-needle aspirates) and examine the molecular basis of *in vitro* response to cyclopamine in human pancreatic cancer cell lines.

Our first specific aim was to examine the differential expression of 46 genes directly associated with the HH pathway or influenced by HH signaling in PAC clinical specimens and uninvolved pancreas. The rationale behind this analysis was that potentially novel tumor-specific targets could be identified, a prerequisite to the future development of prognostic, diagnostic or targeted anti-HH therapies. It should be noted that the 46 genes examined were chosen based upon extensive literature reviews of the HH molecular pathway (Ingham & McMahon, 2001; Nusse, 2003; Pasca di Magliano & Hebrok, 2003; Roy & Ingham, 2002). Because of the relatively recent discovery of this pathway, its true molecular portrait remains to be fully elucidated and genes involved in HH signaling continue to be discovered. Therefore, the 46 genes examined in this dissertation should not be taken as a definitive analysis of the entirety of the HH pathway. To quantify the expression of all 46 genes, we used TLDA, a recently developed technique that expands upon the capabilities of real-time quantitative PCR by allowing the user to examine multiple, user-defined genes simultaneously in a single RNA sample. However, before analysis of pancreatic tissues could commence, it was necessary to validate the TLDA methodology. Throughout the course of this validation study, it was found that there is a low inter- and intra-assay variation (<5%) which would allow the determination of less than 2-fold differences in gene expression levels using TLDA. In addition, multivariate gene expression analysis can be performed using TLDA in archival paraffin-

embedded clinical samples (the most widely available specimens at most treatment/research facilities and cooperative groups). Further, unique gene expression profiles were lost after RNA amplification, a method commonly employed to increase the pool of mRNA isolated from micro-dissected tissues. This study is (to our knowledge) the first publication validating the use of multivariate gene expression analysis using TLDA in archival paraffin-embedded tissues. Collectively, the development and validation of TLDA represents a significant methodological advance which, ultimately, allowed subsequent examination of HH gene expression in matched surgically resected pancreatic cancer (both snap-frozen and paraffin-embedded) and endoscopic ultrasound-guided fine-needle aspirate (EUS-FNA) biopsies.

Definitive gene expression analysis of PAC remains difficult due to the host desmoplastic stromal interaction and subsequent tumor heterogeneity. Neoplastic cells often represent only a small percentage of the cellular content (<40%) in many parts of the tumor mass which can necessitate difficult and time-intensive procurement of malignant epithelia from within heterogeneous tissues (Crnogorac-Jurcevic et al., 2002). In addition, tumor heterogeneity can vary between patients and the type of biopsy collected (snap-frozen, paraffin-embedded or fine-needle aspirates) further complicating genetic analyses of this tumor type. Although EUS-FNA biopsies are increasingly being used to diagnose PAC in unresectable patients, their usefulness as a tissue source in evaluating gene expression profiles remains unknown. Having validated TLDA, we were able to use this method to examine the expression of 46 genes directly associated with or influenced by the HH pathway within a series of matched heterogeneous PAC samples that included snap-frozen, paraffin-embedded and EUS-FNA biopsies. In addition, differen-

tial expression in tumor cells was evaluated by comparative analysis with uninvolved pancreas and macro-dissected (tumor cell enriched) PAC specimens. Further, an independent set of EUS-FNA biopsies obtained before and 2 weeks after chemoradiation was examined. A significant finding of this study was that there is a statistically significant concordance in gene expression between matched snap-frozen, archival paraffin-embedded and EUS-FNA biopsies (Figure 1, page 71). This concordance demonstrates that all available clinical PAC specimens are suitable for genetic analyses.

Another finding was that HH pathway genes were significantly overexpressed in all clinical PAC specimens examined in comparison to uninvolved pancreas (Table 1, page 72). This overexpression was the most pronounced in macro-dissected (cancer cell enriched) PAC tissues in comparison to heterogeneous EUS-FNA biopsies suggesting that HH signaling is localized to neo-plastic cells (this observation was confirmed using immunohistochemistry (Figures 2 and 3, pages 74-75)). Of note, macro-dissected tissues were used in this study instead of micro-dissected tissues to gather enough pancreatic cancer cells for mRNA analysis so as not to have to resort to RNA amplification, a method we showed alters gene expression profiles. As shown in Table 1 (page 131), several HH pathway genes are significantly overexpressed (bolded text) in macro-dissected PAC tissues (n = 16) relative to uninvolved pancreas (n = 9). Some of these genes have previously been reported as being overexpressed in PAC (*SHH*, *IHH*, *PTCH1*, *SMO* and *GLI1*) (Kayed et al., 2004; Thayer et al., 2003) while others (*DHH*, *DISP2*, *PTCH2*, *GLI2* and *GLI3*) have not. Interestingly, the expression of *KIF27*, a homolog of the Drosophila Costal-2 gene and an inhibitor of Gli transcription factors, was markedly decreased (nearly 40-fold) in PAC compared to uninvolved pancreas suggesting down-regulation of

this gene may play a role in aberrant HH signaling. The expression of another Gli inhibitor, *SUFU*, however, was not significantly different between PAC and uninvolved pancreas. Alternatively, gene expression of other inhibitors of HH signaling including *LRP2*, *RAB23* and *GSK3B* was significantly increased in PAC. *LRP2* encodes for the receptor Megalin, which binds to HH ligands with high affinity (McCarthy et al., 2002). While this process is believed to have a negative effect on HH signaling, it has recently been suggested there might be a positive role for the receptor in trafficking HH ligand to other parts of the cell membrane (Morales et al., 2006). The GTPase encoded by *RAB23* also plays a role in cellular trafficking and may have similar positive and negative influences on HH signaling (Evans, Ferguson, Wainwright, Parton, & Wicking, 2003). The serine/threonine kinase encoded by *GSK3B*, however, possesses a larger degree of complexity because of its involvement in several different cell signaling pathways, not just HH (Cohen & Frame, 2001). It could be the influence of these other pathways that accounts for the overexpression of *GSK3B* in PAC. Previous reports, however, suggest that down-regulation of Gsk-3β plays a role in carcinogenesis since it phosphorylates and deactivates several oncogenic factors such as c-Myc, β-catenin, Snail and Gli (Jope, Yuskaitis, & Beurel, 2007). Whether this is the case for Gsk-3β in pancreatic cancer has yet to be determined.

