HEDGEHOG SIGNALING IN HEPATOCELLULAR CARINOMA

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ABSTRACT

ISAAC S. CHAN: Hedgehog Signaling in Hepatocellular Carcinoma
(Under the direction of Dr. Anna Mae Diehl and Dr. Albert Baldwin)

Hepatocellular carcinoma (HCC) is a primary malignancy of the liver and one of the most common and deadly tumors worldwide. Most HCC cases are secondary to cirrhosis of the liver resulting from chronic liver injury states and dysregulated wound healing. While treatment of the cancer often involves resection, high recurrence rates are common, suggesting that cells outside the tumor play an important role in tumorigenesis. While the microenvironment is a key contributor to carcinogensis, the molecular mechanisms underlying their involvement remain unknown. Hedgehog (Hh) signaling has been found to play a critical role in liver regeneration after injury and Hh pathway activity is overexpressed in the pre-neoplastic and injured liver environment, leading us to hypothesize that it plays an essential role in the development and progression of HCC. This dissertation addresses the role of Hh signaling in HCCarcinogenesis and, specifically, the liver tumor microenvironment. Advances in this subject can lead to new therapeutic options for patients suffering from the burden of this disease.

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HCC	Hepatocellular carcinoma
HSC	Hepatic stellate cell
MF	Myofibroblasts
SHH	Sonic hedgehog

CHAPTER 1

INTRODUCTION

1.1. Hepatocellular carcinoma

Hepatocellular carcinoma (HCC) is a primary malignancy of the liver and is the third leading cause of cancer-related deaths worldwide. The incidence of HCC is nearly identical to the number of deaths per year (Jemal, Bray et al. 2011). In 2008, the incidence of HCC was estimated to be 16 cases per 100,000 individuals (Forner, Llovet et al. 2012), and has risen to over 700,000 cases diagnosed each year (Ferlay, Shin et al. 2010).

1.1.1 – Risk factors for HCC

The single largest risk factor for HCC is fibrosis and cirrhosis (Schuppan and Afdhal 2008). Patients with compensated liver cirrhosis have a 1-5% annual risk of HCC and over 56% of patients with HCC have undiagnosed cirrhosis (Forner, Llovet et al. 2012). Because all chronic liver diseases lead to cirrhosis, all are risk factors for HCC. The most common of these is viral hepatitis, caused by either hepatitis B virus, accounting for over half of HCC, or hepatitis C virus, accounting for a third of all cases in the US (Sherman 2010). Given the current positive response rates to antiviral therapy in the US, the incidence of HCC due to viral hepatitis is plateauing (Davis, Alter et al. 2010). Yet unexpectedly, overall incidence has increased by almost 50% in the past two decades, suggesting that other factors are contributing to HCC development (Forner, Llovet et al. 2012).

An emergent risk factor for HCC is non-alcoholic fatty liver disease (NAFLD). There is growing evidence of a correlation between HCC, NAFLD, and NALFD-associated conditions, such as obesity and diabetes: The yearly incidence of HCC in NALFD patients is 2.7% (Ascha, Hanouneh et al. 2010). Patients with a baseline body-mass index over 40

have five times higher risk for HCC development and in certain studies, diabetes patients are 4.5 times more likely to develop HCC (Adami, Chow et al. 1996; Calle, Rodriguez et al. 2003). While the exact mechanism underlying NAFLD HCC carcinogenesis remains unknown, it is thought that steatoic changes in NAFLD induces inflammatory and wound-healing responses that lead to cirrhosis and HCC. Other risk factors for HCC include alcoholic liver disease: Compared to teetotalers, patients who regularly consume alcohol are 3.6 times more likely to develop HCC on cirrhosis (Ascha, Hanouneh et al. 2010). Thus, regardless of the etiology of the underlying liver disease, most HCCs arise from an established background of cirrhosis.

1.1.2 – Molecular characteristics of HCC

Analysis of HCC tumors has revealed alterations in several genes causing multiple genetic networks to become dysregulated, reflective of a heterogenous molecular profile. These genetic alterations can include somatic gene mutations linked to cell proliferation and cell cycle regulation. The most frequently mutated gene in HCC is p53 and some reports have found it to be mutated in as many as 67% of HCCs (Coursaget, Depril et al. 1993). Other HCC associated gene mutations include both tumor suppressors and oncogenes such as p16 (Hui, Sakamoto et al. 1996), Rb (Zhang, Xu et al. 1994), Pten (Fujiwara, Hoon et al. 2000), Met (Wang, Ferrell et al. 2001), PI3K (Lee, Soung et al. 2005), B-catenin (Ishizaki, Ikeda et al. 2004), and Smoothened (Sicklick, Li et al. 2006). In addition, HCC harbors amplifications of Vegf (Miura, Miyazaki et al. 1997) and angiogenesis associated genes (Torimura, Ueno et al. 2004). The abundance of studies identifying key drivers of HCC merely point to the many genetic alterations that can result in the activation and dysregulation of a variety signaling pathways including Wnt, Hedgehog, MTOR, and Ras. These dysregulated signaling pathways have been implicated in supporting abnormal proliferation, angiogenesis, and metastasis in HCC.

1.1.3 – Diagnosis and treatment of HCC

In patients with increased risk for developing HCC (i.e. cirrhotic patients), the preferred methods for monitoring and diagnosis are ultrasonography (the gold standard) and serum alpha-fetoprotein (AFP) levels. Sensitivity for ultrasonography imaging ranges between 60-80% with a specificity exceeding 90%. However, the accuracy of AFP monitoring has been questioned. The sensitivity of AFP serum monitoring is at best 66% (Marrero, Feng et al. 2009) and even when combined with ultrasonography, detection rates improve by only 8% while considerably raising the number of false positives compared to ultrasonography alone (Zhang and Yang 1999). These results raise the need for the identification of better and more sensitive biomarkers that predict the presence of HCC.

Once identified, HCC is staged (Pons, Varela et al. 2005) and management is based on tumor stage and liver function. Management of HCC may involve ablation, resection, transplantation, chemoembolizastion, or chemotherapy. Unfortunately for HCC patients, few randomized control trials have been run comparing different treatment options and the collective data are conflicting (Llovet, Di Bisceglie et al. 2008). For early stage tumors (single nodules < 2 cm), first-line therapy often involves ablation, which has a 5-year survival of 50-70% (Cho, Kim et al. 2009). However, ablation techniques become increasingly less effective as nodule size and multifocality increases. Although hepatic resection is preferred treatment for noncirrhotic patients, it is estimated that only 5% of patients in the US qualify, and after 5 years, tumor recurrence occurs in over 70% of cases (Llovet, Schwartz et al. 2005). For patients with non-metastatic HCC and cirrhosis, liver transplantation is often curative for both, making HCC unique as the only solid tumor that can be “cured” through organ transplantation. According to one study, patients with one HCC nodule smaller than 5 cm or patients with up to three nodules of less than 3 cm treated with liver transplantation had four-year survival rates of 75% and less than 15% with recurrence (Mazzaferro, Regalia

et al. 1996). Despite these outstanding results, liver transplantation is limited as an option due to the shortage of organs available for transplant (Bruix and Sherman 2011). Furthermore, liver transplantation is feasible in only 5% of HCC patients due to underlying liver disease (Rougier, Mitry et al. 2007). Therefore, while surgical procedures are the first line for early stage HCC, many patients fail to qualify.

Currently, no systemic chemotherapies (e.g. doxorubicin) or hormonal therapies (e.g. tamoxifen, flutamide) have been shown to improve overall patient survival (Lai, Wu et al. 1988; Chao, Chan et al. 1996; Nowak, Findlay et al. 2004). The only FDA-approved therapeutic agent for late stage HCC is Sorafenib, an orally administered multikinase inhibitor that inhibits Raf signaling, VEGF, PDGF, and c-Kit (Bruix and Sherman 2011). Sorafenib has been shown to significantly increase overall survival by about three months (10.7 months vs. 7.9 months in placebo group) (Llovet, Ricci et al. 2008). Still, despite progress towards better therapies for HCC patients, more can be done. While this section is not meant to be an exhaustive listing of every HCC treatment tested in clinical trials, it highlights the very limited treatment options that are currently offered. There is a real patient-driven need for novel treatment therapies and understanding the molecular events in the pre-neoplastic environment leading to HCC could reveal new targets for better treatment.

1.2. Cirrhosis: the pre-neoplastic environment

Since the leading risk factor for HCC is fibrosis and cirrhosis, appreciating the pre-neoplastic state of the liver is important for the prevention and treatment of HCC. Chronic liver injury leads to fibrosis and cirrhosis, outcomes resulting from dysregulated (i.e., excessively fibrogenic) repair of liver injury. Initially part of the normal wound healing process, fibrosis can accumulate and become pathologic due to sustained signals

associated with chronic liver injury, leading to progressive accumulation of collagen matrix (Figure 1.1). As a result, liver function becomes impaired.

Liver scar tissue is composed of extracellular matrix comprised primarily of collagen. While several cells in the liver can produce collagen (Sedlaczek, Jia et al. 2001), liver myofibroblasts (MF) are the major producers of collagen in injured livers (Friedman 2008). Liver MF accumulation is stimulated by various injury-associated growth factors/cytokines, including platelet derived growth factor (PDGF), transforming growth factor (TGF) beta, and Hedgehog signaling (Pinzani, Knauss et al. 1991; Okuno, Moriwaki et al. 1997; Omenetti and Diehl 2008). Although different studies have shown that liver MF can be derived from many different sources including portal fibroblasts (Knittel, Kobold et al. 1999), hepatocytes (Zeisberg, Yang et al. 2007), cholangiocytes (Rygiel, Robertson et al. 2008), and bone marrow fibrocytes (Abe, Donnelly et al. 2001), sinusoidal hepatic stellate cells (HSC), which reside in the space of Disse (Figure 1.2), are the major source of MF in the liver (de Leeuw, McCarthy et al. 1984; Friedman 2008).

1.2.1. Cellular interactions contributing to fibrosis and cirrhosis

Many groups have focused their research on understanding the mechanisms that drive and maintain fibrogenic repair, which includes the interplay between a range of different liver cell populations. In particular, paracrine signaling between liver MF and other cells in the injured liver microenvironment can initiate and sustain fibrosis. Stressed and apoptotic hepatocytes can release signals such as CXC chemokines (Faouzi, Burckhardt et al. 2001; Canbay, Higuchi et al. 2002) or Hedgehog ligands (Rangwala, Guy et al. 2011) that can promote accumulation of liver MF. Other liver epithelial cells, such as cholangiocytes, can also promote MF accumulation during liver injury via PDGF secretion (Grappone, Pinzani et al. 1999). Reciprocally, liver MF can stimulate liver epithelial cells to release chemokine CXCL16 to recruit inflammatory NKT cells to perpetuate fibrosis

(Omenetti, Syn et al. 2009). In addition to producing collagen, MF can further contribute to the fibrotic environment by initiating paracrine signaling with liver epithelial cells to promote Hedgehog signaling (Omenetti, Porrello et al. 2008). MF also play an important role in angiogenesis (Ankoma-Sey, Matli et al. 1998; Ankoma-Sey, Wang et al. 2000), matrix remodeling through secretion of MMPs (Han, Yan et al. 2007), and the direct secretion of inflammatory signals such as TGF-β (Bachem, Meyer et al. 1992) or osteopontin (Syn, Choi et al. 2011) to help to sustain the inflammatory response associated with fibrosis.

1.2.3. The role of liver progenitors in injury and repair

Liver progenitors also play a major role in liver repair, although the source of these cells is still up for debate. In multiple chronic liver diseases, observations of an expansion of cells in the bile ducts and Canals of Herring (Figure 1.2) suggested that this ductular reaction was directly linked to liver progenitor proliferation (Tan, Hytiroglou et al. 2002; Richardson, Jonsson et al. 2007). Analysis of these cells in multiple species reveal they express multipotent stem cell markers (Cardinale, Wang et al. 2011), and Hedgehog (Omenetti, Yang et al. 2007), Notch (Fabris, Cadamuro et al. 2007), and Wnt (Apte, Thompson et al. 2008) signaling pathways have been implicated in their regulation. Others have shown by lineage tracing that HSC could be the source of liver progenitors (Yang, Jung et al. 2008). In this study, Yang et al. created a double transgenic mouse created by crossing a mouse with a HSC marker driving Cre and a LoxP reporter mouse. After liver injury, sinusoidal cells, ductular cells, and hepatocytes all expressed the reporter tag, meaning that liver epithelial cells could be the progeny of HSC cells or derived from the same lineage. The function of progenitors during liver injury is also disputed. Different groups have indicated that progenitors can give rise to hepatocytes during injury (Yang, Wang et al. 2008) or that they contribute to fibrosis by differentiating into myofibroblasts (Loo and Wu 2008). Paracrine signaling between activated HSC and progenitor cells can

also promote the expansion and accumulation of the progenitor population (Lin, Tang et al. 2008).

It is often thought that cancer is wound healing gone awry (Dvorak 1986). In injured livers, molecular signals not present in healthy livers become activated. As mentioned above, liver MF are heavily influential in the fibrogenic repair process via the paracrine signals they receive and secrete. These same signals are also pertinent in cancer. Given that HCCs emerge from this microenvironment, it is likely that cells and signals from the injured liver microenvironment contribute to progression of HCC.

1.3. The tumor microenvironment

A majority of research in the past 30 years has focused on the properties of tumor cells: sustaining proliferation, resisting apoptosis, evading growth suppressors, supporting angiogenesis, achieving replicative immortality, and eventual invasion and metastasis (Hanahan and Weinberg 2011). Our understanding of cancer cells has grown exponentially as we continue to develop new models and tools, such as whole genome sequencing (Cancer Genome Atlas Research Network 2008; Cancer Genome Atlas Research Network 2011; Cancer Genome Atlas Research Network 2012), to further appreciate how tumor cells acquire and maintain their neoplastic phenotypes. As a result, novel systemic therapies have been developed in response to our knowledge of a cancer cell's aberrant properties. Although some of these therapies are successful (e.g. imatinib), a majority are transient and moderately effective, while metastasis and recurrence looms in the future and dooms patient survival. These clinical obstacles underscore the multiple collaborations tumor cells have with their microenvironment, by recruiting or using neighboring cells to further drug resistance and malignancy.

1.3.1. A historical understanding of the tumor microenvironment

Early on, study of the tumor microenvironment focused on the ECM and endothelial cells. Specifically, it was thought that both corrupted components of the ECM (Dvorak, Dvorak et al. 1979) or corrupt endothelial cells (Folkman 1971) promoted an environment suitable for angiogenesis. In a study by Fukumura et al., orthotopic liver tumors or spontaneous breast tumors were transplanted or induced in mice expressing GFP under the control of the promoter for the angiogenic factor VEGF. In both tumor models, VEGF-GFP positive cells accumulated in the mesenchymal compartment of the tumors, demonstrating that the VEGF production occurs in fibroblasts, not tumor cells (Fukumura, Xavier et al. 1998). Since these initial studies, our understanding of the tumor microenvironment has broadened. Over the past decade, there is an evolving sense that the many cells that constitute the microenvironment can influence tumors beyond angiogenesis.

1.3.2. The role of the tumor microenvironment

The tumor microenvironment consists of a heterogenous mix of different cells that interact with the tumor cell to promote malignant phenotypes. One commonly studied example is the stromal production of mitogens that sustain the proliferative capacity of tumor cells: In a variety of tumors, virtually all studied stromal cell types, from immune cells to endothelial cells to fibroblasts, can secrete mitogenic and trophic factors such as EGF (Wang, Zhang et al. 2011), TGF-β (Rosenthal, McCrory et al. 2004), BMP2 (Mathieu, Sii-Felice et al. 2008), HGF (Kuperwasser, Chavarria et al. 2004; Verras, Lee et al. 2007), and FGF (Yan, Fukabori et al. 1992). Immune cells, endothelial cells, and fibroblasts also promote other malignant properties in tumor cells through similar juxtacrine signaling (Pietras and Ostman 2010). While some studies have revealed very direct relationships between stromal cells and cancer cells, other studies suggest a more intricate relationship. For instance, the secretion of both EGF by tumor associated macrophages and CSF-1 by

breast tumor cells is required for either cell to migrate, suggesting an almost commensal relationship between the two (Wyckoff, Wang et al. 2004). In another example, elevated VEGF signaling from stromal cells in the tumor microenvironment (Fukumura, Xavier et al. 1998) triggers angiogenesis and the creation of microvessels, which disrupt cellular tight junctions lining the vasculature, and leads to pericyte depletion and worse patient outcomes (Yonenaga, Mori et al. 2005). As a result, a pericyte-depleted microenvironment leads to increased hypoxic and EMT signaling within breast cancer cells, which promotes metastatic behavior (Cooke, LeBleu et al. 2012). Thus, interactions between cells in the tumor microenvironment are not restricted to one-dimensional signaling, but can be reciprocal and multi-layered.

1.3.3. Tumors as wounds that fail to heal

Since dysregulated wound healing is thought to advance malignancy, there has been renewed interest in the role of the cancer associated fibroblast (CAF). It was posited that since fibroblasts and their cousins, myofibroblasts (MF) have major roles in wound healing (i.e. they are key cells involved in matrix remodeling), these cells would also have cancer nourishing properties. One observation supporting this hypothesis is that following normal wound healing, the number of activated fibroblasts and MF decrease (Tomasek, Gabbiani et al. 2002) . Yet in the tumor microenvironment, fibroblasts and MF maintain a constant presence, perhaps as a corollary of organ fibrosis (Kalluri and Zeisberg 2006).