In addition to HH pathway genes, genes that are known to be influenced by HH signaling were also examined in PAC. The caveat to this examination is that differences in gene expression between PAC and uninvolved pancreas may not be due to HH signaling since most of the genes analyzed influence, and are themselves influenced by, other cellular pathways. One such pathway is the cell cycle, which has previously been shown

to interact with HH signaling (Roy & Ingham, 2002). *CCNB1*, *CCNE1*, *CCNE2*, *CDKN1A* and *E2F1*, key mediators of cell cycle progression, were all significantly over-expressed in PAC. These results are in agreement with previous studies performed in pancreatic cancer (Biankin et al., 2001; Ito et al., 2002; Yamazaki et al., 2003; Yue, Yu, Zhao, Song, & Feng, 2003). Mitogenic factors such as *PDGFRA*, *TGFA* and *TGFB1*, whose expression can be influenced by Gli transcription factors, were also overexpressed in PAC. These results are also in agreement with previous reports (Barton, Hall, Hughes, Gullick, & Lemoine, 1991; Ebert et al., 1995; Jonson et al., 2001). *EGFR* and *EGF* expression has previously been shown to be increased in PAC (Yamanaka et al., 1993); however, in this study, it was found that *EGFR* expression was not significantly different between PAC and uninvolved pancreas and the expression of *EGF* was significantly decreased (almost 30-fold). It is possible that since these genes are expressed in the desmoplastic stroma of pancreatic cancers (Korc, 2007), their expression was altered when we macro-dissected PAC tissues to enrich cancer cell content. This may also explain the unexpected decrease in *MYC* expression, which has previously been shown to be increased in PAC (Lin, Liu, & Li, 1988). Of note, EGFR inhibitors have shown limited to no benefit in the treatment of pancreatic cancer (Moore et al., 2007; Xiong et al., 2004). Other genes more closely regulated by the HH pathway, including the transcription factors *EN2*, *SNAI1*, *SNAI2* and *CTNNB1*, were all overexpressed in PAC with *EN2* having not been previously identified in pancreatic cancer. Expression of the K-*ras* oncogene, a well-defined key mediator of pancreatic carcinogenesis and the potential "trigger" for aberrant HH signaling (Ji et al., 2007), was also overexpressed in PAC. Collectively, the data presented in Table 1 agrees with previous reports on gene expression profiles in

PAC and indicates that the expression of several HH pathway genes and downstream targets of HH signaling are significantly increased in PAC compared to uninvolved pancreas.

In completing our first specific aim, we found that molecular analysis of HH pathway expression using TLDA was affected by tissue heterogeneity but not by different methodologies used for tissue collection (surgical resection versus fine-needle aspiration) or storage (snap-frozen versus archival paraffin-embedded). The ability to analyze EUS-FNA biopsies is important since only 10-15% of PAC patients are candidates for surgical resection and examination of EUS-FNA biopsies would allow future studies to include patients presenting with more advanced, unresectable disease. As demonstrated by the chemoradiation study in which it was found that HH pathway gene expression is not significantly altered within the first 2 weeks of chemoradiotherapy (Figure 4, page 76), EUS-FNA biopsies can be used for multiple sampling from a single patient to determine the modulation of gene expression before, during and after treatment. In addition, the significant overexpression of HH pathway members in all PAC specimens examined as well as the localization to neo-plastic cells suggests this pathway may be a valuable tumor-associated therapeutic target.

Our second specific aim focused on identifying genes associated with response and/or resistance to HH pathway inhibition. The identification of such genes could ultimately be used to develop a molecular basis for the rational selection of newly diagnosed pancreatic cancer patients who may be candidates for HH inhibitor therapy. Currently, specific genes in the HH pathway that could be used as molecular markers of response remain unknown. The completion of the second aim was initially approached by deter-

mining differential response (IC_{50} values) to cyclopamine, a known HH antagonist, in a panel of 11 pancreatic cancer cell lines. Subsequent studies focused on obtaining the expression profile of 46 genes directly associated with or influenced by the HH pathway for each cell line (prior to treatment). Genes associated with response were identified by a significant statistical concordance between gene expression and IC_{50} values. To evaluate their role as indicators of response to HH pathway inhibition, the expression of these genes was modulated *in vitro* using siRNA and subsequent changes in response to cyclopamine were examined. The completion of these studies led to several significant findings. A total of 10/11 pancreatic cancer cell lines demonstrated a dose-dependent decrease in cell proliferation following treatment with cyclopamine (1-30 μM). Tomatidine, a structurally related compound that does not inhibit HH signaling (Cooper et al., 1998), had little to no effect on cell proliferation suggesting that the decrease in cell proliferation induced by cyclopamine results from inhibition of the HH pathway. IC_{50} values varied > 5-fold from 8.79 to 45.09 μM (for HPAF-2 and S2013 cells, respectively) with no measurable response observed in MiaPaCa-2 cells (Table 1, page 108). *SMO*, *GLI3*, *EN1* and *GSK3B* mRNA levels significantly correlated with cyclopamine IC_{50} values (Figure 4, page 109). Knockdown of *SMO*, *EN1* and *GSK3B* mRNA levels using commercially available siRNA was achieved (as determined by Taqman PCR). However, these modifications did not have a significant impact on cyclopamine sensitivity. It is possible that the decrease in the mRNA levels of these genes was not sufficient to reduce their corresponding protein levels to such a degree that response to cyclopamine is altered. If these proteins are relatively stable and not easily degraded, they may have a long half-life and knockdown of protein expression could potentially take longer than the

amount of time used in these studies (24 hours siRNA pre-treatment + 96 hours cyclopamine treatment = 120 hours total). Alternative approaches to transient siRNA transfection, such as lentiviral delivery of shRNA directed against a gene of interest, could be used to more effectively knock down both mRNA and protein expression. In addition, these genes could be overexpressed, either transiently or stably, *in vitro* and then the effect this genetic modification has on cyclopamine response could be measured. Knockdown or overexpression would be confirmed at the protein level in order to delineate specific versus off-target effects.

Sustained knockdown (up to a 75% decrease) of *GLI3* mRNA levels, using 2 different siRNA sequences, was achieved in Panc-1 cells for the entire duration of cyclopamine treatment (Figure 5A, page 110). Interestingly, this resulted in a 2 to 3-fold increase in the sensitivity of Panc-1 cells to cyclopamine in comparison to mock transfection and siRNA controls (Figure 5B, page 110). This change in response is assumed to be the result of decreasing Gli-3 mRNA and protein expression and not due to an off-target effect. In order to verify that Gli-3 protein is decreased with siRNA treatment, it will be necessary to detect expression using Western blot analysis. The results from these siRNA experiments suggest that Gli-3 does play a functional role in mediating *in vitro* response to HH pathway inhibition. The molecular mechanisms by which this occurs, however, remains unclear. It could be speculated that increased expression of Gli-3, in its full-length, transcriptional activator form, promotes increased HH signaling that cannot be overcome by cyclopamine. It has been shown that in the presence of HH ligand, full-length Gli-3 translocates into the nucleus where it binds to the promoter of Gli-1 thus leading to transcriptional activation of HH target genes (Dai et al., 1999). Al-

ternatively, Gli-3 may influence other cellular pathways that provide protection against cell death (such as apoptosis) as suggested by the induction of caspase-3 cleavage following knockdown of *GLI3* expression (Figure 5C, page 111). There is some evidence to support this potential mechanism as studies have suggested that Gli-3 can regulate apoptosis during mammalian limb development (Aoto, Nishimura, Eto, & Motoyama, 2002; Bastida et al., 2004).

The completion of our second specific aim led us to conclude that HH gene expression profiles as well as response to cyclopamine varied in human pancreatic cancer cell lines and these differences allowed for the identification of potential indicators of response. Although correlative studies suggested four genes (*SMO*, *GLI3*, *EN1* and *GSK3B*) may be indicators of response to cyclopamine, only modulation of *GLI3* expression resulted in a significant change in response. These results indicate that Gli-3 appears to be a previously unknown indicator of response to cyclopamine To fully elucidate the molecular basis for Gli-3's role in mediating resistance to cyclopamine, additional studies, including those examining Gli-3 and its connection to programmed cell death, are warranted. One of these studies could be examining the effect of *GLI3* knockdown in other pancreatic cancer cell lines (possibly another cyclopamine-resistant cell line or a moderately cyclopamine-sensitive cell line) in order to determine if a similar increase in cyclopamine response, as that observed in Panc-1 cells, is achieved. Another study could focus on providing further evidence for Gli-3's role in regulating apoptosis. The caspase-3 cleavage observed after GLI3 knockdown is only one marker of apoptosis. Other markers such as cleavage of caspases-8 and -9, Bid and PARP could also be examined

using Western blot analysis. In addition, an independent assay such as Annexin V staining could be used to verify the occurrence of apoptosis.

To identify genes associated with *in vitro* response to cyclopamine, mRNA levels were quantified in cell lines prior to treatment. In order to carry out our third specific aim, which focused on examining the physiological and molecular changes that result from HH pathway inhibition in pancreatic cancer cells, it was necessary to examine gene expression after treatment as well. Elucidation of these molecular changes that result in a physiological response to HH pathway inhibition could ultimately provide a mechanistic basis for combining HH antagonists with other therapeutic agents to improve clinical outcome (e.g. synergistic effects). Since activation of the HH pathway appears to be an early event in the development of pancreatic cancer, these analyses may also be useful in understanding tumor etiology. Initial studies focused on determining changes in DNA synthesis and apoptosis in cells treated with cyclopamine by examining BrdU incorporation and caspase activation, respectively. To evaluate the molecular basis of response, subsequent studies examined changes in the expression of genes directly associated with or influenced by the HH pathway after cyclopamine treatment. The completion of these studies led to several significant findings. Cyclopamine, administered at its IC_{50} concentration, markedly reduced DNA synthesis in sensitive HPAF-2 cells ($IC_{50} \sim 8$ μM) (Figure 2A, page 106). This effect was also observed in Panc-1 cells ($IC_{50} \sim 30$ μM) treated at both 8 and 30 μM cyclopamine but to a lesser extent (Figure 2B, page 106). Western blot analyses showed that HPAF-2 cells had activation of caspases (-3, -8 and -9) and Bid and a marked decrease in the protein expression of PARP in response to 8 μM cyclopamine (IC_{50}) (Figure 3, page 107). The unexpected result of reduced PARP protein ex-

pression is in contrast to what has previously been reported to occur to this enzyme during apoptosis, which is cleavage of full-length PARP into an 86-kDa fragment (Ivana Scovassi & Diederich, 2004). One explanation for this discrepancy is that the cleavage fragment has a relatively short protein half-life and is therefore difficult to detect using Western blot analysis. Another explanation is that PARP is not being cleaved in cyclopamine-treated HPAF-2 cells. Instead, cyclopamine may be affecting the transcription or translation of PARP through an unknown mechanism, which results in reduced protein expression. Regardless of either explanation, the absence of PARP is important in terms of targeting cancer cells since this enzyme is critical for DNA damage repair. Panc-1 cells demonstrated no markers of apoptosis after exposure to 8 μM cyclopamine; however, exposure to 30 μM cyclopamine (IC_{50}) did result in some markers of apoptosis (i.e. cleavage of caspases-8 and -3) (Figure 3, page 107).