In more recent years, our understanding of interactions between fibroblasts and tumor cells has expanded and we now know that their contributions to tumorigenesis are multifaceted. In addition to their impact on tumor cell proliferation, CAFs can directly stimulate tumor angiogenesis (Orimo, Gupta et al. 2005), chemoresistance (Wang, Li et al. 2009), and metastasis (Jedeszko, Victor et al. 2009) through secretion of different factors and cytokines. CAFs also promote malignant and metastatic phenotypes through

reorganization and remodeling of ECM (i.e. increased fibrotic injury). In a prospective observational study of 439 patients with breast cancer, levels of fibrosis were found to be a potential prognostic parameter. Patients with increased fibrosis had poorer survival, higher tumor recurrence, and higher distant organ metastasis (Hasebe, Sasaki et al. 2002). Additional studies in glioblastoma (Huijbers, Iravani et al. 2010), breast cancer (Levental, Yu et al. 2009), and HCC (Zhao, Cui et al. 2010) suggest that increased ECM stiffness and increased fibrosis accompanies cancer progression and can induce invasive properties of cancerous epithelia. Moreover, the genetic or pharmacologic disruption of fibrosis prevented tumor progression in mouse models of breast cancer (Levental, Yu et al. 2009). Similarly, in a mouse model of pancreatic ductal adenocarcinoma, Olive et al. increased therapeutic delivery by ablating CAFs in the tumor microenvironment, which resulted in depleted desmoplasia surrounding the tumor and improved survival (Olive, Jacobetz et al. 2009). These studies support the idea that CAFs contribute to the tumor microenvironment through dysregulated wound healing and increased fibrosis, which supports a more aggressive tumor phenotype.

1.3.4. The tumor microenvironment in HCC

Recent studies in the liver have begun to unravel the relationship between liver MF, liver fibrosis, and HCC progression. In 2008, a large study undertaken by Hosida et al. analyzed tissues from 307 patients with HCC. Gene expression profiling was performed to identify a gene signature unique to patients with poor survival and time-to-recurrence. While profiling of the tumors failed to generate a consensus gene signature able to predict survival time, profiling of liver tissue surrounding the tumor led to a reproducible signature predictive of prognosis, indicative that stromal tissue plays a role in HCC progression (Hoshida, Villanueva et al. 2008). Furthermore, this poor-prognosis signature included genes commonly associated with liver MF, such as Fibrillin1, Col4a1, and Mmp7. Mikula et al.

demonstrated that liver MF can augment hepatocellularcarcinogenesis: Simultaneous subcutaneous injection of malignant hepatocytes with liver MF in mice resulted in a more aggressive xenografts than malignant hepatocytes alone, due to induction of TGF-β and epithelial-to-mesenchymal transitions in tumor cells (Mikula, Proell et al. 2006). Microarray analysis of hepatocytes in co-culture with liver MF revealed increased expression of genes associated with inflammatory cytokines and motility (Coulouarn, Corlu et al. 2012). In the same co-culture, analysis of liver MF reaffirmed previous studies that demonstrated increased angiogenic potential (VEGFA) and matrix remodeling (MMP9). While these studies demonstrate that crosstalk between malignant hepatocytes and liver MF are bidirectional and dynamic, much is still unknown about how liver MF drive hepatocellularcarcinogenesis and how malignant hepatocytes influence liver MF.

The complexity of the numerous relationships between stromal cells and tumor cells underscores the difficulty of fully comprehending the role of the microenvironment in tumor progression. To address this question, it is necessary to first understand how tumor cells and stromal cells can communicate. Research has been directed towards secreted factors that can initiate and engage paracrine signaling, such as TGF-β or various growth factors and cytokines. Other possibilities include morphogenic signaling pathways (e.g. Wnt, Notch, Hedgehog) and their ligands (β-catenin, Jagged, HH ligands), which play pivotal roles in fetal development, mediate crosstalk between epithelial and stromal compartments (Kolterud, Grosse et al. 2009), and become reactivated during adult injury and wound healing (Arwert, Hoste et al. 2012). One candidate pathway that fits these criteria is Hedgehog signaling pathway, which is essential during development and becomes reactivated during organ injury and dysregulated in cancer.

1.4. Hedgehog signaling, an overview

Our understanding of Hedgehog signaling came from humble and small beginnings. In 1980, Nüsslein-Volhard and Wieschaus set out to understand the genes regulating *Drosophila* development. To accomplish this, they used a chemical screen to generate mutants with malformed bodies, and as a result, first identified Hedgehog as a critical gene for body polarity and segmentation (Nusslein-Volhard and Wieschaus 1980). The gene mutation corresponded to an abnormal dentricle formation that produced flies which looked like hedgehogs, thus granting it its apropos name. In 1992, three labs independently cloned the Drosophila *hh* gene and showed that it encodes a secreted peptide, which paved the road for future discoveries of Hedgehog's role in development and disease (Lee, von Kessler et al. 1992; Mohler and Vani 1992; Tabata, Eaton et al. 1992).

Since then, we have found Hedgehog signaling to be a remarkably conserved pathway, critical in the development and patterning of many multicellular organisms (Ingham and McMahon 2001). Hedgehog is also well known for its role in limb (Riddle, Johnson et al. 1993; Harfe, Scherz et al. 2004), neural tube (Roelink, Porter et al. 1995), and organ development (Motoyama, Liu et al. 1998). The crucial role of Hedgehog signaling is validated by developmental defects that occur when Hedgehog signaling malfunctions. Mutations in the gene encoding SHH ligand in developing mammals can lead to holoencephalopathy (Belloni, Muenke et al. 1996). A similar malformation occurs after exposure to the teratogen cyclopamine, which inhibits Hedgehog signaling activity (Binns, James et al. 1962). Thus, proper Hedgehog signaling is required for normal development.

1.4.1. Hedgehog ligands

Under certain conditions, HH-ligand producing cells generate and secrete HH ligands into the environment. In mammals, three homologous Hh ligands have been identified, Sonic Hedgehog (SHH), Indian Hedgehog (IHH), and Desert Hedgehog (DHH). These

ligands are first synthesized as propeptides, cleaved to generate an N-terminal fragment (Lee, Ekker et al. 1994), and undergo further lipid modifications (Porter, Young et al. 1996). The biological purpose for cells to secrete HH ligands into the environment still remain obscure but seem to depend on specific environmental stimuli. For example, the liver and ventral pancreas both emerge from the ventral foregut endoderm, due to signaling from the cardiac mesoderm (Douarin 1975). Deutsch et al. demonstrated that FGF signaling from cardiac mesoderm directs ventral foregut endoderm to express genes for liver development and stimulates SHH ligand expression in order to suppress pancreatic development genes (Deutsch, Jung et al. 2001). The secretion of different mammalian HH ligands is also affected by location specific signals. Pathi et al. found that SHH expression was required for digit duplication and lateralization, whereas IHH was not (Pathi, Pagan-Westphal et al. 2001). However, in mice with constitutively active Hh signaling, IHH ligands, not SHH, are expressed by colonic epithelium (van Dop, Uhmann et al. 2009). Overexpression of DHH and SHH ligands in basal cells of mouse skin results in a common phenotype different from IHH overexpression. While IHH overexpression did not show any overt phenotype, epidermal progenitor cell hyperplasia, loss of epidermal tissue renewal, and spontaneous development of basal carcinoma lesions followed DHH and SHH overexpression, suggesting that these two ligands are responsible for regulating epidermal stem cell fate (Adolphe, Narang et al. 2004). Signals from injured tissue can also drive HH ligand production: Both SHH and IHH ligands are expressed the environments of injured liver (Sicklick, Li et al. 2005), lung (Watkins, Berman et al. 2003), and kidney (Ding, Zhou et al. 2012).

1.4.2. Explaining Patched and Smoothened

After secretion, HH ligands bind to the receptor Patched (PTC), a 12 trans-membrane domain receptor on Hh-responsive cells. All three ligands bind to PTC with

similar affinity and activate the Hh signaling cascade (Figure 1.3). Binding of HH ligands to PTC relieves it of its normal function as an inhibitor of Smoothened (SMO) (Murone, Rosenthal et al. 1999), a 7 trans-membrane domain protein which facilitates Hh signal transduction (Corbit, Aanstad et al. 2005). Although the mechanism by which PTC represses SMO is still unknown, it is thought that PTC inhibits SMO indirectly, possibly through the movement of a SMO-regulating small molecule across the cell membrane. Support for this hypothesis comes from the observation that PTC shares structural similarities to the resistance-nodulation-division (RND) family of bacterial proton-driven transmembrane transporters (Taipale, Cooper et al. 2002). Mutations in the RND-homologous regions of PTC decrease its ability to suppress Hh signaling activity. Furthermore, the Taipale et al. failed to note any specific and direct association between PTC and SMO in Hh-responsive cells, suggesting that a direct physical inhibition of SMO by PTC is unlikely. One candidate for a small molecule regulator of SMO is oxysterols (Dwyer, Sever et al. 2007). Oxysterols induce expression of Hh target genes through indirect activation of SMO. In the model proposed by Dwyer et al., the authors suggest that by regulating SMO exposure to intracellular oxysterols, PTC is able to regulate SMO. Supporting these results is the observation that PTC shares significant homology with NPC1 (which itself has homology with the bacterial RND family), a protein involved in LDL-derived cholesterol transport (Davies, Chen et al. 2000). Other regulators of SMO include vitamin D3 (Bijlsma, Spek et al. 2006). Despite multiple studies addressing this question, further research is needed to fully understand the mechanism behind PTC and SMO interactions.

1.4.3. The glee club: GLI transcription factors

Once activated, SMO localizes to the primary cilia and initiates an intracellular chain-of-events leading to the activation of latent Zinc-finger transcription factors, the Glioma-family of proteins (GLI1, GLI2, GLI3). While GLI1 and GLI2 both function as transcriptional

activators, only GLI2 is required for proper Hh signaling transduction and embryonic development (Ding, Motoyama et al. 1998; Bai, Auerbach et al. 2002). Regulation of GLI activity occurs through protein phosphorylation and nuclear localization (Figure 1.3). All GLI proteins are negatively regulated through phosphorylation by PKA, GSK3b, or CK1 (Kaesler, Luscher et al. 2000; Riobo, Lu et al. 2006; Varjosalo, Bjorklund et al. 2008; Pan, Wang et al. 2009). Activated SMO promotes Hh signaling by preventing the phosphorylation and ubiquitination of GLI proteins through a mechanism that seems to involve the AKT antagonism of PKA activity (Riobo, Lu et al. 2006). In mammals, there are conflicting studies on whether phosphorylation of GLI can also influence nuclear-cytoplasmic shuttling (Sheng, Chi et al. 2006). Suppressor of Fused (SUFU), which binds to all three GLI proteins, is thought to act as a negative regulator of GLI nuclear localization (Stone, Murone et al. 1999). The exact mechanism by which SUFU regulates GLI proteins is not yet understood but several studies suggest that SUFU keeps GLI proteins in the cytosol (Dunaeva, Michelson et al. 2003; Merchant, Vajdos et al. 2004), possibly via regulation of a leucine-rich nuclear export signal region on GLI (Kogerman, Grimm et al. 1999) or via recruitment of a histone deacetylase to competitively inhibit GLI binding sites in the nucleus (Cheng and Bishop 2002). SMO counteracts these repressive activities by promoting the rapid ubiquitination of SUFU (Yue, Chen et al. 2009).

1.4.4. Negative feedback control of Hedgehog signaling

In the nucleus, GLI proteins bind to Hh-target genes, such as ptch1, gli1, gli2, gli3, opn, foxf1, foxm1, and hhip (Marigo, Johnson et al. 1996; Teglund and Toftgard 2010). The transcription and translation of HHIP (Hedgehog interacting protein) provides an additional layer of negative feedback: HHIP competitively competes with HH ligands for binding to PTC and reduces pathway activity (Chuang and McMahon 1999). Another repressor/regulator of Hh signaling is GLI3 (Tole, Ragsdale et al. 2000) and loss-of-function mutants develop

similar phenotypes as mice with overexpressed Hh signaling. The GLI3 repressor is formed after cleavage and degradation of the C-terminal domain following sequential phosphorylation by PKA, GSK3b, and CK1 (Tempe, Casas et al. 2006). SHH ligand downregulates the formation of the GLI3-repressor by inhibiting its phosphorylation (Wang, Fallon et al. 2000; Bai, Stephen et al. 2004).

Although certain aspects of Hh signaling remain obscure, what is clear is that environmental context contributes to the activity of Hh signaling. In development, active Hh signaling facilitates the crosstalk between epithelial and mesenchyme cell populations (Ingham and McMahon 2001). Similar patterns of Hh signaling emerge after tissue injury. In the liver, following injury, Hh signaling is triggered, becomes reactivated, and plays an important role in repair. Sustained and dysregulated Hh signaling in the liver leads to fibrosis, cirrhosis, and cancer.

1.5. The role of Hedgehog signaling in liver disease

After liver development, Hedgehog signaling becomes silenced. In both *in vitro* (e.g. mouse embryonic stem cells vs. well differentiated hepatocytes) and *in vivo* (e.g. Ptc-lacZ mice) models, qRT-PCR and IHC analysis reveals that the Hh pathway is activated and present in early development but becomes silenced in mature cells (Sicklick, Li et al. 2006). In uninjured livers, there is little to no expression of HH ligands and evidence of Hh signaling activity (Sicklick, Li et al. 2006; Yang, Wang et al. 2008). One possible explanation for suppressed Hh signaling in healthy livers is the high expression of HHIP, a Hh signaling repressor, by sinusoidal cells, observed in both *in vitro* (Yang, Wang et al. 2008) and *in vivo* (Choi, Omenetti et al. 2009) models.

1.5.1. Hedgehog signaling is required for liver repair and regeneration

Hepatic injury triggers a wound healing response to regenerate the liver. This response is regulated by autocrine and paracrine signals within and between hepatic cells and includes a reactivation of Hh signaling pathway. Transient activation of Hh signaling is required after acute injury for livers to mount a proper wound healing response and once the injury signal is removed, the liver resolves the insult and Hh signaling is down-regulated (Omenetti, Popov et al. 2008). For example, treating mice with a Hh inhibitor after partial hepatectomy (a model of acute liver injury) results in a lack of activated Hh signaling, impaired liver regeneration and epithelial cell proliferation, significantly reduces accumulation of liver MF and progenitors, and results in the death of mice after 72 hours (Ochoa, Syn et al. 2010).

1.5.2. Clinical evidence for Hedgehog signaling in liver disease

Sustained Hh activity, however, is widely observed in multiple chronic human liver diseases and is not limited to one single etiology. Rather, sustained Hh signaling generally occurs in livers with faulty tissue regeneration. The level of hepatic Hh signaling correlates with severity of liver disease: Both hepatocytic SHH ligand expression and stromal GLI2 expression correlate with fibrosis stage (Guy, Suzuki et al. 2012). Compared to healthy livers, patients with primary biliary cirrhosis, a chronic cholestatic liver disease, have livers that harbor many more cells expressing Hh signaling activity (i.e. PTC, GLI2, IHH expression) (Jung, McCall et al. 2007). Additionally, the liver environments of patients with non-alcoholic fatty liver disease and non-alcoholic steatohepatitis (Syn, Jung et al. 2009), alcoholic liver disease (Jung, Brown et al. 2008), and viral hepatitis (Pereira Tde, Witek et al. 2010) all harbor elevated HH ligand, PTC, and GLI2 expressing cells. Using liver sections from patients with alcoholic liver disease or viral hepatitis, Syn et al. and Pereira et al. further characterize the GLI2-positive, PTC-positive cells and demonstrate that these cells co-

express alpha smooth muscle actin (ASMA) and Vimentin (VIM), both mesenchymal and myofibroblastic markers. These observations suggest that the liver MF population is Hh-responsive and Hh signaling promotes their hepatic accumulation. Hh-responsive cells continued to be expressed in HCC, where they surround the tumor nodule (Pereira Tde, Witek et al. 2010). Thus, strong clinical evidence suggests that continuous Hh signaling promotes liver fibrosis and worsen liver injury. These clinical observations have been verified experimentally in mice with haploinsufficiency of ptc. When these mice, which overexpress Hh signaling, are subjected to cholestatic liver injury (Omenetti, Porrello et al. 2008) or hepatotoxic diets (Syn, Jung et al. 2009), liver MF accumulation is enhanced and fibrosis is worsened as compared to WT mice.

1.5.3. Hepatocyte injury initiates Hedgehog signaling in the liver

Since Hh signaling is quiescent in healthy, normal livers, the molecular initiators of Hh signaling must occur during liver injury. Clinical observations that hepatocytes undergoing ER-stress related injury express high levels of SHH ligands implicated them as a potential culprit for initiators of hepatic Hh signaling (Rangwala, Guy et al. 2011). In essence, stressed and apoptotic hepatocytes release SHH and IHH ligands via an Akt dependent mechanism. This event initiates paracrine Hh signaling between wounded liver epithelia and surrounding stroma. Two key experiments led to this current understanding of how Hh signaling is initiated in liver disease. First, hepatocytes were isolated from transgenic $Ikk\beta^{fl/fl}$ mice. *In vitro* disruption of the NFkβ pathway, by treating isolated hepatocytes with Cre recombinase, resulted in caspase-3 activation (i.e. indicating cell apoptosis) along with increased intracellular levels of SHH and IHH proteins (Jung, Witek et al. 2010). Second, isolated primary hepatocytes from WT mice were treated with tunicamycin to induce ER stress and found to release biologically active HH ligands possibly by an AKT related mechanism (Rangwala, Guy et al. 2011).

1.5.4. Hedgehog signaling activation affects multiple liver cell types

While hepatocytes are not themselves Hh responsive, neighboring stromal cells are Hh responsive and include hepatic stellate cells (HSC), hepatic progenitors, immune cells, cholangiocytes, and endothelial cells. These cells activate Hh signaling in response to HH ligands, which sets off a cascade of intracellular events to support the wound healing process. HH ligands activate Hh signaling in HSC, which promotes pro-survival pathways. Reducing the bioavailability of HH ligands in culture significantly reduces proliferation and increases HSC apoptosis (Sicklick, Li et al. 2005). In addition, HSC require active Hh signaling as they undergo EMT to transform into liver MF (Choi, Omenetti et al. 2009). Culture activated HSC upregulate Hh pathway genes (e.g. ptc, smo, and gli2) and downregulate Hh pathway inhibitors (e.g. hhip), whereas pharmacologic inhibition of Hh signaling with SMO antagonists reduces HSC differentiation into MF (Yang, Wang et al. 2008; Choi, Omenetti et al. 2009).

Liver progenitors are also Hh responsive and accumulate after liver injury (Sicklick, Li et al. 2006; Jung, Brown et al. 2008; Ochoa, Syn et al. 2010). HH ligands enhance the growth, proliferation, and viability of liver progenitors (e.g. oval cells, immature ductular cells) that become mobilized to regenerate the liver after injury (Sicklick, Li et al. 2006; Omenetti, Yang et al. 2007), but these responses are blunted with Hh signaling inhibition (Ochoa, Syn et al. 2010). Furthermore, in transgenic mice with unrestrained Hh signaling, the accumulation of injury-related Hh-responsive liver progenitors is enhanced.

HH ligands fuel the inflammatory response by stimulating ductular epithelial cells to produce various chemokines that serve as chemoattractants for various liver immune cells, including NKT cells (Omenetti, Syn et al. 2009). Activated Hh signaling in NKT cells results in enhanced proliferation, decreased apoptosis, and increased secretion of pro-fibrogenic cytokines, IL-13 and IL-4 (Syn, Witek et al. 2009). Both NKT cells (Syn, Witek et al. 2009)

and activated cholangiocytes, (Witek, Yang et al. 2009) can also produce additional HH ligands, which further enhance Hh signaling in the injured liver microenvironment. Given the broad impact of activated Hh signaling in cells that respond to liver injury, it is not surprising that Hh signaling inhibition potentially interferes with the activities of fibrosis-causing cells. Treating mice after partial hepatectomy with the Hh inhibitor cyclopamine results in a dramatic reduction in fibrosis (Ochoa, Syn et al. 2010), providing evidence that pharmacologic inhibition of Hh signaling can reverse fibrogenic repair.

Taken as a whole, the result of chronic liver injury is a Hh-rich microenvironment that promotes continuous wound healing, caused in part by sustained Hh signaling. Dysregulated and sustained Hh signaling promotes a pro-fibrotic phenotype, which is an important risk factor for liver cancer. Thus, it is reasonable to hypothesize that Hh signaling is also active in the liver tumor microenvironment. In other solid tumors, increased Hh signaling leads to tumor progression, either in a ligand independent (i.e. malignant epithelia harbor Hh pathway mutations) or ligand dependent (i.e. dysregulated paracrine Hh signaling between the tumor cell and its environment) fashion.

1.6. Over the hedge: Hedgehog signaling in cancer

In 1996, the link between Hedgehog signaling and cancer was established in patients with basal cell nevus syndrome (BCNS, also known as Gorlin syndrome), an autosomal dominant disease predisposing patients to the development of basal cell carcinomas (BCC). Linkage studies of patients with BCNS mapped the locus carrying the mutant gene to the chromosome 9q22 and later to the Ptc1 gene (Hahn, Wicking et al. 1996; Johnson, Rothman et al. 1996). Mice haploinsufficient for Patched-1 showed similar phenotypes as patients with BCNS but oddly, did not develop BCC (Hahn, Wojnowski et al. 1998). However, after exposure to ionizing radiation, these mice progressed to develop BCC (Aszterbaum, Epstein et al. 1999). Targeted deletion of Ptc-1 in skin progenitors also

resulted in BCC lesions (Adolphe, Hetherington et al. 2006). Together, these results suggested that 1) Ptc1 is a tumor suppressor, 2) a two-hit mechanism to Ptc-1 is required for cancer development, and 3) abnormal Hh signaling in the pre-neoplastic setting promotes malignancy.

Since then, there has been mounting evidence that abnormal Hh signaling is involved in multiple cancers. In prostate cancer, high levels of Hh signaling have been observed (Karhadkar, Bova et al. 2004) and a positive correlation exists between the level of Hh signaling activity and the severity and aggressiveness of the disease (Sheng, Li et al. 2004). Elevated Hh signaling has been observed in glioblastomas (Verhaak, Hoadley et al. 2010), pancreatic cancer (Thayer, di Magliano et al. 2003), renal cell carcinoma (Dormoy, Danilin et al. 2009), ovarian cancer (Bhattacharya, Kwon et al. 2008), esophageal cancer (Ma, Sheng et al. 2006), colon cancer (Varnat, Duquet et al. 2009), lung cancer (Watkins, Berman et al. 2003; Yuan, Goetz et al. 2007), melanoma (Stecca, Mas et al. 2007), and liver cancer (Sicklick, Li et al. 2006). Knockdown of Hh signaling in various cancer cell lines appears to decrease cell malignancy.

1.6.1. Ligand independent Hedgehog signaling in cancer

Abnormal Hh signaling works in both ligand independent and ligand dependent modes to drive carcinogenesis. Direct evidence for dysregulation of ligand independent Hedgehog signaling (i.e. endogenous mutations of core Hh signaling components) has been shown in BCC (Reifenberger, Wolter et al. 2005), medulloblastoma (Wolter, Reifenberger et al. 1997; Taylor, Liu et al. 2002), breast cancer (Nessling, Richter et al. 2005; Fiaschi, Rozell et al. 2009), pancreatic cancer (Thayer, di Magliano et al. 2003; Pasca di Magliano, Sekine et al. 2006), colon cancer (Xie, Johnson et al. 1997), and liver cancer (Sicklick, Li et al. 2006). Aberrant Hh signaling has been shown to provide several advantages to the tumor cell, such as promoting cell proliferation and regulating cell cycle progression (Adolphe,

Hetherington et al. 2006). GLI consensus binding sites have been found on cyclin D1 (Yoon, Kita et al. 2002) and overexpression of GLI results in elevation of cell cycle regulator foxm1 (Teh, Wong et al. 2002). Hh signaling also promotes cell survival, another cancer hallmark. In BCC cell lines, binding sites for GLI were found on bcl2, a negative regulator molecule of apoptosis, and transfection of gli1 pcdna corresponded with dose dependent increases of bcl2 activity and decreased cellular apoptosis (Bigelow, Chari et al. 2004). In gastric cancer cells, downregulating GLI activity by blocking SMO resulted in decreased bcl2 expression (Han, Lee et al. 2009). Thus, the association between GLI1 and blc2 appears to be conserved among multiple cancer types.

Hh signaling is also an established effector of EMT, a process involved in tumor invasiveness and metastasis. In ovarian cancer cell lines, overexpression of GLI1 increased cell mobility and invasiveness along with EMT genes such as vimentin and mmp1 (Liao, Siu et al. 2009). Hh inhibition in pancreatic cancer cell lines resulted in downregulation of mesenchymal programming and invasiveness and upregulated epithelial genes such as E-cadherin (Feldmann, Dhara et al. 2007). Notably, overexpression of GLI1 in pancreatic cancer cells resulted in increased invasiveness (Feldmann, Dhara et al. 2007; Inaguma, Kasai et al. 2011). Similar observations are reported in breast cancer cells (Souzaki, Kubo et al. 2011), glioma cells (Wang, Pan et al. 2010), and gastric cancer cells (Yoo, Kang et al. 2008). Together, these studies help to explain the effectiveness of targeting GLI proteins to disrupt tumor xenograft growth of various cancers (Thayer, di Magliano et al. 2003; Lauth, Bergstrom et al. 2007).

1.6.2. Ligand dependent, paracrine Hedgehog signaling in cancer

While evidence for a ligand-independent mechanism for Hh signaling first advanced understanding of this pathway in cancer, a growing number of studies have challenged this assertion and provide data that tumorigenesis is driven by ligand-dependent paracrine Hh

signaling. First, some animal models of oncogenic Hh signaling fail to drive tumor development. For example, in two models of pancreatic ductal adenocarcinoma (PDAC) and prostate cancer, oncogenic SMO, despite being localized to malignant epithelial cells, was insufficient to drive the development of neoplastic lesions (Mao, Ligon et al. 2006; Tian, Callahan et al. 2009). Secondly, these studies were followed by additional reports in both human breast (O'Toole, Machalek et al. 2011), pancreatic (Bailey, Mohr et al. 2009; Tian, Callahan et al. 2009) and prostate cancers (Fan, Pepicelli et al. 2004; Shaw, Gipp et al. 2009) that while the HH ligand producing cell is the tumor epithelial cell, they themselves are not Hh-responsive. Instead, these studies suggest that the Hh-responsive cells in tumors are the tumor associated stroma. In fact, malignant HH-ligand producing pancreatic epithelia were found to be devoid of primary cilia, signifying that they lack a crucial component of autocrine Hh signaling (Bailey, Mohr et al. 2009; Seeley, Carriere et al. 2009). Yet orthotopic implantation of pancreatic cancer cells overexpressing SHH ligands resulted in tumors that were sensitive to Hh inhibition (Bailey, Mohr et al. 2009). The authors showed that tumor stromal fibroblasts, not malignant epithelia, expressed primary cilia, SMO translocation into the cilia, and high Hh pathway activity. Finally Yauch et al. used a large panel of over 100 colon, pancreatic, and prostate tumor epithelial cell lines to demonstrate that sensitivity to an antagonist of SMO did not correlate with Hh signaling activity (Yauch, Gould et al. 2008). These landmark results suggested that previous studies demonstrating reduction in malignancy by SMO antagonists could have resulted from off-target drug effects rather than a true knockdown of Hh signaling. Instead, these tumor epithelial cell lines were refractory to treatment while mesenchymal cell lines were two times more sensitive to the Hh inhibitor than the most sensitive cancer cell line. However, these same malignant epithelial cell lines expressed large amounts of SHH and IHH ligands compared to normal epithelial cells. Co-injection of HH-ligand producing tumor cells with fibroblasts harboring a genetic deletion of Smo produced reduced tumor burden in a mouse xenograft model,

suggesting that disruption of the paracrine Hh signaling between epithelial tumor cells and stromal cells can prevent carcinogenesis.

1.6.3. Paracrine Hedgehog signaling promotes the hallmarks of cancer

What are some potential advantages paracrine Hh signaling provide the tumor? A number of papers have implied that that malignant epithelia use paracrine Hh signaling to recruit/activate Hh-responsive fibroblasts to promote fibrosis. In an orthotopic model of pancreatic cancer, Bailey et al. used transformed pancreatic cancer cell lines overexpressing SHH ligands to demonstrate epithelial-secreted ligands contributed to the formation of desmoplasia and promotes the proliferation and differentiation of pancreatic fibroblasts (Bailey, Swanson et al. 2008). Pancreatic cancers are notorious for being refractory to standard chemotherapies. Olive et al. demonstrated that combinatorial treatment of a genetically engineered mouse model of PDAC with a SMO antagonist and the chemotherapeutic, Gemcitabine, resulted in decreased tumor burden (Olive, Jacobetz et al. 2009). These authors concluded that treatment with the Hh inhibitor both reduces desmoplasia and fibrosis surrounding the tumor nodule and increases the therapeutic drug delivery of Gemcitabine. Finally, when compared to normal fibroblasts, BCNS patient-derived fibroblasts heterozygous for Patched-1 expressed higher levels of pro-fibrotic molecules MMP1, MMP3, and tenacin C when cultured in vitro, adding more support that dysregulated Hedgehog signaling in tumor-associated fibroblasts contributes to increased fibrogenesis. Aside from increasing fibrosis surrounding the tumor, paracrine Hh signaling also promotes tumor growth. In two separate xenograft models, created from fibroblasts with increased Hh pathway activity combined with malignant epithelial cells overexpressing SHH ligands, grew significantly faster than their controls (Fan, Pepicelli et al. 2004; Shaw, Gipp et al. 2009).

1.6.4. Hedgehog signaling in HCC

The question of how does Hh signaling contribute to liver cancer is still largely unknown. Several studies would suggest that autocrine Hh signaling in liver tumor cells is elevated and is a major pathway responsible for tumor proliferation, viability, chemoresistance and invasion (Sicklick, Li et al. 2006; Cheng, Xu et al. 2009; Chen, Lingala et al. 2011; Chen, Lin et al. 2011; Lu, Zhao et al. 2012). However most of these studies describing the advantages conferred by Hh signaling were performed in cell lines. Furthermore, evidence for overexpression of Hh signaling was found in whole liver tissue and does not account for cell-specific localization. Addressing this, Pereira et al. performed immunostaining on human HCC samples and found that PTC(+) and GLI2(+) cells were localized to the stromal compartment surrounding the tumor nodule, suggesting a paracrine mechanism might exist for liver cancer to advance (Pereira Tde, Witek et al. 2010).

1.6.5. Development of clinical inhibitors of Hedgehog signaling

The prevalence of Hh pathway activity in a variety of cancers has led to drug development of Hh signaling inhibitors. Interestingly, the development of Hh inhibitors began prior to its discovery in *Drosophila* or cancer. Observations that sheep that ate the wild corn lily, *Veratrum californicum*, gave birth to one-eyed lambs, led to the isolation and synthesis of the compound cyclopamine. Three decades later, it was found to inhibit Hh signaling (Cooper, Porter et al. 1998; Incardona, Gaffield et al. 1998). Just like every major signaling pathway that has been found in pre-clinical models to drive cancer progression, the proof is in the pudding, or as it relates to patients, the effectiveness of inhibitors in clinical trials. As of April 2012, there are seven Hh inhibitors under clinical development and all seven inhibitors antagonize SMO (Rudin 2012). Six have already entered phase I/II clinical trials for various cancers, including BCC, brain cancers, CML, pancreatic cancers, bone cancers, head and neck cancers. Vismodegib (GDC-0449) was the first targeted

inhibitor of Hh signaling to be approved for clinical treatment of BCC (Guha 2012). Vismodegib is considered a competitive inhibitor of SMO, and belongs to a class of cycopamine-derivatives that outcompete cyclopamine for binding to SMO. However, to date, reproducible clinical responses to SMO inhibitors have only been reported in BCC (Sekulic, Migden et al. 2012) and medulloblastomas (Rudin, Hann et al. 2009). In fact, a 199 patient Phase II, randomized, double-blind, placebo-controlled study evaluating GDC-0449 as first line therapy for previously untreated metastatic colorectal cancer yielded unsatisfactory results: Not only were there no statistically significant differences in outcomes between the two arms, but the hazard ratio for progression free survival actually favored the placebo group (Rudin 2012). And despite significant responses in a Phase II trial in advanced BCC (overall disease control rate was over 86%), the median duration of response was 7.6 months and median progression free survival was 9.6 months. Acquired resistance is attributed to mutations that alter SMO to prevent the binding of GDC-0449 to SMO (Yauch, Dijkgraaf et al. 2009). Unique challenges remain to determine whether Hh signaling inhibition will provide therapeutic benefit to patients.

Questions persist about therapeutic potential of Hh signaling inhibition in cancers because the method by which Hh signaling provokes cancer progression is still yet to be determined in multiple cancers. Combination therapy might be a more favorable strategy if paracrine signaling between stromal and malignant epithelial cells is found to be a main driver of cancer progression. But the broad evidence of both ligand-independent and ligand-dependent mechanisms of Hh activity in many tumors, including liver cancer, suggests that Hh signaling is a potential and exciting novel target for pharmaceutical therapy. And while the role of Hh signaling in pre-neoplastic livers is established, the lack of knowledge about the role of the Hh-responsive microenvironment in HCC underscores the necessity of additional research. The results of preclinical Hh inhibition in liver cancer cell lines is

promising and with clinical inhibitors against Hh signaling being developed, understanding the mechanism of Hh signaling in HCC could open up new avenues for medical treatment.

1.7. Metabolism in physiology and cancer

Normal differentiated cells metabolize biochemical energy to synthesize adenosine triphosphate (ATP). The energy released during metabolic respiration is stored as ATP. While both aerobic respiration and anaerobic respiration are processes that transform glucose into ATP, aerobic respiration is 15 times more efficient at generating ATP than anaerobic respiration. Initially, both pathways begin with glycolysis, but during aerobic respiration, the two pyruvate generated at the end of glycolysis are further oxidized in the tricarboxylic acid (TCA) cycle and oxidative phosphorylation. In glycolysis, glucose is transported into the cell via glucose transporters and trapped in the cytoplasm by glucose phosphorylation. During glycolysis, a series of catabolic reactions converts one glucose molecule into two molecules of pyruvate, two NADH, and two net ATP (Figure 1.4). In the presence of oxygen, pyruvate is further oxidized into acetyl-CoA by the pyruvate dehydrogenase complex and enters the TCA cycle. This process oxidizes acetyl-CoA to carbon dioxide and generates additional units of the reduced coenzyme, NADH. NADH is a highly reduced species that releases energy when oxidized. To utilize this energy, a set of enzymes within the inner membrane of the mitochondria removes electrons from NADH and passes them to the terminal electron acceptor, oxygen. This process is termed oxidative phosphorylation and establishes a proton gradient across the membrane of the mitochondria used by ATP synthase to drive the production of ATP, where the cell ultimately stores the energy released by respiration. However, when oxygen is limited, anaerobic respiration occurs. In anaerobic conditions, oxygen is not available as a final electron acceptor, and pyruvate, instead of entering the TCA cycle, is converted by lactate dehydrogenase into lactate (Figure 1.5). In addition to generating ATP from glycolysis, this process is

necessary to recycle NADH back to NAD+, a required coenzyme for glycolysis. When oxygen is resupplied, lactate is converted back into pyruvate to enter the aerobic respiration.

1.7.1. Cancer metabolism

Over 90 years ago, Otto Warburg first noted that the growth of cancer cells (from human skin, throat, intestine, penis and nose) relies upon enhanced anaerobic glycolysis, even when sufficient oxygen is available for oxidative phosphorylation (Warburg 1956). Warburg recognized that despite aerobic conditions, cancer cells (as opposed to normal cells) produce energy by high rates of glycolysis and lactic acid fermentation, yet have low rates of oxidative phosphorylation in the mitochondria (Figure 1.6). Thus, cancer cells follow a pattern of glycolytic activity that mimics the activity of normal cells under hypoxic (anaerobic) conditions. This observation was later termed the Warburg effect (Racker 1972). Warburg hypothesized that this was due to impaired respiration in the tumor cell (mitochondrial defects), although we now know this is not necessarily the case (Dang 2012). The FDG-PET scan is the direct clinical application of the Warburg Effect. Briefly, a radiolabeled hexokinase substrate, 2-18F-2-deoxyglucose (FDG), is incorporated into the cell by glucose transporters and phosphorylated by hexokinase. In oncology, this technique is used to label and monitor tumors and track metastatic growth on the assumption that glycolytic tumor cells have a high uptake of glucose (Ben-Haim and Ell 2009).