Cyclopamine altered the expression of genes involved in cell cycle maintenance that are known to be influenced by HH signaling (Roy & Ingham, 2002) (Figure 6, page 112). Sensitive HPAF-2 cells had significant decreases in *CCND1*, *CCND3*, *CCNE1*, *CCNE2* and *E2F1* (all of which mediate G1/S phase transition) mRNA levels that appeared to be transient after 36 hours of cyclopamine exposure. The decrease in expression of these genes (with the exception of *CCND1*), however, continued even after 96 hours of cyclopamine treatment in relatively resistant Panc-1 cells. In addition, mRNA levels of *CDKN1A*, an inhibitor of the cell cycle, increased in treated HPAF-2 but not Panc-1 cells. Collectively, these data suggest that HPAF-2 cells could be undergoing cell cycle arrest earlier than Panc-1 cells as a result of cyclopamine exposure with apoptosis occurring later. This might explain why HPAF-2 cells appear to be farther along than

Panc-1 cells in terms of apoptotic events after exposure to cyclopamine (Figure 3, page 107). Cell cycle arrest and apoptosis combined could therefore account for the decreased proliferation of both treated HPAF-2 and Panc-1 cells with apoptosis possibly playing a larger role in the physiological response of HPAF-2 cells to cyclopamine. It could also be suggested that proliferation of Panc-1 cells is not affected by cyclopamine to the extent that HPAF-2 cells are because *CCND1* and *CDKN1A* expression do not change and *CCND2* expression markedly increases (up to 15-fold) after treatment. Additional studies will need to be carried out in order to substantiate these findings. These include: (1) validating the Western blot analysis of apoptosis markers, possibly using an independent assay such as Annexin V staining, (2) determining if the decreased gene expression of cell cycle mediators observed after cyclopamine exposure correlates with decreased protein expression (Western blot analysis) since post-translational modification can have a profound effect on cell cycle activity and (3) examining the physiological and molecular effects of cyclopamine treatment in a moderately sensitive cell line (e.g. Panc 2.03 or AsPC-1) in order to better understand the differences in cyclopamine effects between HPAF-2 and Panc-1 cells.

Previous reports have shown that cyclopamine decreases expression of HH target genes *in vitro*, in particular *PTCH1* and *GLI1* (Berman et al., 2002; Feldmann et al., 2007; Mukherjee et al., 2006). Similar decreases were observed in this study for Panc-1 but not HPAF-2 cells (Figure 7, page 113). In addition, cyclopamine significantly decreased the expression of other HH pathway genes that have not been previously examined in treated pancreatic cancer cells. These include the HH ligands *DHH* and *IHH*, the HH receptors *PTCH2* and *SMO*, the HH transcription factors *GLI2* and *GLI3* and the HH

transcriptional target *EN1*. While there was no apparent decrease in *PTCH1* and *GLI1* mRNA levels in treated HPAF-2 cells, there was a decrease in *PTCH2* and *GLI2* expression indicating that cyclopamine may suppress HH signaling differently in different cell lines (i.e. cell line-specific effects). Interestingly, cyclopamine decreased the expression of genes identified as possible indicators of response, *SMO*, *GLI3* and *EN1*, further suggesting their importance in the study of variable response to HH pathway inhibition. Of note, in studies conducted with tomatidine, the expression of these 3 genes in addition to *PTCH1* and *GLI1* did not significantly change in treated (30 μM) Panc-1 cells (data not shown). Collectively, these data suggest that cyclopamine targets members of the HH signaling pathway, which is in agreement with previous studies on this HH antagonist (Chen et al., 2002; Chen et al., 2002; Cooper et al., 1998).

The completion of our third specific aim led us to conclude that cyclopamine decreases pancreatic cancer cell proliferation through both decreased DNA synthesis and apoptosis. Further, treatment with cyclopamine appears to negatively influence cancer cell proliferation by regulating the expression of cell cycle mediators. A simplified mechanism by which this might occur as well as the role Gli-3 might play in mediating response to cyclopamine is illustrated in Figure 1 (page 132). The overexpression of cyclins B1, E1 and E2 and E2F1 in clinical PAC specimens (Table 1, page 131) combined with the finding that cyclopamine appears to target the cell cycle creates an interesting prospect in terms of potential therapeutic strategies for pancreatic cancer. Cyclopamine's effect on pancreatic tumors is potentially two-fold in that it could target both aberrant HH signaling and cell cycle progression. There is also the potential for combination therapy in terms of pairing HH inhibitors with compounds that selectively target cell cycle activ-

ity. For example, roscovitine and flavopiridol inhibit the cell cycle by interfering with cyclin-dependent kinase activity (De Azevedo et al., 1997; Kaur et al., 1992). Both of these compounds have shown promise as anti-cancer agents in the treatment of pancreatic cancer (Iseki, Ko, Xue, Seapan, & Townsend, 1998; Jung, Motwani, & Schwartz, 2001) and may be appropriate to combine with HH inhibitors. Future studies (both *in vitro* and *in vivo*) evaluating potential synergistic effects between HH antagonists and other therapies may, ultimately, provide a basis for future clinical applications.