1.7.2. Regulators of the Warburg Effect

Warburg's observation about tumor glycolysis renewed interest in the regulation of metabolism by the tumor cell. Several associations between oncogenes and metabolic enzymes have been made (Fan, Dickman et al. 2010). In this study, Fan et al. discovered that conditional activation of AKT leads to upregulation of glucose import and the Warburg effect. Inhibiting PI3K signaling in a mouse model of lung adenocarcinoma leads to

decreased detection by FDG-PET, suggesting that disruption of PI3K/AKT signaling impacts tumor glucose transport (Engelman, Chen et al. 2008). Other oncogenes, such as RAS and SRC also increase glucose uptake and the phosphorylation of glycolytic enzymes (Cooper, Esch et al. 1984; Flier, Mueckler et al. 1987). A study by Matoba et al. showed that p53 is coupled to mitochondrial respiration. Specifically, through Synthesis of Cytochrome c Oxidase 2 (SCO2), p53 regulates the cytochrome c oxidase complex, essential for oxidative phosphorylation. In p53 deficient cells, the authors observed a shift towards cellular utilization of glycolytic pathways (Matoba, Kang et al. 2006). The metabolic reprogramming of tumor cells is also attributed to HIF1α, a transcription factor that is stabilized in response to hypoxia. Multiple tumor cells have shown to have elevated HIF1α signaling (Sutter, Laughner et al. 2000) and HIF1α activation suppresses mitochondrial function through activating Pdk1 (Kim, Tchernyshyov et al. 2006; Papandreou, Cairns et al. 2006) and directly increases glycolytic-associated genes hexokinase II (Mathupala, Rempel et al. 2001). HIF1α binding sites have been found on the M2 isoform of pyruvate kinase (PKM2), which further promotes the idea that HIF1α regulates aspects of glycolytic metabolism in cells (Kim, Tchernyshyov et al. 2006).

Pyruvate kinase, which catalyzes the conversion of phosphoenolpyruvate into pyruvate, has faced greater scrutiny as a key mediator of enhanced glycolysis in cancer cells (Christofk, Vander Heiden et al. 2008). Christofk et al. discovered that tumor cells exclusively express PKM2, and switching cells from the M2 isoform to the M1 isoform of pyruvate kinase resulted in a "reversal" of the Warburg effect. This phenotype included reduced lactate production, increased oxygen consumption, and a reduced ability to form tumors in nude mice xenografts. Intriguingly, PKM2 was found to be necessary to stabilize HIF1α, suggesting that it participates in a positive feedback loop to promote HIF1a activation and also glucose metabolism in tumor cells (Luo, Hu et al. 2011). Upregulation of PKM2 can alter the malignant epithelial cell in many ways, including modifying histones and

regulating epigenetic changes (Yang, Xia et al. 2012), increasing tumor growth (Christofk, Vander Heiden et al. 2008), increasing bioavailability of nucleic acids (Ye, Mancuso et al. 2012), and even further altering the metabolism of the cancer cell by promoting lipogenesis (Panasyuk, Espeillac et al. 2012).

1.7.3. The tumor microenvironment and cancer metabolism

Although the amount of information being uncovered about tumor metabolism is growing, questions still remain about how the tumor microenvironment influences the metabolic state of the cancer. Two groups in ovarian and breast cancer have reported that the growth of tumor cells is enhanced by metabolic end-products of the surrounding stroma. In an co-culture model of stromal adipocytes and ovarian cancer cells, Nieman et al. demonstrated that adipocytes directly transfer lipids to ovarian cancer cells to promote in vitro and in vivo growth and migration (Nieman, Kenny et al. 2011). Another group suggested that stromal fibroblasts in breast cancer are glycolytic themselves and secrete lactate into the microenvironment to promote growth and metastasis of breast cancer cells (Bonuccelli, Tsirigos et al. 2010). The idea that metabolic, and specifically, glycolytic end products such as lactate, from stromal cells can support tumorigenesis is intriguing and demonstrates yet another method by which the microenvironment influences the growth of tumors.

1.7.4. The impact of glycolytic end product lactate on tumor progression

Elevated lactate levels in both plasma and within the tumor itself have also been associated with poor prognosis and overall survival in several types of cancer (Walenta, Wetterling et al. 2000). Lacate itself has been shown to confer advantages to tumor cell viability and proliferation (Sonveaux, Vegran et al. 2008) and can enhance motility and migration of tumor cells (Goetze, Walenta et al. 2011). Lactate also signals macrophages to

release mitogenic and angiogenic factors, indirectly contributing to tumor proliferation and angiogenesis (Jensen, Hunt et al. 1986). The impact of lactate on the tumor microenvironment is reinforced by additional evidence that tumor cells express higher levels of monocarboxylate transporter 1 (MCT1), a lactate importer, than non-malignant cells (Koukourakis, Giatromanolaki et al. 2007; Sonveaux, Vegran et al. 2008). Inhibition of MCT1 causes antitumor effects, including reduced growth and increased necrosis of tumor xenografts, suggesting that targeting lactate associated transporters has therapeutic benefit. Another study which suggests that endothelial cells uptake lactate in the tumor microenvironment to promote angiogenesis would seemingly rule them out as a source of lactate production (Sonveaux, Copetti et al. 2012).

1.7.5. Glycolytic regulation of liver MF

In the pre-neoplastic environment of the liver, the metabolism of hepatic stellate cells is an important regulator of their fibrogenic potential. In recently published work, our lab revealed a previously unsuspected "metabolism-centric" mechanism governing the fate of hepatic stellate cells (Chen, Choi et al. 2012). We demonstrate that during culture (and in injured livers), the trans-differentiation of quiescent HSC into myofibroblasts is mediated via a metabolic switch that favors glucose consumptive processes, and show that this global change in HSC metabolism is controlled by Hedgehog signaling. Hh signaling orchestrates this myofibroblastic reprogramming of HSC by directing HIF1α-dependent induction of glycolytic enzymes, resulting in cellular accumulation of the glycolytic end-product, lactate. In HSC themselves, lactate accumulation was shown to orchestrate global phenotypic changes that cause those cells to become myofibroblastic, thereby enhancing their wound healing capabilities.

These results suggest that in the pre-neoplastic liver, HSC glucose metabolism plays

an important role in development of fibrosis, and is mediated by Hh signaling. It is possible that hypoxic environments upregulate glucose metabolism in fibrotic and pre-neoplastic livers, which later contribute their end-products to promote tumorigenesis. Thus, the extent of metabolic contribution by liver cancer associated fibroblasts towards HCCarcinogenesis remains largely unknown and highlights the need for further study on the metabolism of liver cancer as a whole.

1.8. Summary

Hepatocellular carcinoma is a disease that afflicts many patients worldwide. Unfortunately for many patients with HCC, therapeutic options are extremely limited, creating a real patient-driven need for better therapies. The largest risk factor for HCC is cirrhosis, and HCC commonly recurs in cirrhotic livers after tumor ablation. The pre-neoplastic cirrhotic microenvironment may promote the outgrowth of malignant hepatocytes, but the mechanisms involved remain obscure. Because deregulated, excessively fibrogenic repair of liver injury causes cirrhosis itself, one possibility is that stromal-epithelial interactions fuel HCC growth. In multiple tumors, communication between stromal cells in the tumor microenvironment and tumor cells nurtures malignant phenotypes. An important signaling pathway that regulates crosstalk between stroma and epithelia is Hedgehog signaling, which plays critical roles in development, wound healing, and cancer. This pathway is especially important in liver regeneration and is a potential candidate for therapeutic targeting in liver cancer. Sustained Hedgehog signaling worsens liver fibrosis and regulates the behaviors of multiple cells involved in wound-healing. Dysregulation of this pathway continues as liver cancer develops, which warrants investigation into a) whether active Hh signaling is required for tumor growth, b) does Hh signaling regulate communication between malignant hepatocytes and Hh-responsive liver MF, and if so, c) how these communications promote HCC.

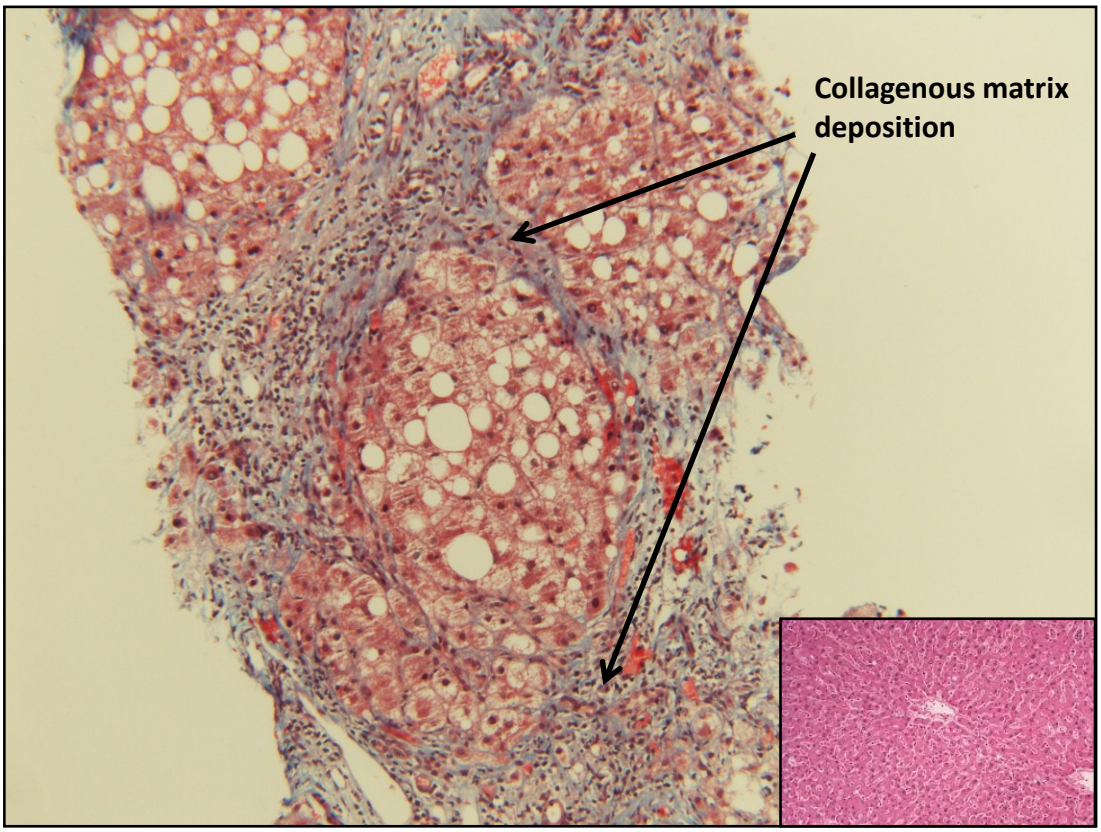

Figure 1.1. Masson trichrome stained core liver biopsy of NAFLD with cirrhosis.

Cirrhosis is characterized by the replacement of normal liver parenchyma with fibrous tissue composed of collagenous matrix. Here fibrotic bands are stained blue by trichrome staining. Histologically, cirrhosis contains nodular remnants of normal liver architecture (portal triads and central veins) that are separated by wide scars and thin fibrous septa. Normal liver architecture is shown in the insert. Note the lack of fibrogenic matrix in normal liver.

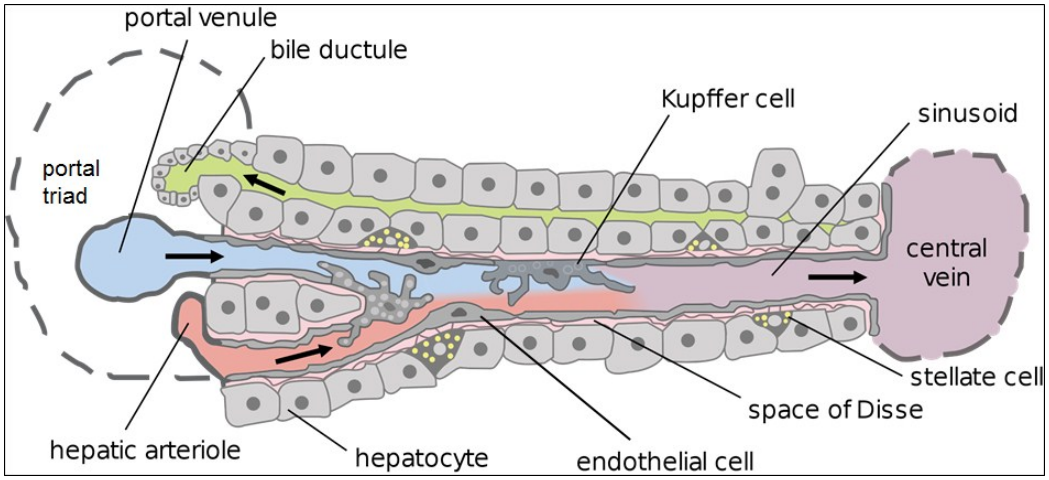

Figure 1.2. Graphical representation of the liver architecture. The liver receives blood from the portal vein and the hepatic artery, which run beside the common bile duct and form the portal triad. The hepatic artery receives arterial blood and the portal vein receives blood from the gastrointestinal tract. Blood is passed through the hepatic sinusoids and out of the liver through hepatic venules. The central vein is a branch of the hepatic vein. Hepatocytes are organized into cords that are separated by sinusoids. Endothelial cells line the sinusoids and lie directly adjacent to hepatocytes. The space between the endothelium and hepatocytes is the space of Disse. Hepatic stellate cells occupy this perisinusoidal space. Kupffer cells are specialized macrophages in the liver and contribute to the inflammatory response during wound healing.

(Figure is adapted from *Wikipedia*, n.d., Retrieved August 21, 2012, from https://en.wikipedia.org/wiki/File:Hepatic_structure2.svg.)

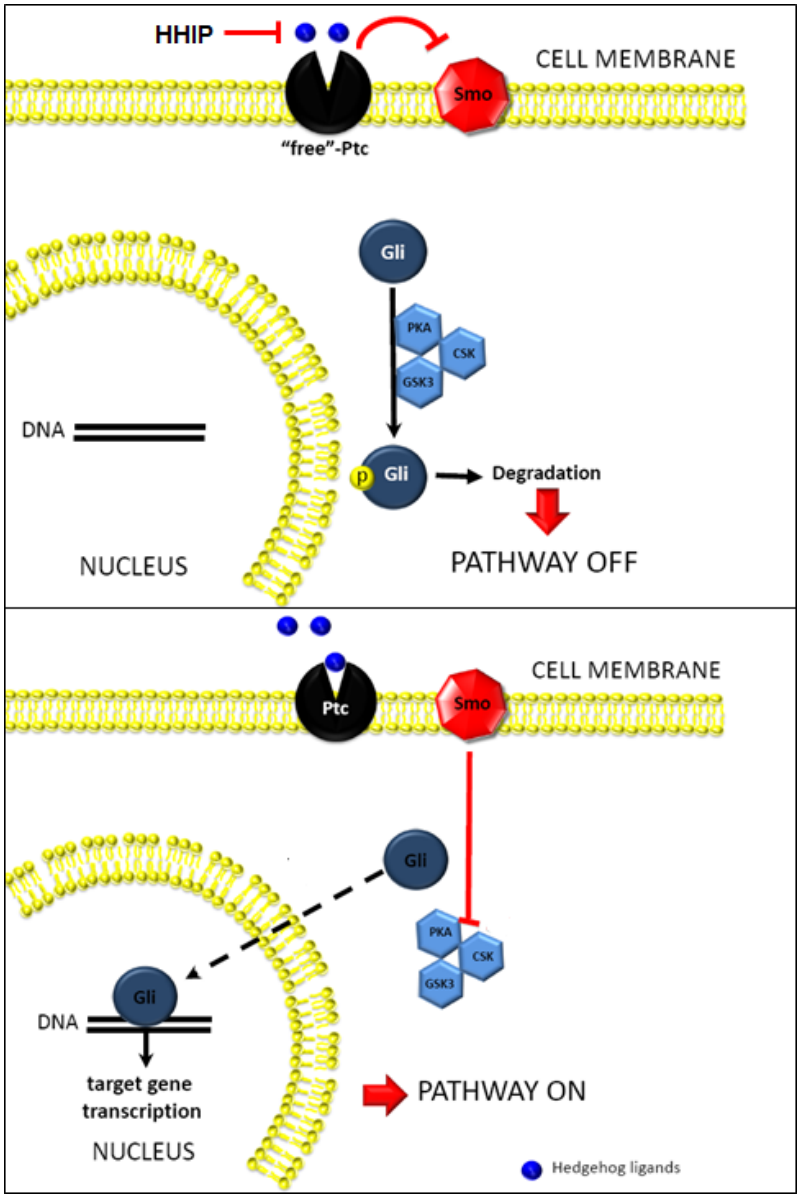

Figure 1.3. Overview of the Hedgehog signaling pathway.

Figure 1.3. Overview of the Hedgehog signaling pathway. Hedgehog ligands interact with the Hh receptor, Patched (Ptc) which relieves Ptc-mediated repression of Smoothened (Smo), permitting Smo activation. Activated Smo prevents the phosphorylation and ubiquitination of Glioma (Gli)-family proteins, leading to their accumulation and nuclear localization. In the nucleus, Gli proteins bind to Hh-target genes and regulate the transcription of Hh-associated genes. Factors like Hedgehog-interacting protein (HHIP) competitively inhibit Hh signaling by binding to HH ligands.

(Figure is adapted from *Hedgehog signaling in the liver*. J Hepatol. 2011 Feb;54(2):366-73.)