Future Directions

Specificity of Cyclopamine

A major concern of this dissertation research is the specificity of cyclopamine for inhibiting HH pathway activity since micromolar concentrations are required to elicit physiological responses *in vitro* (i.e. decreased cell proliferation and induction of apoptosis). In order to address this concern, it is important to mention that other anti-cancer agents (e.g. cisplatin, doxorubicin and etoposide) require similar concentrations to inhibit cell growth and/or induce apoptosis in pancreatic cancer cell lines (Muerkoster et al., 2005; Shafaee, Schmidt, Du, Posner, & Weichselbaum, 2006; Zhao et al., 2006). Also important are previous studies that have demonstrated the selectivity of cyclopamine for Smo, an essential component of HH signaling. It has been demonstrated that a mutated, constitutively activated form of Smo (W539L; SmoA1) diminishes cyclopamine's ability to inhibit HH pathway activity, as measured by a GLI-dependent luciferase reporter assay, in comparison to wild-type Smo (Taipale et al., 2000). In addition, it has been demonstrated that SAG, a chlorobenzothiopene-containing Smo agonist, antagonizes cyclo-

pamine's inhibition of HH pathway activity as determined by the GLI-luciferase reporter assay (Chen et al., 2002). This assay, commonly used in conjunction with quantifying *GLI1* mRNA levels, is used to measure HH pathway activity since *GLI1* is a transcriptional target of HH signaling (Taipale et al., 2000). Currently, this is the only functional assay used to measure HH pathway activity (Riobo & Manning, 2007).

It is important to note, however, that a decrease in GLI-reporter activity does not always correlate with the physiological effects observed after cyclopamine treatment, such as decreased cell proliferation. The converse of this is also true in that a decrease in cell proliferation is not always accompanied by a decrease in GLI-reporter activity after cyclopamine treatment. Both of these effects were reported by Mukherjee et al. (2006) in a study of HH signaling and response to cyclopamine in breast cancer cell lines. Therefore, in experiments designed to test the selectivity of cyclopamine for Smo, it will be necessary to demonstrate not only changes in HH pathway activity (as determined by the GLI-reporter assay) but also changes in physiological response (i.e. cell proliferation and/or viability). One experiment testing the ability of cyclopamine to target HH signaling could involve overexpressing the constitutively active form of Smo (SmoA1) in a pancreatic cancer cell line relatively sensitive to cyclopamine (e.g. HPAF-2) and determining if the effects of this compound (i.e. decreased cell proliferation and induction of apoptosis) are lessened or abolished. Another experiment could involve pre-treating pancreatic cancer cells with SAG and then treating these cells with cyclopamine in order to determine, once again, if the effects of cyclopamine can be abrogated. A complimentary experiment could involve pre-treating cells with cyclopamine and then treating them with SAG to determine if the actions of SAG (e.g. increased GLI-reporter activity) can be an-

tagonized. Yet another experiment could involve knocking down Smo expression (both gene and protein) *in vitro* and determining if pancreatic cancer cells undergo changes similar to those observed after cyclopamine treatment.

KRAS as a Mechanism of Resistance to Cyclopamine

Another concern of cyclopamine is that targeting the HH pathway through Smo may not be sufficient to inhibit HH signaling. In a study performed by Ji et al. (2007), it was demonstrated that oncogenic, constitutively active KRAS can signal through the RAF/MEK/MAPK pathway to up-regulate GLI expression *in vitro*, thereby activating HH signaling independent of ligands and/or receptors. The results of this study suggest that cell lines with mutant KRAS could potentially bypass the inhibitory effects of cyclopamine. This issue is briefly mentioned by Ji et al.; however, the matter has yet to be investigated further. Therefore, the role of KRAS in HH pathway activation should be considered as a possible mechanism of resistance to cyclopamine. In order to test this mechanism, experiments would focus on pancreatic cancer cell lines that are relatively resistant to cyclopamine. One cell line would have mutant KRAS (e.g. Panc-1) and another, wild-type KRAS (e.g. BxPC-3) to use as a positive and negative control, respectively. Both cell lines could be pre-treated with siRNA directed specifically against mutant KRAS (commercially available) and then treated with cyclopamine to determine if targeting constitutively active KRAS increases sensitivity to cyclopamine (possibly through a synergistic mechanism). To confirm the results of this experiment, another experiment could involve pre-treating these same cell lines with inhibitors of MEK (e.g. U0126 and PD98059), a downstream component of the Ras signaling cascade (Van Aelst,

Barr, Marcus, Polverino, & Wigler, 1993), and then treating them with cyclopamine to determine if a similar effect is observed as in the mutant KRAS knockdown experiments.

Functional Role of Gli-3 in Mediating Resistance to Cyclopamine

The possible role of KRAS in mediating resistance to cyclopamine is important to consider for this dissertation research. However, the genes identified as potential indicators of response to cyclopamine in these studies (*SMO*, *GLI3*, *EN1* and *GSK3B*) could be equally as important. In particular, *GLI3* gene expression (prior to treatment) was found to correlate with response to cyclopamine (IC_{50} values) in pancreatic cancer cell lines (Figure 4, page 109) and was significantly decreased (up to 3.14-fold) after cyclopamine treatment (Figure 7, page 113). Knockdown of *GLI3* mRNA levels using siRNA significantly increased *in vitro* sensitivity to cyclopamine (Figure 5B, page 110) indicating that Gli-3 appears to be a previously unknown indicator of response to this compound. This change in response is assumed to be the result of decreasing Gli-3 mRNA and protein expression and not due to an off-target effect. In order to verify that Gli-3 protein is decreased with siRNA treatment, it will be necessary to detect expression using Western blot analysis. Detection of Gli-3 protein by our laboratory has been unsuccessful since commercially available antibodies were found to be non-specific. Efforts are currently underway to develop a specific antibody capable of detecting endogenous Gli-3 protein.