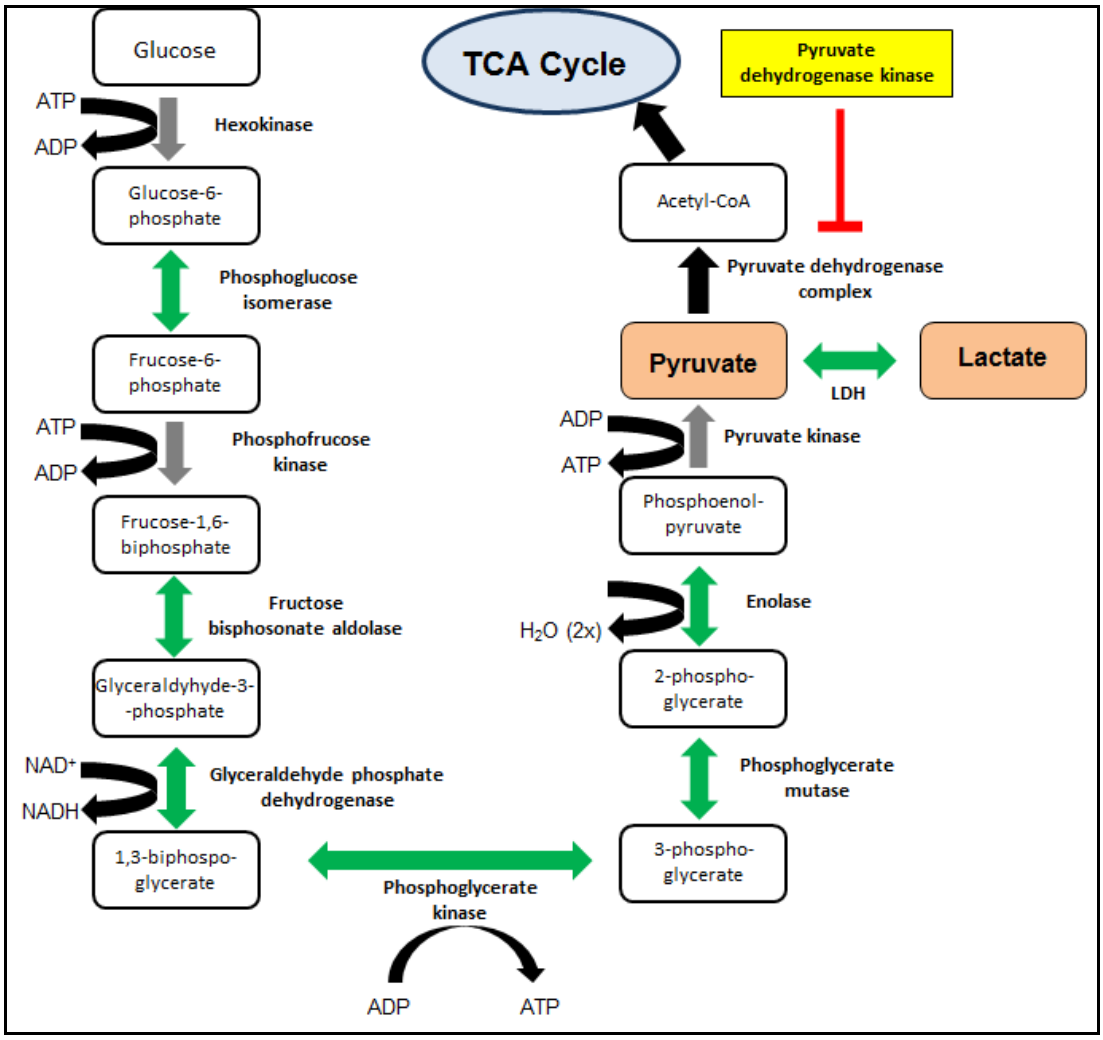

Figure 1.4. Overview of glycolysis.

Figure 1.4. Overview of glycolysis. Glycolysis begins when glucose is transported into the cell via glucose transporters. Glucose is metabolized to pyruvate through a series of catalyzed reactions that occur during glycolysis. In aerobic conditions, pyruvate dehydrogenase catalyzes the reaction that converts pyruvate to acetyl CoA which then enters the TCA cycle and is further oxidized in the mitochondria during oxidative phosphorylation. The activity of pyruvate dehydrogenase is regulated by the enzyme pyruvate dehydrogenase kinase. In anaerobic conditions, pyrvuate is coverted to lactate via the enzyme lactate dehydrogenase (LDH) during lactic acid fermentation. Gray arrows indicate irreversible reactions.

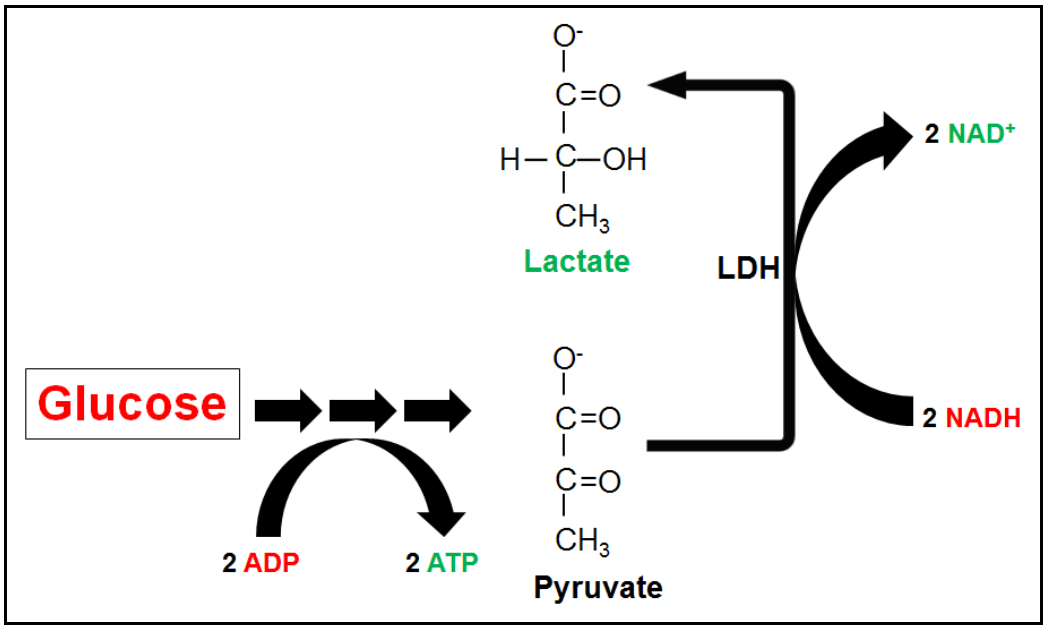

Figure 1.5. Anaerobic glycolysis. In anaerobic conditions, glucose is converted to pyruvate but because of decreased oxygen availability, does not enter the TCA cycle. Instead, pyruvate is converted to lactate by lactate dehydrogenase (LDH) and as a result of this process, recycles NADH to NAD^+. The generation of 2 NAD^+ molecules allows glycolysis to continue and thus the cell relies primarily on glycolysis for the production of ATP.

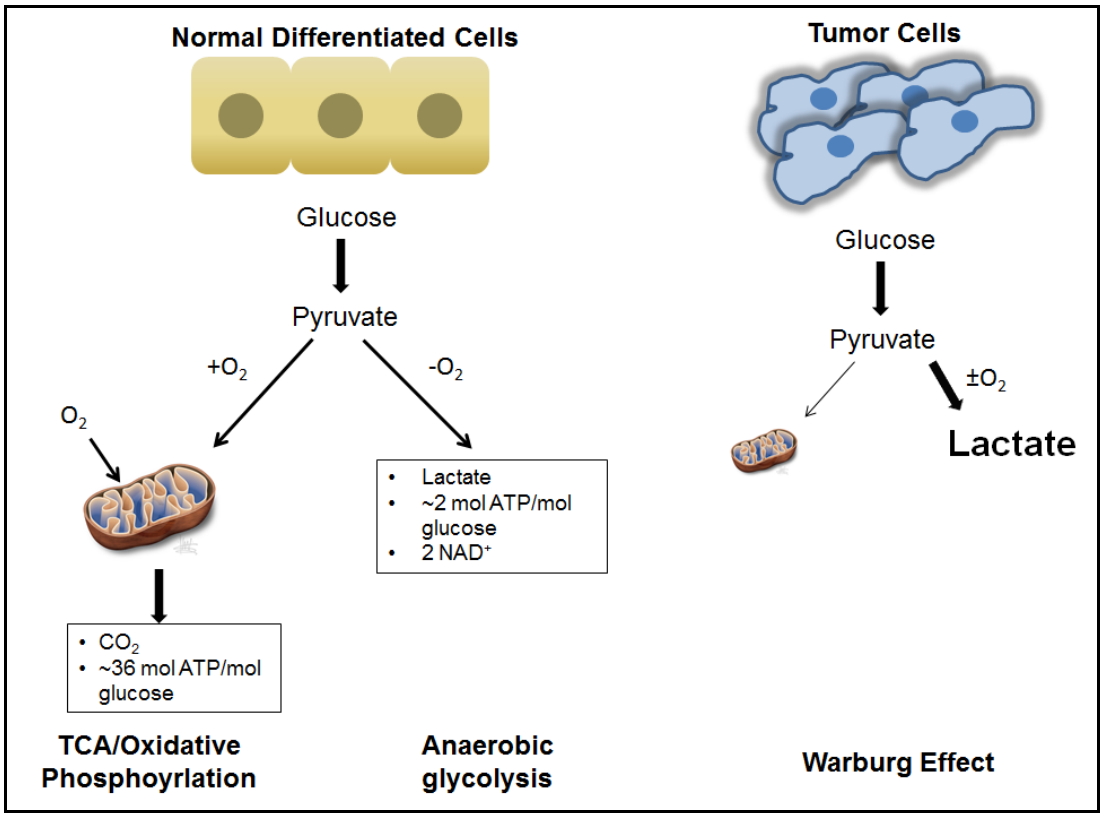

Figure 1.6. Overview of the Warburg Effect. As discussed, cells metabolize glucose to pyruvate during glycolysis. In the presence of oxygen, normal differentiated cells use oxidative phosphorylation to produce ATP. However, cancerous or proliferative cells downregulate their use of oxidative phosphorylation in the mitochondria and instead produce ATP using primarily glycolysis. As a result, lactate is often a side product of the Warburg effect.

(Figure is adapted from *Understanding the Warburg Effect: The Metabolic Requirements of Cell Proliferation*. Science. 2009 May 22;324(5930):1029-33.)

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CHAPTER II

HEDGEHOG SIGNALING ANTAGONIST PROMOTES REGRESSION OF BOTH LIVER FIBROSIS AND HEPATOCELLULAR CARCINOMA IN A MURINE MODEL OF PRIMARY LIVER CANCER

Summary

Objective: Chronic fibrosing liver injury is a major risk factor for hepatocarcinogenesis in humans. Mice with targeted deletion of Mdr2 (the murine ortholog of MDR3) develop chronic fibrosing liver injury. Hepatocellular carcinoma (HCC) emerges spontaneously in such mice by 50-60 weeks of age, providing a model of fibrosis-associated hepatocarcinogenesis. We used $Mdr2^{-/-}$ mice to investigate the hypothesis that activation of the hedgehog (Hh) signaling pathway promotes development of both liver fibrosis and HCC. Methods: Hepatic injury and fibrosis, Hh pathway activation, and liver progenitor populations were compared in $Mdr2^{-/-}$ mice and age-matched wild type controls. A dose finding experiment with the Hh signaling antagonist GDC-0449 was performed to optimize Hh pathway inhibition. Mice were then treated with GDC-0449 or vehicle for 9 days, and effects on liver fibrosis and tumor burden were assessed by immunohistochemistry, qRT-PCR, Western blot, and magnetic resonance imaging. Results: Unlike controls, $Mdr2^{-/-}$ mice consistently expressed Hh ligands and progressively accumulated Hh-responsive liver myofibroblasts and progenitors with age. Treatment of aged Mdr2-deficient mice with GDC-0449 significantly inhibited hepatic Hh activity, decreased liver myofibroblasts and progenitors, reduced liver fibrosis, promoted regression of intra-hepatic HCCs, and decreased the number of metastatic HCC without increasing mortality. Conclusions: Hh pathway activation promotes liver fibrosis and hepatocarcinogenesis, and inhibiting Hh signaling safely reverses both processes even

when fibrosis and HCC are advanced.

Introduction

Hepatocellular carcinoma (HCC) is an insidious cancer that accounts for up to 1 million deaths a year and is the third leading cause of cancer deaths worldwide (Rahbari, Mehrabi et al. 2011). Cirrhosis, a consequence of progressive liver injury and fibrosis, is the single largest risk factor for HCC (Luedde and Schwabe 2011). An altered wound healing response to chronic liver injury, with resultant dysregulated activation of myofibroblasts and progenitor cell populations, has been implicated in cirrhosis pathogenesis and eventual carcinogenesis (Schuppan and Afdhal 2008).

Loss-of-function mutations in the hepatocyte canalicular phospholipid flippase MDR3 (ABCB4) have been associated with a wide range of human biliary diseases including progressive familial intrahepatic cholestasis type 3 (PFIC3), cholestasis of pregnancy, drug induced cholestasis and an adult biliary cirrhosis with features similar to primary sclerosing cholangitis (PSC) (Jacquemin 2001; Trauner, Fickert et al. 2007; Trauner, Fickert et al. 2008; Gonzales, Davit-Spraul et al. 2009). Mice with a targeted deletion of Mdr2 (the murine ortholog of MDR3) lack the liver-specific P-glycoprotein that transports phosphatidylcholine (PC) across the canalicular membrane. The absence of phospholipids in bile results in progressive sclerosing cholangitis with accompanying portal inflammation, ductular proliferation and portal fibrosis. Liver injury manifests shortly after birth and hepatocellular carcinomas emerge spontaneously between 50 to 60 weeks of age (Frijters, Ottenhoff et al. 1996; Van Nieuwkerk, Elferink et al. 1996; De Vree, Ottenhoff et al. 2000; Fickert, Zollner et al. 2002; Fickert, Fuchsbichler et al. 2004; Popov, Patsenker et al. 2005; Baghdasaryan, Fickert et al. 2008). Unlike xenograft models that are widely utilized to examine mechanisms of- and treatments for- HCC, $Mdr2^{-/-}$ mice provide a model that parallels the natural evolution of HCC on a background

of chronic inflammation, liver injury and fibrosis (Katzenellenbogen, Mizrahi et al. 2007).

Gene expression analyses in Mdr2-deficient mice and heterozygote controls have demonstrated robust and sustained induction of multiple adaptive mechanisms that control cellular responses related to oxidative stress, inflammation, lipid metabolism, and proliferation, prompting speculation that these processes contribute to hepatocarcinogenesis (Katzenellenbogen, Pappo et al. 2006). Although not formally assessed by earlier studies of Mdr2-deficient mice, another pathway that might play a role in fibrosis-associated hepatocarcinogenesis is Hedgehog (Hh), because Hh signaling has been implicated in both fibrogenic repair of liver injury and HCC.

The Hh pathway is an evolutionarily conserved signaling pathway that is activated when Hh ligands (Sonic hedgehog and Indian hedgehog) bind to Patched (Ptc), a transmembrane receptor that is expressed on the surface of Hh-responsive cells. Upon ligand binding, Ptc is inactivated, relieving its repression of Smoothened (Smo), a trans-membrane protein that mediates Hh signaling inside the cell. Smo activation culminates in the nuclear localization of Gli-family transcription factors, Gli1, Gli2, and Gli3, which, in turn, regulate downstream gene expression. The pathway is quiescent in normal liver (Ramalho-Santos, Melton et al. 2000; Berman, Karhadkar et al. 2003), but becomes reactivated as a repair mechanism in chronic liver injury (Omenetti, Yang et al. 2007; Omenetti and Diehl 2008; Omenetti, Popov et al. 2008; Omenetti, Porrello et al. 2008; Syn, Jung et al. 2009). Hh ligands promote the growth and viability of myofibroblasts, the accumulation of which leads to abnormal liver repair, fibrosis and eventual cirrhosis (Sicklick, Li et al. 2005; Choi, Omenetti et al. 2009). Hh ligands also serve as viability and proliferative factors for liver epithelial progenitors, and expansion of this compartment has been linked to the formation and maintenance of hepatocellular carcinomas (Huang, He et al. 2006). The possibility that Hh pathway activation contributes to hepatocarcinogenesis is supported by the fact that Sonic hedgehog (Shh)

ligand expression is noted in approximately 60% of human HCCs, and expression of the Hh-regulated genes, Gli1 and Ptc, occurs in 50% of human tumors (Patil, Zhang et al. 2006; Sicklick, Li et al. 2006; Pereira Tde, Witek et al. 2010; Chen, Tang et al. 2011).

Based on these observations, we postulated that Hh pathway activation contributes to the pathogenesis of both liver fibrosis and fibrosis-associated HCC. To test this hypothesis, we treated aged Mdr2-deficient mice with the Hh pathway inhibitor, GDC-0449, a small-molecule inhibitor that binds to Smoothened (SMO). This agent was selected because of its human safety profile in phase 1 trials, as well as its effectiveness in solid organ tumors like basal cell carcinoma (BCC) and medulloblastoma (Rudin, Hann et al. 2009; Von Hoff, LoRusso et al. 2009; Lorusso, Rudin et al. 2011). Our aims were to determine whether or not mice with advanced liver disease and HCCs would tolerate Hh pathway inhibition and experience improvements in liver fibrosis and/or tumor burden.

Methods

Mice. Mdr2$^{-/-}$ mice were a gift from Dr. Detlef Schuppan (Beth Israel Deaconess Medical Center, Boston, MA). Age matched FVB/NJ wild type mice were obtained from Jackson Laboratory (Bar Harbor, ME). All mice were housed with a 12-h light-dark cycle and given water and standard chow *ad libitum*. At ages, 2, 4, 12, 36 52 and 62-weeks, mice were sacrificed under general anesthesia. Liver weight and body weight were recorded, serum and liver tissue were collected. Animal studies were approved by the Institutional Animal Care and Use Committee as governed by the National Institute of Health's "Guide for the Care and Use of Laboratory Animals", Duke University Animal Welfare Assurance Number A3195-01.

Serum AST/ALT determination. Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured using kits commercially available from Biotron Diagnostics (66-D and 68-D respectively; Hemet, CA) according to the manufacturers' instructions.

Western Blot. Total protein was extracted from snap frozen whole liver tissue in RIPA buffer. Samples were pooled by age (2-4 samples per age group) except as noted. Western blot analysis was performed as previously described. Membranes were probed with the following primary antibodies: Sonic hedgehog (sc-9024; Santa Cruz Biotechnology, Santa Cruz, CA), Indian hedgehog (ab39634; Abcam), β-actin (sc-47778, Santa Cruz Biotechnology), Gli2 (18-732-292462**,** Genway, San Diego, CA). All antibodies were diluted 1:1000 and were incubated at 4^{o}C overnight. Western blot images were acquired using a FluorChem HD2 digital darkroom system (Cell Biosciences, Santa Clara, California).