The results from these siRNA experiments suggest that Gli-3 does play a functional role in mediating *in vitro* response to HH pathway inhibition. The molecular mechanisms by which this occurs, however, remains unclear. It could be speculated that increased expression of Gli-3, in its full-length, transcriptional activator form, promotes

increased HH signaling that cannot be overcome by cyclopamine. It has been shown that in the presence of HH ligand, full-length Gli-3 translocates into the nucleus where it binds to the promoter of Gli-1 thus leading to transcriptional activation of HH target genes (Dai et al., 1999). In addition, Haycraft et al. (2005) demonstrated that cilia formation may be required for the processing and function of Gli-3 in order to sustain proper HH signaling during embryological development. While the role of cilia in cancer has yet to be explored, studying these organelles may provide a unique opportunity to elucidate the mechanisms of Gli-3 activity. Alternatively, Gli-3 may influence other cellular pathways (other than HH) that provide protection against cell death (such as apoptosis) as suggested by the induction of caspase-3 cleavage following knockdown of *GLI3* expression (Figure 5C, page 111). There is some evidence to support this potential mechanism as studies have suggested that Gli-3 can regulate apoptosis during mammalian limb development (Aoto et al., 2002; Bastida et al., 2004).

To further investigate the importance of Gli-3 in mediating response to HH pathway inhibition, it will be necessary to address the issues of (1) cyclopamine specificity and (2) the potential role of mutant KRAS in activating HH signaling. In terms of the first issue, if cyclopamine is not a good model for HH antagonism, then the role of Gli-3 as an indicator of response to HH pathway inhibition is called into question. In order to address this concern, experiments could be performed to determine if knockdown of *GLI3* increases sensitivity to other, known inhibitors of HH signaling. HH inhibitor compounds developed by the pharmaceutical companies Myriad and Genentech would be available for these experiments. In terms of the second issue, mutant KRAS might account for resistance to HH pathway inhibition rather than Gli-3. It is possible that KRAS

and Gli-3 work through different mechanisms in order to confer resistance to HH antagonism and, therefore, Gli-3 is still a valid indicator of response. This is further indicated by the finding in this dissertation that cell lines sensitive to cyclopamine have mutant KRAS (e.g. HPAF-2) but have low to undetectable gene expression levels of *GLI3*. Despite this finding, the issue of mutant KRAS-mediated resistance to cyclopamine and its possible relationship to Gli-3 should be addressed. Experiments could be performed to determine if knockdown of *GLI3* increases sensitivity to HH pathway inhibition in pancreatic cancer cell lines with either mutant or wild-type KRAS. Experiments in this dissertation focused on knocking down *GLI3* in Panc-1 cells (Figure 5, page 110), which are known to have mutant KRAS (Sipos et al., 2003). These experiments could be repeated in BxPC-3 cells, which have wild-type KRAS (Sipos et al., 2003), are relatively resistant to cyclopamine (Table 1, page 108) and, interestingly, have the most *GLI3* expression of the 11 pancreatic cancer cell lines examined, in order to determine if mutant KRAS can affect the ability of *GLI3* knockdown to increase sensitivity to HH pathway inhibition.

If these experiments further suggest that Gli-3 is an important mediator of resistance to HH pathway inhibition, then it will be necessary to determine the molecular basis by which this occurs. This could be approached, initially, by reconstituting *GLI3* mRNA levels in pancreatic cancer cell lines that have little or no expression of this gene (e.g. HPAF-2). Currently, the transcriptional targets of Gli-3 remain unknown so it would be interesting to examine the changes in gene expression that are occurring as a result of *GLI3* modulation and determine if these changes provide a molecular basis for resistance to HH pathway inhibitors such as cyclopamine. Changes in the expression of genes involved in a variety of pathways, including cell cycle, apoptosis and HH, could be

examined using TLDA. Alternatively, the molecular changes resulting from *GLI3* knockdown could also be examined using TLDA. These initial studies would be validated at the functional (protein) level and would require *in vivo* modeling as a basis for future clinical applications.

Gli-3 as a Potential Therapeutic Target for Pancreatic Cancer

In addition to Gli-3's potential role as an indicator of response to HH pathway inhibition, there is a separate aspect of Gli-3 to consider that is unrelated to cyclopamine and HH antagonism. Gli-3 might be an important component of pancreatic cancer cell growth and/or viability. This is suggested, in particular, by the finding that knockdown of *GLI3* alone decreased proliferation of (Figure 5B, page 110) and induced caspase-3 cleavage in (Figure 5C, page 111) Panc-1 cells. These results will certainly need to be demonstrated in other pancreatic cancer cell lines. In addition, future studies will require focusing on the cellular mechanisms by which Gli-3 potentially dictates cancer cell fate. These mechanisms could initially be identified by overexpressing *GLI3* and determining what changes in gene expression are occurring using TLDA (as suggested above). The importance of determining if Gli-3 plays a crucial role in pancreatic cancer becomes evident when considering that *GLI3* gene expression was found to be significantly increased (7.11-fold) in clinical PAC specimens in comparison to uninvolved pancreas (Table 1, page 131). This suggests that expression of Gli-3 is tumor-associated and that targeting this molecule may prove useful in developing potentially novel treatments for pancreatic cancer.