Immunohistochemistry. 4μm formalin-fixed, paraffin-embedded samples were dewaxed and rehydrated. To evaluate tissue architecture, slides were stained with hematoxylin and eosin (H&E) and Sirius red per standard protocol. For immunohistochemistry, slides were incubated in 3% hydrogen peroxide/methanol. Antigen retrieval was performed by heating in 10mM sodium citrate buffer or 0.25% pepsin (K19; Invitrogen, Carlsbad, CA) for 10 minutes. Sections were blocked (Dako Envision, Carpinteria, CA) and incubated with primary antibodies overnight at 4^{o}C: glioblastoma-2 (Gli-2; 18-732-292462; 1:2000; Genway, San Diego, CA); cytokeratin 19 (Troma-III; Hybridoma Bank, Iowa City, IA; 1:500); α-fetoprotein (AFP) (Dako, 1:1000); Indian hedgehog (Abcam; 1:750, Cambridge, MA); Polymer-HRP anti-rabbit (K4003; Dako) or anti-mouse (K40011; Dako) or MACH3 mouse AP polymer kit (MP530, Biocare Medical, Concord, California, USA)

were used as secondary antibodies. 3,3'-Diaminobenzidine (DAB) Substrate Chromogen System (K3466; Dako) and/or Ferangi Blue (Biocare) was employed in the detection procedure. Omitting primary antibodies from the reactions eliminated staining which demonstrated staining specificity. Images were acquired on an Olympus IX71 (Tokyo, Japan) inverted microscope using the DP2-BSW (Olympus) image acquisition software system.

Quantitative Real-Time Reverse Transcription PCR. RNA was isolated from whole liver, as well as from resected tumor specimens had standard TriZol extraction as has been previously described (Choi, Omenetti et al. 2009). A table of primers has been included in the Supplementary Materials.

Morphometry. Formalin-fixed liver and tumor sections were stained for CD44 and osteopontin as described above, and were quantified by morphometric analysis with MetaView software (Universal Imaging, Downington, PA). A minimum of 5 randomly selected 10x or 20x fields/section were evaluated for each mouse.

Quantitative Immunohistochemical Analysis. Formalin-fixed liver and tumor sections were costained for Gli2 and CK19. A minimum of 5 ductular regions, 20x fields per section, were evaluated for each mouse by counting the number of cells co-labelled.

Hepatic hydroxyproline assay. The hydroxyproline content in whole liver specimens was quantified colorimetrically as previously reported (Witek, Stone et al. 2009).

GDC-0449 treatment. In the initial, dose-finding cohort of animals, 16 $Mdr2^{-/-}$ mice (age 57-68 weeks) were assigned to treatment with vehicle control (DMSO, n=5), 20mg/kg

GDC-0449 (n=5), or 40mg/kg GDC-0449 (n=6). Male-female ratios were balanced between the groups. GDC-0449 (Selleck Chemicals, Houston, TX) was freshly constituted daily in DMSO. All mice were given a daily intraperitoneal injection for 9 days. On day 10, animals were sacrificed. Samples were collected as above. A second cohort of 20 $Mdr2^{-/-}$ mice (age 51-59 weeks of age) were subjected to whole body magnetic resonance imaging to assess tumor burden and then pairs of mice with comparable tumor burdens were assigned to treatment with either DMSO (control, n=10) or 40mg/kg GDC-0449 (n=10). Drugs were delivered as described above; MRI scanning was repeated after the 9^{th} day of treatment; mice were sacrificed for necropsy and tissue harvest.

Pathology. H&E and sirius red staining of liver sections was evaluated by a board-certified pathologist. Representative sections of tumor and non-tumor tissue were examined. Tumor tissue was defined as grossly visible nodules that were at least 10mm in size and resectable.

Abdominal magnetic resonance imaging. Animals were assigned a blinding code, which was maintained during magnetic resonance (MR) data acquisition and analysis. MR mouse liver imaging was performed on a 7T Bruker Biospec 70/30 horizontal bore system (Billerica, MA). Animals were lightly anesthetized under isofluorane with continuous monitoring and maintenance of physiological parameters throughout the imaging session (~60 min for each animal). Axial and coronal 2D T2-weighted fast spin echo images (TURBO-RARE, TE/TR = 11 /4200 ms with 1 mm slice thick, matrix = 256 X 256 and FOV of 2.4 cm X 2.4 cm, 5 averages, 0.0 mm interslice gap) images were first obtained for screening purposes and supplemental anatomic information. For directed tumor volumetric analysis, 64 contiguous 500 μM thick 3D FSE proton density images

biased towards T1 weighting (TURBO-RARE, TE/TR = 9 /1500 ms, matrix = 256 X 256 X 64 and FOV 2.2 cm X 2.2 cm X 2.2 cm, 25 minutes duration) were acquired. All imaging sequences were performed using respiratory gating.

Volumetric analysis from MR data sets was performed in Osirix software, an open source image processing application developed and maintained by Pixmeo (Geneva, SUI). Liver tumors were manually segmented in each animal by a board-certified radiologist (blinded to treatment) post-treatment. Selected areas were reviewed for consistency on coronal and sagittal representations, and cross-correlated with axial 2D FSE images. For volumetry, two separate volume measurements were obtained for each lesion, with the average volume then taken. Interrater reliability (kappa value) was = 0.97. T1 and T2-weighted scanning sequences were performed.

Statistical analyses. Results expressed as mean±SD. Significance established using the Student's t-test. Differences were considered significant when $p<0.05$.

Results

Progressive accumulation of Hh-responsive cells in Mdr2$^{-/-}$ mice.

Various forms of acute and chronic liver injury induce hepatic expression of Hh ligands and activation of Hh responsive cells. Serum levels of AST and ALT were consistently higher in Mdr2$^{-/-}$ mice than age-matched controls (Figure 2.S1), confirming that the knockout mice had chronic liver injury. Thus, we surveyed livers from Mdr2$^{-/-}$ mice for Hh pathway activation. Compared to liver protein lysates from wild type controls, lysates from Mdr2$^{-/-}$ mice demonstrated an increase in Sonic hedgehog (Shh) and Indian hedgehog (Ihh) by Western blot analysis. This was apparent at the first time point examined (2 weeks after birth) and maintained throughout the lifespan of the animals (up to 64 weeks of age) (Figure 2.1A). Immunohistochemistry for the Hh-

regulated transcription factor, Gli2, demonstrated progressive, age-related accumulation of cells with nuclear Gli2 staining in $Mdr2^{-/-}$ mice. Such Gli2-positive cells included stromal cells, as well as hepatocytic and ductular cells (Figure 2.1B-C). Together, these data suggest that the Hh pathway is activated in chronically injured livers of Mdr2-deficient mice, resulting in progressive expansion of various Hh-responsive cell populations.

Treatment of $Mdr2^{-/-}$ mice with the Smoothened inhibitor, GDC-0449, is safe and decreases Hedgehog pathway signaling.

To determine the appropriate dose of GDC-0449, a pilot study was done with 16 aged (57-68 week old) $Mdr2^{-/-}$ mice. Animals were given a daily intraperitoneal injection of 20mg/kg GDC-0449 (n =5), 40mg/kg GDC-0449 (n=6), or DMSO vehicle control (n= 5) for 9 days and then sacrificed. There was no statistical difference in mortality between the control and high dose (40 mg/kg) GDC-0449 groups. At sacrifice, liver/body weight ratios of mice in the three groups were also similar**,** suggesting that a relatively short course of systemic treatment with GDC-0449 at 40mg/kg is well tolerated by mice with advanced liver disease and HCC (Figures 2.1.S2A-B**)**. Western blot analysis of liver lysates from this cohort of animals demonstrated that GDC-0449 treatment caused a decrease in Shh ligand levels at the higher dose, and Gli2 expression attenuation at both doses (Figure 2.S2C).

Hedgehog signaling inhibition with GDC-0449 abrogates effects of Hh signaling on target gene expression.

Based on the data acquired in our dose-finding study, a second cohort of $Mdr2^{-/-}$ mice (51-59 weeks of age) were assigned to treatment with vehicle (DMSO; n=10) or 40mg/kg GDC-0449 (n=10) via daily i.p injection for 9 days. Overall survival between

the two groups in the second cohort was equal, with no deaths in the DMSO treatment group and 1 death in the GDC-0449 treatment group secondary to iatrogenic injury. Both the liver parenchyma and tumors of treated mice showed decreased expression of the Hh pathway target Gli2 (Figures 2.2A-B). Real-time PCR analysis of resected tumors revealed that GDC-0449 treatment released the inhibitory effects of Hh signaling on PPARγ, causing a significant increase in its gene expression (Figure 2.2C). Treatment with GDC-0449 also caused a significant decrease in expression of Gli1, another Hh target gene (Figure 2.2D).

Hedgehog signaling inhibition with GDC-0449 reduces liver fibrosis.

$Mdr2^{-/-}$ mice demonstrated progressive age-related increases in hepatic expression of alpha-smooth muscle actin (α-sma), a marker of myofibroblastic hepatic stellate cells (Figure 2.3A, top panel). This was accompanied by enhanced expression of pro-fibrogenic factors, such as transforming growth factor TGF-β and platelet derived growth factor PDGF-β (Figures 2.S3A-B), and progressive fibrosis, as evidenced by Sirius red staining (Figure 2.3A, bottom panel) and quantification of the hepatic hydroxyproline content (Figure 2.3B). Treatment with GDC-0449 decreased α-sma-expressing myofibroblastic cells, hepatic expression of TGF-β and PDGF-β, Sirius red staining, and hydroxyproline content, demonstrating that Hh pathway inhibition reduced liver fibrosis (Figures 2.3C-D and Figures 2.S3C-D).

Hedgehog signaling inhibition with GDC-0449 decreases accumulation of liver progenitor cells.

In response to injury, progenitor populations in the liver proliferate. Hence, immunohistochemical analysis for progenitor markers, such as cytokeratin-19 (CK-19) and α-fetoprotein (AFP), showed age-related increases in $Mdr2^{-/-}$ mice (Figure 2.4A). Co-

staining for CK19 and Gli2 confirmed that the progenitor population was Hh responsive (Figure 2.4B). Upon treatment with GDC-0449, progenitor markers decreased (Figure 2.4C), indicating that Hh signaling was required to maintain progenitor populations during tumorigenesis, and that Hh inhibition was sufficient to shrink the size of progenitor populations even in mice with advanced HCC.

Decreasing Hedgehog signaling reduces expression of osteopontin and prevents accumulation of osteopontin-responsive (CD44-positive) cells.

Stem/progenitor cell populations for many types of cancer, including HCC, are thought to be enriched with cells that express CD44, a receptor for the stem cell growth factor, osteopontin (Yang, Fan et al. 2008; Orian-Rousseau 2010). Osteopontin expression is regulated by Hh signaling (Syn, Choi et al. 2011). Therefore, we examined the effects of GDC-0449 on osteopontin and its receptor, CD44. Immunohistochemistry and quantitative morphometry demonstrated that inhibition of the Hh pathway with GDC-0449 significantly decreased osteopontin staining within primary liver tumors (Figure 2.5A). Similar treatment-related decreases in CD44+ tumor cells were also noted (Figure 2.5B). Gene expression analysis showed that expression of both osteopontin and CD44 mRNAs also tended to decrease in GDC-0449-treated mice (Figure 2.5C). Together, these results suggest that Hh signaling may regulate putative liver cancer stem/progenitor cells by modulating availability of osteopontin.

MRI and histological evidence of liver tumor involution following GDC-0449 treatment.

To evaluate the potential impact of changes in matrix and progenitor cells on HCC, pre- and post-treatment tumor volumes were analyzed using magnetic resonance imaging (Figures 2.6A-B). An analysis of tumors in mice without overt metastasis demonstrated that tumor volumes decreased in mice that received a 9 day course of

GDC-0449-treatment, while vehicle-treated animals evidenced persistent tumor growth (Figure 2.6C; -6.7% +/- 11.7% vs 22.7% +/-9.1%, p = 0.03). This data correlated with necropsy findings: Only 56% of GDC-0449-treated mice had visible liver tumor nodules, compared to 80% of the DMSO mice (Table 2.1). Furthermore, both MRI and necropsies showed a decreased number of metastasis in GDC-0449-treated mice compared to vehicle-treated controls (Table 2.1). Histological analysis of H&E-stained liver sections was also performed on all animals from both cohorts. If tumor nodules were not grossly visible or greater than 10 mm in size, the samples were excluded from analysis. Microscopic tumor nodules in GDC-0449 treated animals demonstrated increased rates of hemorrhagic infarct (20% vs 0%; Figure 2.6D, top panel), microvesicular steatosis (40% vs 0%, Figure 2.6D, middle panel), acidophilic necrosis and degenerative cytoplasmic changes (70% vs 40%, Figure 2.6D, bottom panel) in comparison to tumors from vehicle treated animals. Thus, findings on MRI, necropsy, and liver histology were consistent and demonstrated that Hh pathway inhibition caused significant regression of primary and metastatic HCC.

Discussion

We studied $Mdr2^{-/-}$ mice to determine whether or not activation of the Hh pathway contributes to hepatocarcinogenesis during chronic fibrosing liver injury. Mdr2-deficient mice lack a phospholipid flippase that is required for normal bile formation, and consequently exhibit liver injury and ductular proliferation from a young age. All afflicted mice eventually develop significant liver fibrosis and metastatic HCC. Our results demonstrate that this pathology is accompanied by progressive activation of the Hh pathway. Introduction of a specific inhibitor of the key Hh signaling intermediate, Smoothened, reduced pathway activity and proved that sustained Hh signaling was required to maintain the expanded populations of liver myofibroblasts and progenitors

that had accumulated in the damaged livers. Moreover, treating aged Mdr2-deficient mice (which already had advanced liver fibrosis and HCC) with the Hh pathway inhibitor significantly reduced liver fibrosis and tumor burden, demonstrating for the first time that inhibiting Hh signaling has clinically-relevant, therapeutic value for both liver fibrosis and HCC. Even more exciting is evidence that the prohibitive effect on hepatic tumor growth *in vivo* pertains to both intra-hepatic HCC and distant metastasis.

In addition, our results provide insight into some of the underlying mechanisms involved. Hepatic production of Hh ligands was increased in mice that developed progressive liver fibrosis and invasive HCC. These mice also demonstrated consistently higher serum aminotransferase levels, in keeping with other evidence that Mdr2 deficiency provokes chronic hepatocyte injury (Smit, Schinkel et al. 1993; Mauad, van Nieuwkerk et al. 1994). Various stressors that reduce hepatocyte viability have been shown to induce production and release of Hh ligands by wounded hepatocytes (Jung, Witek et al. 2010; Rangwala, Guy et al. 2011). Thus, hepatocyte injury is likely one of the factors that increases Hh ligand generation in mdr2-deficient livers. Myofibroblastic cells and ductular-type progenitors that progressively accumulate in chronically injured livers also produce Hh ligands, as well as other factors, such as PDGF-β, that stimulate autocrine-paracrine synthesis of Hh ligands (Omenetti, Popov et al. 2008; Yang, Wang et al. 2008). Relative to age/gender matched healthy control mice, aged mdr2-deficient mice with ductular reactions and liver fibrosis demonstrated higher hepatic mRNA levels of PDGF-β, suggesting a mechanism by which the fibrogenic repair response itself might perpetuate excessive hepatic accumulation of Hh ligands. The latter concept is supported by evidence that inhibiting Hh signaling with GDC-0449 reduced accumulation of myofibroblasts and ductular-type progenitors, decreased expression of PDGF-β, and suppressed hepatic expression Hh ligands in mice that remained at risk for hepatocyte injury due to genetic deficiency of Mdr2.

Chronic over-production of Hh ligands has important pathobiological implications because mice that generated increased Hh ligands also demonstrated excessive activation of the Hh pathway in their livers. This was evidenced by larger numbers of cells with nuclear staining for the Hh-regulated transcription factor, Gli2, and enhanced expression of various Hh-target genes, including Gli1 and osteopontin. In such animals, we showed that treatment with a highly specific antagonist of Smoothened was able to suppress all of these responses, consistent with published evidence that activation of Smoothened in Hh-responsive cells promotes nuclear localization of Gli2, and consequent induction of Gli1 and osteopontin transcription (Das, Harris et al. 2009; Omenetti, Choi et al. 2011). Osteopontin, in turn, has been shown to promote myofibroblast accumulation and liver fibrosis in mice (Machado and Cortez-Pinto 2011; Syn, Choi et al. 2011). It also acts via its receptor, CD44, to enhance the viability and growth of certain types of liver progenitors, including liver cancer stem/progenitor cells (Haramaki, Yano et al. 1995; Yang, Fan et al. 2008; Orian-Rousseau 2010; Rangwala, Omenetti et al. 2011). Hence, the Hh pathway might be modulating both liver fibrosis and HCC outgrowth by regulating the autocrine/paracrine availability of osteopontin.

Myofibroblasts and ductular progenitor cells are also important sources of other pro-fibrogenic factors (e.g., PDGF-β and TGF-β) that are capable of activating Gli2 via mechanisms that do not require Smoothened (Dennler, Andre et al. 2007; Friedman 2008; Dennler, Andre et al. 2009). We observed increased hepatic expression of both PDGF-β□□and TGF-β in Mdr2-deficient mice. This suggests that Smoothened-independent mechanisms that re-enforce the effects of the canonical Hh pathway may evolve during fibrogenic repair. In mdr2-deficient mice, however, GDC-0449 significantly reduced expression of TGF-β and tended to suppress expression of PDGF□, demonstrating that canonical Hh signaling with resultant Smoothened activation is ultimately required to fully engage noncanonical pathways that rely on interaction of

TGF-β and PDGF-β with their respective receptors to activate Gli2 in injured, fibrotic livers.

Finally, the fact that liver fibrosis/cirrhosis is a major risk factor for the development of HCC should not be misconstrued to imply that fibrosis *per se* causes cancer. It is conceivable that fibrosis and HCC each result from some adaptive response that occurs during chronic liver injury. Our results demonstrate that injury-related activation of the Hh pathway typifies an adaptive response that is both pro-fibrogenic and pro-carcinogenic. This discovery, in turn, provides novel evidence that helps to explain why fibrosis and HCC often develop in the same livers. As such, it has immediate clinical relevance to the many cirrhotic patients with HCC. Additional research will be required, however to delineate the precise down-stream mechanism(s) involved and to determine which (if any) of those subsequent fibrogenic and carcinogenic processes are inter-dependent, and which are totally independent of each other.