Table 1

Differences in Gene Expression Between Uninvolved Pancreas and PAC

Gene Name (Symbol)	Fold Change*	*p* -value†
Hedgehog Pathway Genes		
Sonic Hedgehog (SHH)	**74.25 ↑**	**0.0005**
Indian Hedgehog (IHH)	**3.62 ↑**	**0.0327**
Desert Hedgehog (DHH)	**7.26 ↑**	**0.0078**
Hedgehog acyl transferase (HHAT)	1.34 ↓	0.5852
Dispatched homolog 1 (DISP1)	1.52 ↓	0.4787
Dispatched homolog 2 (DISP2)	**5.97 ↑**	**0.0491**
Tout velu homolog 1 (EXT1)	1.95 ↑	0.1617
Tout velu homolog 2 (EXT2)	1.99 ↑	0.1941
Hedgehog interacting protein (HHIP)	1.26 ↓	0.7262
Megalin (LRP2)	**5.28 ↑**	**0.0474**
Open brain homolog (RAB23)	**2.84 ↑**	**0.0060**
Patched 1 (PTCH1)	**6.48 ↑**	**0.0001**
Patched 2 (PTCH2)	**17.61 ↑**	**0.0005**
Smoothened (SMO)	**16.35 ↑**	**0.0000**
Fused homolog (STK36)	1.39 ↑	0.6442
Suppressor of Fused homolog (SUFU)	1.82 ↑	0.2780
Costal-2 homolog (KIF27)	**39.35 ↓**	**0.0000**
Glycogen synthase kinase 3-beta (GSK3B)	**3.69 ↑**	**0.0010**
Glioma-associated oncogene 1 (GLI1)	**18.62 ↑**	**0.0002**
Glioma-associated oncogene 2 (GLI2)	**11.35 ↑**	**0.0000**
Glioma-associated oncogene 3 (GLI3)	**7.11 ↑**	**0.0011**
Genes Influenced By Hedgehog Signaling		
Cyclin B1 (CCNB1)	**16.07 ↑**	**0.0000**
Cyclin D1 (CCND1)	1.64 ↓	0.3325
Cyclin D2 (CCND2)	3.55 ↓	0.0576
Cyclin D3 (CCND3)	1.74 ↑	0.2603
Cyclin E1 (CCNE1)	**4.37 ↑**	**0.0431**
Cyclin E2 (CCNE2)	**7.76 ↑**	**0.0009**
p21, Cip1 (CDKNA1)	**4.89 ↑**	**0.0111**
E2F1 transcription factor (E2F1)	**5.69 ↑**	**0.0045**
Retinoblastoma 1 (RB1)	1.36 ↓	0.5692
c-Myc oncogene (MYC)	**3.32 ↓**	**0.0416**
Epidermal growth factor ligand (EGF)	**28.84 ↓**	**0.0002**
Epidermal growth factor receptor (EGFR)	1.16 ↑	0.7481
Platelet-derived growth factor receptor alpha (PDGFRA)	**3.66 ↑**	**0.0001**
Transforming growth factor alpha (TGFA)	**3.59 ↑**	**0.0076**
Transforming growth factor beta-1 (TGFB1)	**7.96 ↑**	**0.0000**
Engrailed homolog 1 (EN1)	3.03 ↑	0.3292
Engrailed homolog 2 (EN2)	**15.44 ↑**	**0.0169**
Notch homolog 1 (NOTCH1)	1.58 ↑	0.4061
Notch homolog 2 (NOTCH2)	1.43 ↑	0.4396
Snail homolog 1 (SNAI1)	**21.13 ↑**	**0.0003**
Snail homolog 2 (SNAI2)	**8.56 ↑**	**0.0000**
K-ras oncogene (KRAS2)	**17.32 ↑**	**0.0000**
Bcl-2 oncogene (BCL2)	1.65 ↓	0.3518
Beta-Catenin (CTNNB1)	**2.02 ↑**	**0.0194**
Archipelago homolog (FBXW7)	1.52 ↑	0.4084

*Relative to uninvolved pancreas

†$p<0.05$ was considered statistically significant; Calculated using Student's *t* -test