Proof that inhibition of Hh signaling substantially reduced the hepatic content of myofibroblasts and progenitors suggests that these cell types promote and/or maintain the outgrowth of malignant hepatocytes. This finding, in turn, provides a starting point for further research. Indeed, others have reported that Smoothened antagonists lead to the involution of pancreatic cancers by influencing tumor angiogenesis (Olive, Jacobetz et al. 2009). Myofibroblasts are known to be an important source of vascular growth factors, such as VEGF (Ankoma-Sey, Wang et al. 2000; Lee, Semela et al. 2007; Witek, Yang et al. 2009), and we found increased hepatic expression of VEGF and its receptor, VEGFR1, in the diseased livers of Mdr2-deficient mice compared to the healthy livers of age/gender-matched controls (data not shown). Hence, it is possible that myofibroblast depletion exerted a negative impact on the hepatic vasculature that ultimately resulted in HCC involution. This concept is supported by evidence that GDC-0449 tended to

suppress hepatic expression of VEGF/VEGFR1 (data not shown) and increased necrosis in the HCC of GDC-0449-treated mice. Hh signaling is also known to maintain various progenitor populations (Katoh and Katoh 2009; Tanaka, Nakamura et al. 2009; Song, Yue et al. 2011). Previously, we reported that Hh ligands function as autocrine and paracrine survival signals for liver progenitors and showed that liver myofibroblasts are an important paracrine source of Hh ligands that serve this purpose (Sicklick, Li et al. 2006; Jung, McCall et al. 2007; Jung, Brown et al. 2008; Yang, Wang et al. 2008; Jung, Witek et al. 2010). Liver progenitors, in turn, generate Hh ligands that provide paracrine signals that re-enforce the growth of liver myofibroblasts (Fleig, Choi et al. 2007; Omenetti, Syn et al. 2009). Given this background, it is not surprising that blocking Hh signaling with a Smoothened antagonist resulted in the mutual depletion of both cell types. To our knowledge, however, by demonstrating marked treatment-related decreases in CD44, cytokeratin-19, and α-fetoprotein within HCC tumors, the current data provide the first evidence that Smoothened antagonists reduce populations of cells that exhibit features of tumor stem/progenitor cells. Further investigation is required to ascertain how aberrant Hedgehog signaling promotes these cancer stem cell compartments.

In conclusion, our findings in the $Mdr2^{-/-}$ mouse model of progressive liver fibrosis and spontaneous hepatocarcinogenesis demonstrate that increased production of Hh ligands and progressive accumulation of Hh-responsive cell types, such as myofibroblasts and liver progenitor cells, precede the emergence of HCC, and persist after the development of HCC. Despite advanced liver fibrosis and HCC, a short course of treatment with a highly-specific and clinically available Hh signaling inhibitor is well-tolerated and demonstrates appreciable anti-tumor effects. Thus, Hh pathway inhibition with GDC-0449 merits evaluation as a potential treatment for HCC arising in cirrhotic patients, providing a novel treatment option for an emerging disease with a poor

prognosis and limited therapeutics.

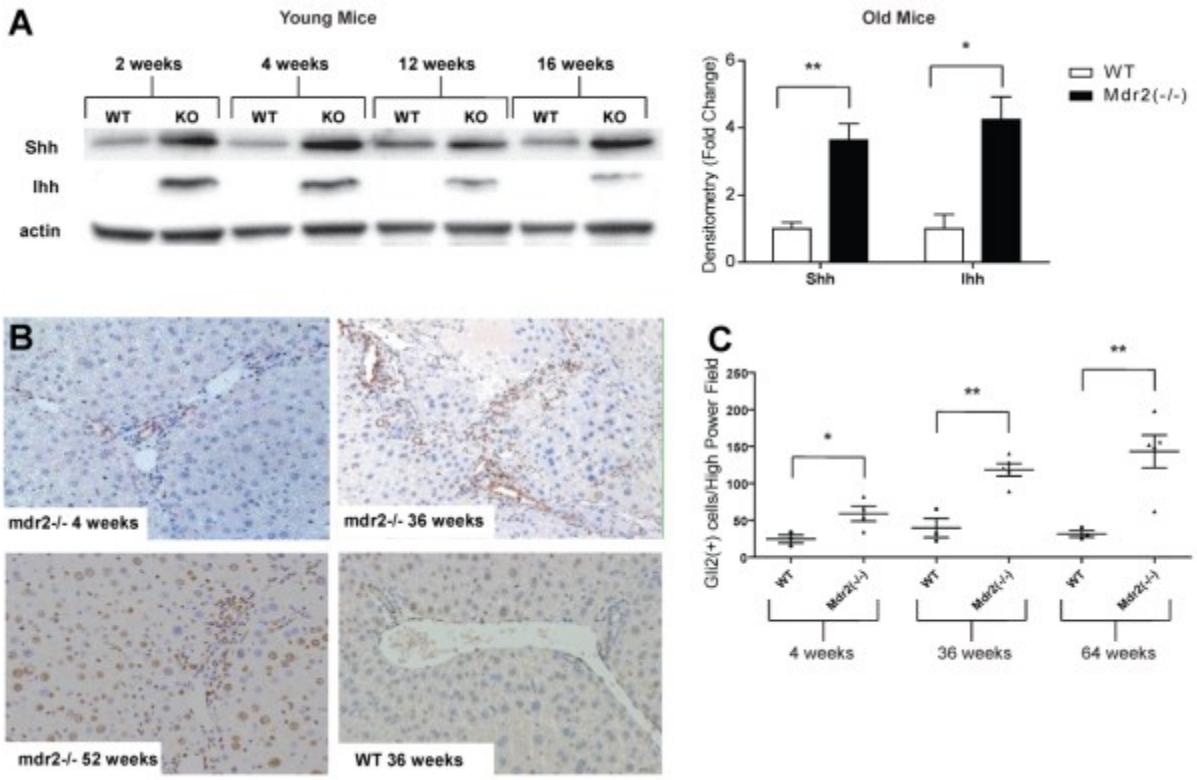

Figure 2.1. Increased hepatic Hedgehog (Hh) pathway activity in Mdr2$^{-/-}$ mice.

Figure 2.1. Increased hepatic Hedgehog (Hh) pathway activity in Mdr2$^{-/-}$ mice.

A. Western blot analysis for Sonic Hedgehog (SHh) and Indian Hedgehog (IHh) in whole liver extracts pooled from young Mdr2$^{-/-}$ mice (2-16 weeks old), old (36-64 weeks old) Mdr2$^{-/-}$ mice and age-matched controls (n=3-5 mice/group/time point). A representative Western blot demonstrating results from young mice is shown. Mean±SEM data from older mice are graphed. **B.** Representative liver sections stained to demonstrate Gli2 from young (4 wk old), middle aged (36 wk old) and old (52 wk old) Mdr2$^{-/-}$ mice. Little Gli2 expression was noted in wildtype mice at any age, so results from a representative 36 wk old wild type mouse is shown. (10x) **C.** Quantitative Gli2 immunohistochemical data. The number of nuclear Gli2(+) cells was counted in at least 5 high power fields (HPF) per liver section in Mdr2$^{-/-}$ mice and wild type controls at 4, 36 and 64 weeks of age (n=3-4 mice/group/time point) and mean±SEM are graphed. *$p<0.05$, **$p<0.01$ in Mdr2$^{-/-}$ groups vs. respective controls

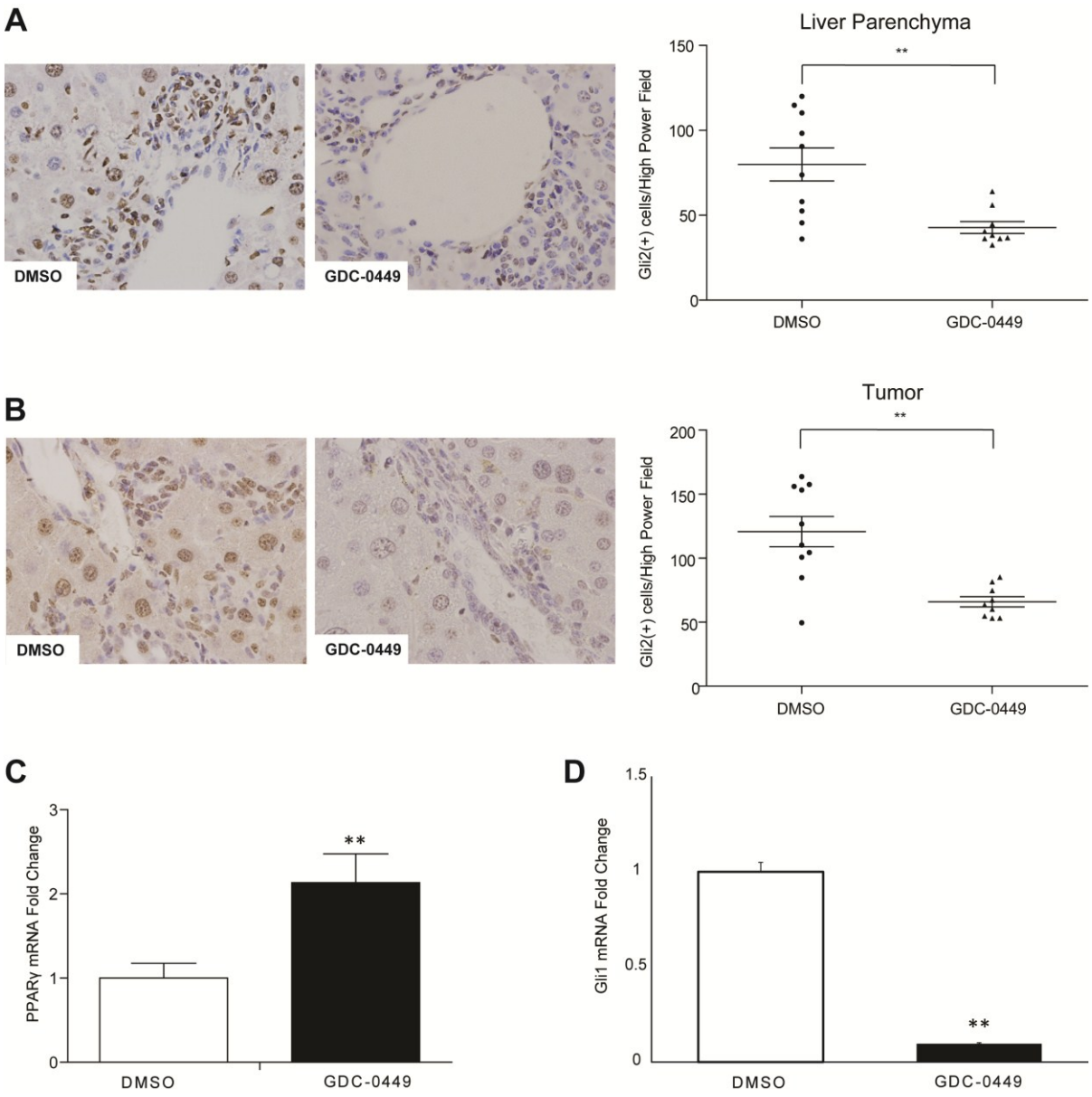

Figure 2.2. Hedgehog (Hh) inhibitor, GDC-0449, abrogates effects of Hh signaling within liver parenchyma and HCC nodules.

Figure 2.2. Hedgehog (Hh) inhibitor, GDC-0449, abrogates effects of Hh signaling within liver parenchyma and HCC nodules. A. Liver sections stained for Gli2 from representative DMSO- and GDC-0449- treated mice (40x). Quantitative Gli2 immunohistochemistry data in non-tumor livers of mice treated with DMSO or GDC-0449 (n=9-10/group) are graphed as mean±SEM *(**p<0.01*. The number of ductular cells with Gli2 positive staining were counted in each portal tract/section under 40x magnification. **B.** Tumor sections from the same mice were also stained to demonstrate Gli2. Results from representative DMSO- and GDC-0449-treated mice are displayed. Quantitative Gli2 immunohistochemistry data were generated by counting nuclear Gli2 positive ductular and hepatocytic cells in tumor sections under 40x magnification. Results are graphed as mean±SEM Gli2-positive cells/40x high power field (**p<0.01*)* **C-D** Quantitative reverse transcription-PCR (qRT-PCR) analysis of whole liver RNA from DMSO-(open bar) and GDC-0449 (black bar) treated mice. **C.** PPAR-γ, a gene that is normally repressed by Hh signaling. **D.** Gli1, a gene that is induced by Hh signaling. Mean±SEM are graphed (**p<0.01).

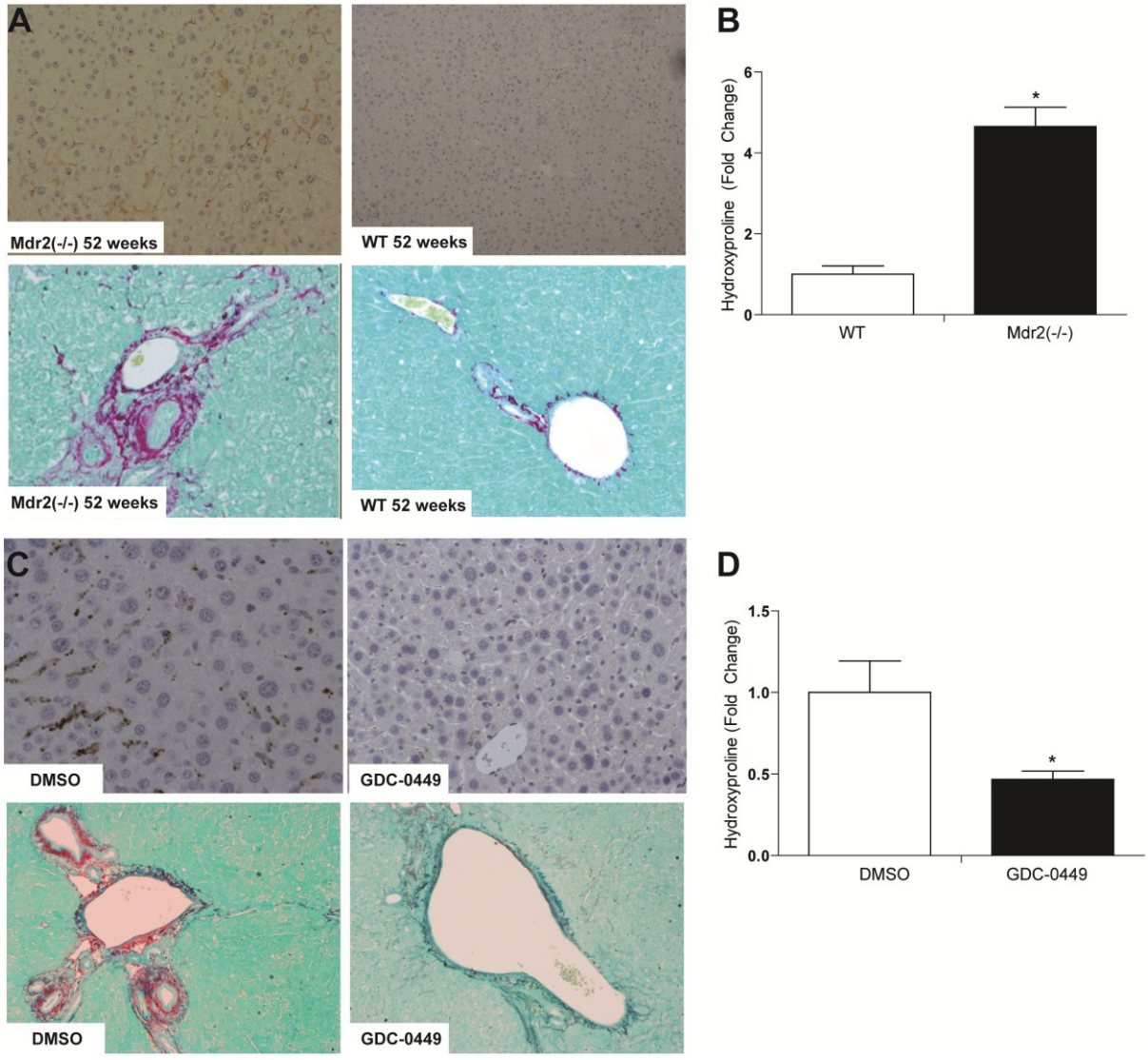

Figure 2.3. GDC-0449 treatment reduces fibrosis in Mdr2$^{-/-}$ mice.

Figure 2.3. GDC-0449 treatment reduces fibrosis in Mdr2$^{-/-}$ mice.

A. Immunohistochemical staining for α-SMA (top panel) and Sirius red (bottom panel) in sections of non-tumor liver from representative age-matched Mdr2$^{-/-}$ and wildtype mice (10x). **B.** Pooled Hepatic hydroxyproline content of 2-*52 wk-old* wildtype (WT) and age-matched Mdr2$^{-/-}$ mice (n=3-5/group). Results in Mdr2$^{-/-}$ mice were normalized to that of age-matched WT mice and graphed as fold change. Data are displayed as mean +/- SD (*p<0.05) **C.** Non-tumor liver sections stained for α-SMA (top panel, 20x) and Sirius red (bottom panel, 10x) in representative DMSO- and GDC-treated Mdr2$^{-/-}$ mice. **D.** Heptic hydroxyproline content of DMSO- and GDC- treated mice (n=9/group). Results in GDC-0449-treated mice were normalized to that of DMSO vehicle-treated mice and graphed as fold change. Data are displayed as Mean +/- SEM (*p<0.05).