Bolded text indicates significant differences in gene expression

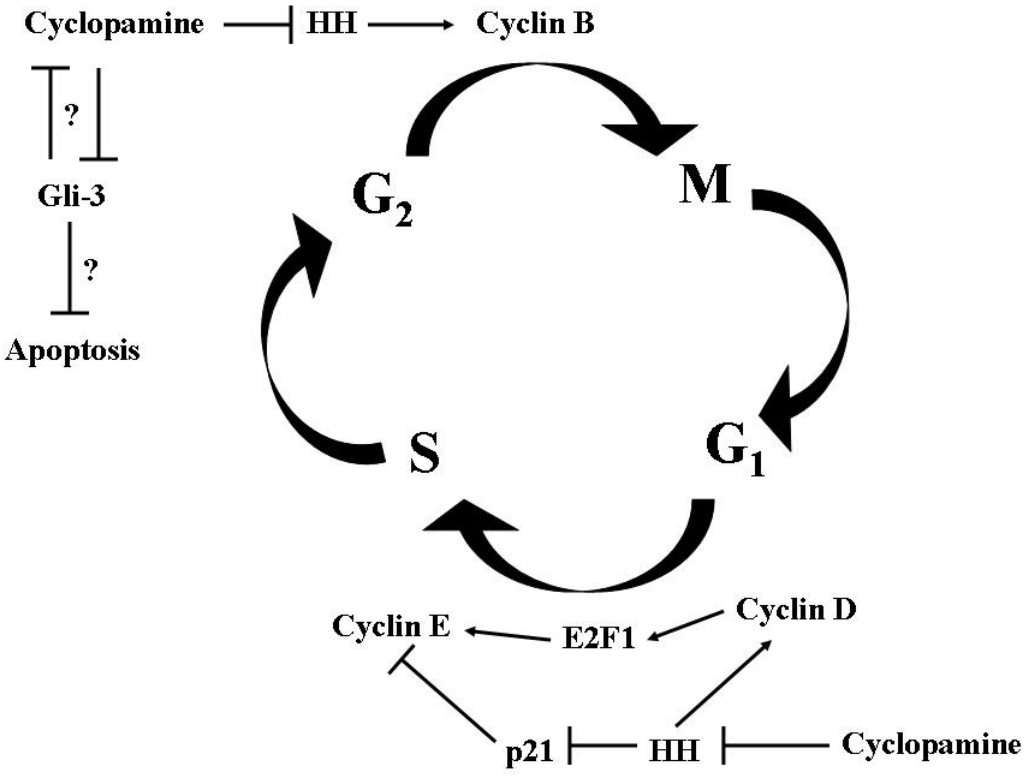

Figure 1. Potential mechanisms by which cyclopamine inhibits pancreatic cancer cell proliferation. Because cell cycle mediators have been shown to be influenced by HH signaling, cyclopamine's antagonism of the HH pathway leads to a decrease in the expression and possibly activity of cyclins B, D and E and E2F1. HH signaling inhibits p21, an inhibitor of cell cycle progression and, accordingly, cyclopamine promotes p21 expression. Increased expression of Gli-3 appears to confer resistance to cyclopamine. Cyclopamine, in turn, can decrease Gli-3 expression. The mechanisms by which these events occur remain unknown. Gli-3 might play an important role in determining cell fate due to its potential connection with apoptosis.

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APPENDIX

INSTITUTIONAL REVIEW BOARD FOR HUMAN USE APPROVAL FORMS

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Review Board for Human Use

Form 4: IRB Approval Form
Identification and Certification of Research
Projects Involving Human Subjects

UAB's Institutional Review Boards for Human Use (IRBs) have an approved Federalwide Assurance with the Office for Human Research Protections (OHRP). The UAB IRBs are also in compliance with 21 CFR Parts 50 and 56 and ICH GCP Guidelines. The Assurance became effective on November 24, 2003 and expires on February 14, 2009. The Assurance number is FWA00005960.

Principal Investigator: STEG, ADAM

Co-Investigator(s):

Protocol Number: **X070917011**

Protocol Title: *Analysis of the Hedgehog Pathway in Pancreatic Adenocarcinoma*

The IRB reviewed and approved the above named project on 10-02-07. The review was conducted in accordance with UAB's Assurance of Compliance approved by the Department of Health and Human Services. This Project will be subject to Annual continuing review as provided in that Assurance.

This project received EXPEDITED review.

IRB Approval Date: 10-02-07

Date IRB Approval Issued: 10-02-07

HIPAA Waiver Approved?: Yes

Marilyn Doss

Marilyn Doss, M.A.
Vice Chair of the Institutional Review
Board for Human Use (IRB)

Investigators please note:

The IRB approved consent form used in the study must contain the IRB approval date and expiration date.

IRB approval is given for one year unless otherwise noted. For projects subject to annual review research activities may not continue past the one year anniversary of the IRB approval date.

Any modifications in the study methodology, protocol and/or consent form must be submitted for review and approval to the IRB prior to implementation.

Adverse Events and/or unanticipated risks to subjects or others at UAB or other participating institutions must be reported promptly to the IRB.

470 Administration Building
701 20th Street South
205.934.3789
Fax 205.934.1301
irb@uab.edu

The University of
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AB 470
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BIRMINGHAM AL 35294-0104

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Review Board for Human Use

PI: STEG, ADAM
Protocol # X070917011

UAB IRB Approval of
Waiver of Informed Consent and/or Waiver of Patient Authorization

☑ **Approval of Waiver of Informed Consent to Participate in Research.** The IRB reviewed the proposed research and granted the request for waiver of informed consent to participate in research, based on the following findings:

1. The research involves no more than minimal risk to the subjects.
2. The research cannot practicably be carried out without the waiver.
3. The waiver will not adversely affect the rights and welfare of the subjects.
4. When appropriate, the subjects will be provided with additional pertinent information after participation.

Check one:
- ☑ **and** Waiver of Authorization (below)
- ☐ **or** Waiver of Authorization (below)
- ☐ Waiver of Authorization not applicable

☑ **Approval of Waiver of Patient Authorization to Use PHI in Research.** The IRB reviewed the proposed research and granted the request for waiver of patient authorization to use PHI in research, based on the following findings:

1. The use/disclosure of PHI involves no more than minimal risk to the privacy of individuals
   - i. There is an adequate plan to protect the identifiers from improper use and disclosure.
   - ii. There is an adequate plan to destroy the identifiers at the earliest opportunity consistent with conduct of the research, unless there is a health or research justification for retaining the identifiers or such retention that is otherwise required by law.
   - iii. There is an assurance that the PHI will not be reused or disclosed to any other person or entity, except as required by law, for authorized oversight of the research study, or for other research for which the use or disclosure of PHI would be permitted.
2. The research cannot practicably be conducted without the waiver or alteration.
3. The research cannot practicably be conducted without access to and use of the PHI.

—OR—

☐ **Full Review**
The IRB reviewed the proposed research at a **convened meeting** at which a majority of the IRB was present, including one member who is not affiliated with any entity conducting or sponsoring the research, and not related to any person who is affiliated with any of such entities. The partial waiver of authorization for screening was approved by the majority of the IRB members present at the meeting.

Date of Meeting

Signature of Chair, Vice-Chair or Designee

Date

☑ **Expedited Review**
The IRB used an **expedited review procedure** because the research involves no more than minimal risk to the privacy of the individuals who are the subject of the PHI for which use or disclosure is being sought. The review and approval of the partial waiver of authorization for screening was carried out by the Chair of the IRB, or by one of the Vice-Chairs of the IRB as designated by the Chair of the IRB.

10-02-07
Date of Expedited Review

Marilyn Davis
Signature of Chair, Vice-Chair or Designee

10-02-07
Date

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