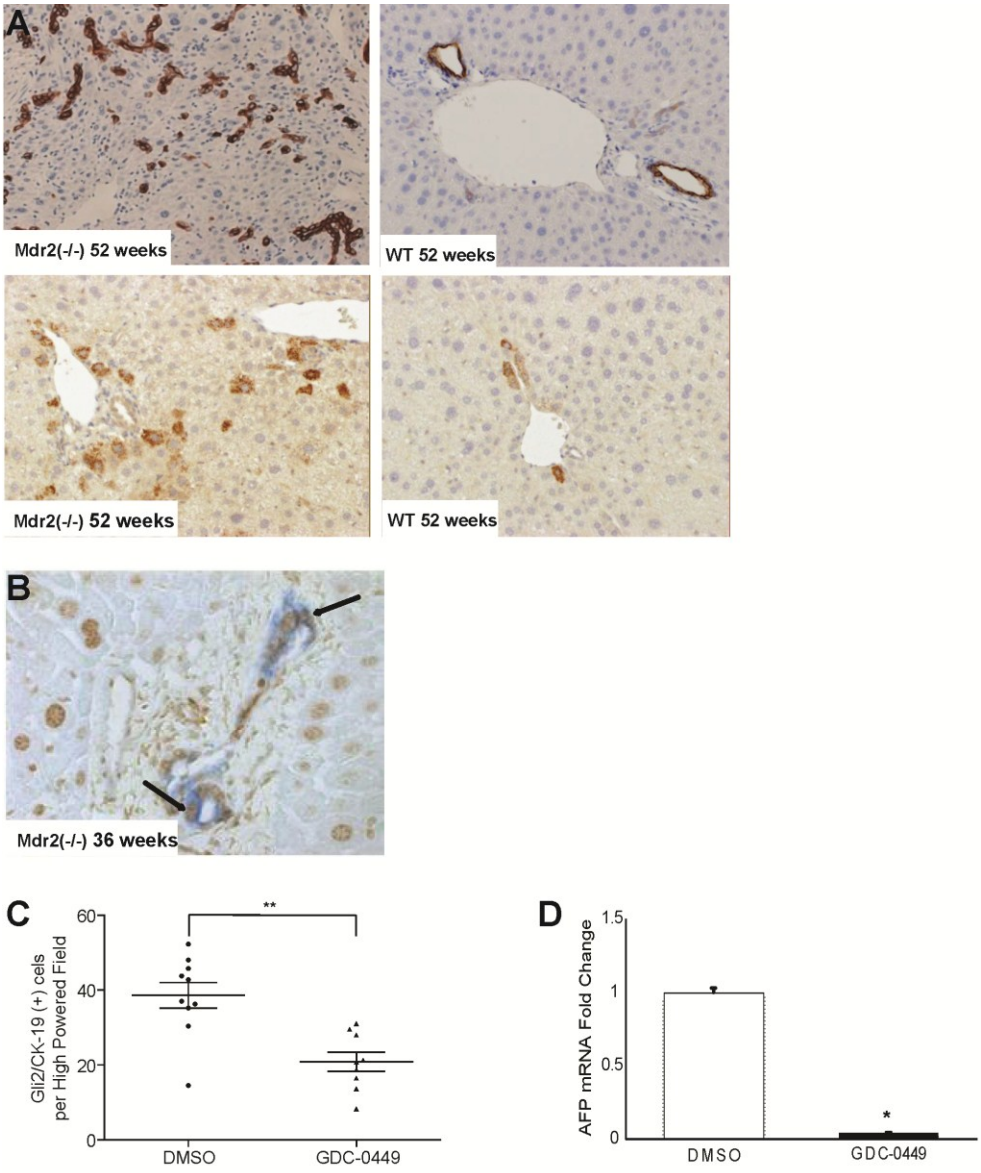

Figure 2.4. Effects of Mdr2 deficiency and Hedgehog (Hh) inhibition on hepatic progenitor populations.

Figure 2.4. Effects of Mdr2 deficiency and Hedgehog (Hh) inhibition on hepatic progenitor populations.

A. Immunohistochemical staining of liver sections from representative, age-matched Mdr2$^{-/-}$ and wildtype mice for progenitor markers, cytokeratin-19 (CK-19) (top panels) and α -fetoprotein (AFP) (bottom panels) (20x). **B.** Representative micrograph of a portal triad in liver of an Mdr2$^{-/-}$ mouse, demonstrating co-localization of CK-19 (blue) and Gli2 (brown) in the ductular compartment (40x). **C**. Quantitative Gli2 and CK19 immunohistochemistry in DMSO- and GDC-0449-treated Mdr2$^{-/-}$ mice (n=9-10/group). The number of Gli2 and CK19 double-positive ductular-appearing cells were counted within tumors under 20x magnification. Mean±SEM double(+) cells t per high power field (HPF) are graphed (* $p < 0.05$) **D.** QRT-PCR analysis of AFP in tumor RNA from mice treated with DMSO (open bar) or GDC-0449 (closed bar) Results in the GDC-0449-treated mice were normalized to that of the mice treated with DMSO vehicle and graphed as Mean±SEM (*$p<0.05$).

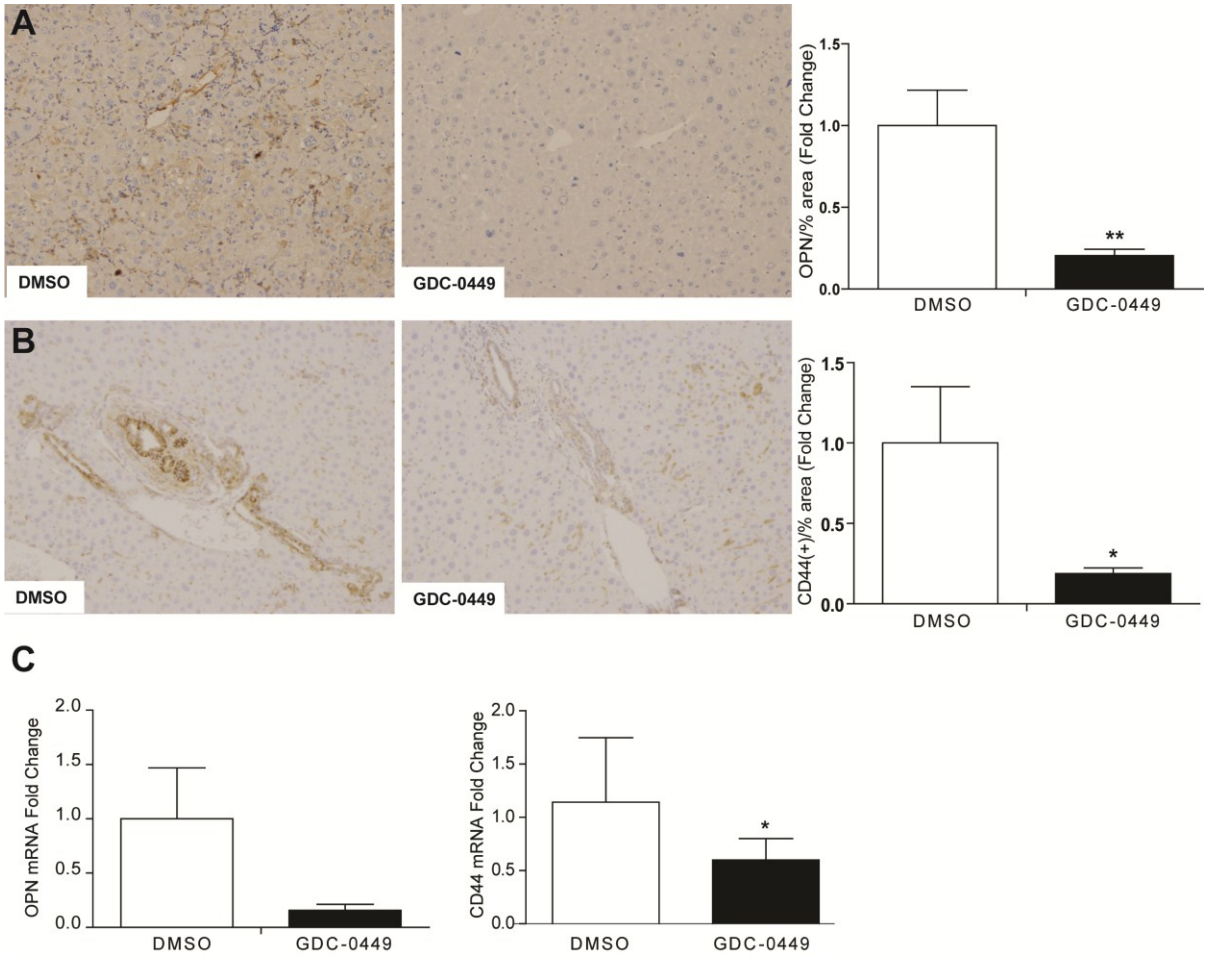

Figure 2.5. Inhibition of Hh signaling decreases osteopontin and osteopontin-responsive (CD44) positive cells in tumors and peri-tumoral tissues of aged Mdr2$^{-/-}$ mice.

Figure 2.5. Inhibition of Hh signaling decreases osteopontin and osteopontin-responsive (CD44) positive cells in tumors and peri-tumoral tissues of aged Mdr2$^{-/-}$ mice.

A. Tumor sections from representative DMSO- and GDC-0449 treated Mdr2$^{-/-}$ mice were stained to demonstrate osteopontin (OPN) Representative sections are displayed (*Right panel*). OPN staining was quantified by morphometric analysis of at least 5 HPF per tumor section using 20x magnification (n=5 mice/group).Results in the GDC-0449-treated group were normalized to that of the group treated with DMSO vehicle and graphed as fold change. Data are displayed as Mean±SEM (**p<0.01). **B.** Immunohistochemical staining for the osteopontin receptor, CD44, in peri-tumoral tissues of representative DMSO- and GDC-0449- treated Mdr2$^{-/-}$ mice. (*Right panel*) CD44 staining was quantified by morphometric analysis as described in A. Results in GDC-0449-treated mice were normalized to those of vehicle-treated controls and graphed as Mean±SEM (**p<0.01). **C.** QRT-PCR analysis of liver tumor RNA from DMSO- (open bar) and GDC-0449- (closed bar) treated Mdr2$^{-/-}$ mice for OPN (left) and CD44 (right). After normalization to results in the DMSO-treated group, Mean±SEM were graphed (*p<0.05).

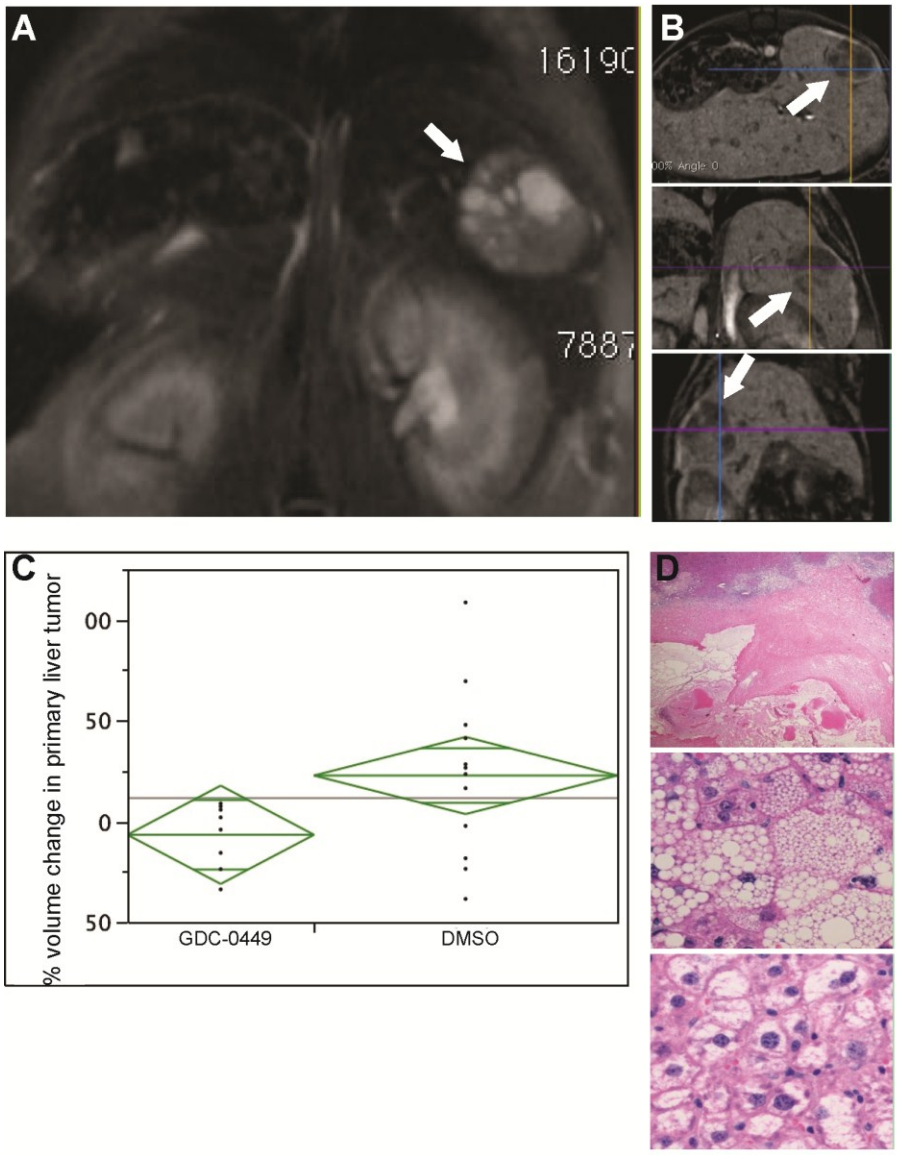

Figure 2.6. Hh pathway inhibition decreases in liver tumor volume by magnetic resonance imaging (MRI) and induces histologic features of tumor involution.

Figure 2.6. Hh pathway inhibition decreases in liver tumor volume by magnetic resonance imaging (MRI) and induces histologic features of tumor involution. A. Representative cross-sectional image of intrahepatic tumor as identified by contrast-enhanced MRI following GDC-0449 treatment. **B.** Representative MRI images of sagittal, coronal, and oblique perspectives used for volumetric analysis. **C.** Change in tumor volume were quantified by computerize generated volumetric measurements and graphed as Mean±SD (*$p<0.05$). **D.** Micrographs of representative histologic changes induced by, GDC-0449, treatment. Top panel shows tumor necrosis; middle panel demonstrates fatty degeneration; bottom panel show vacuolated tumor cells with pyknotic nuclei.

Table 2.1. Effect of GDC-0449 treatment on intrahepatic HCC and HCC metastases.

	Surgical Resection		MRI Imaging	
	Vehicle	**Treatment**	**Vehicle**	**Treatment**
Intrahepatic Tumor Nodules	80%	56%	78%	50%
Metastases	20%	0%	50%	20%

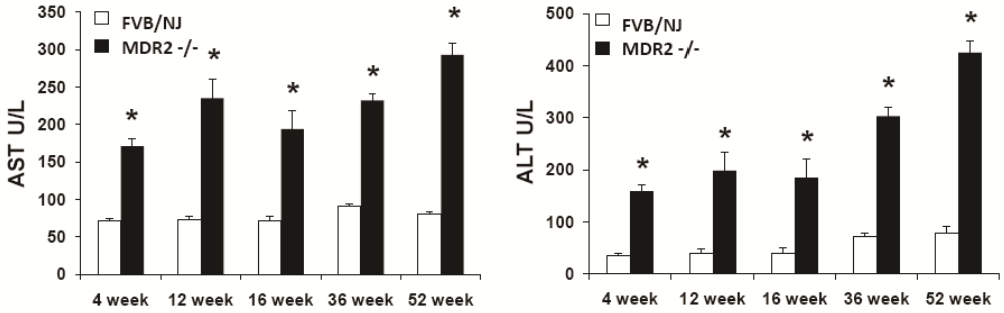

Figure 2.S1. Evidence of ongoing liver injury in Mdr2$^{-/-}$ mice. AST and ALT measurements from Mdr2$^{-/-}$ mice and their age-matched wild type counterparts at various time points. Each data point represents n=2-7 animals and Mean±SD is graphed. (*p< 0.05 vs the age-matched wild-type control).

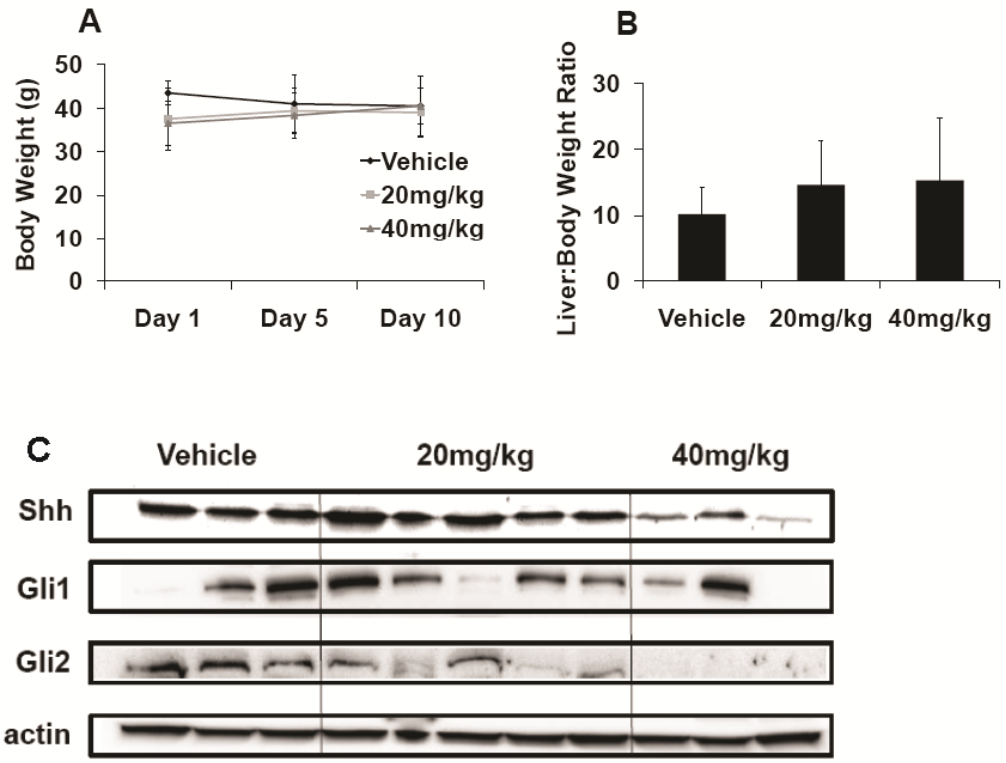

Figure 2.S2. Systemic treatment of GDC-0449 treatment is well tolerated in Mdr2$^{-/-}$ mice with advanced liver disease and HCC. A. Daily body weight measurements were obtained for all animals in each of the three treatment groups and graphed over time. Data expressed as Mean±SD. **B.** Liver to body weight ratios of animals in each of the three treatment groups. Data expressed as Mean±SD. **C.** Western blot analysis for Shh, Gli1, Gli2 and actin (loading control) in whole liver extracts from Mdr2-/- mice treated with vehicle, 20mg/kg GDC-0449, and 40mg/kg GDC-0449. Liver extracts from each treated mouse were loaded individually.

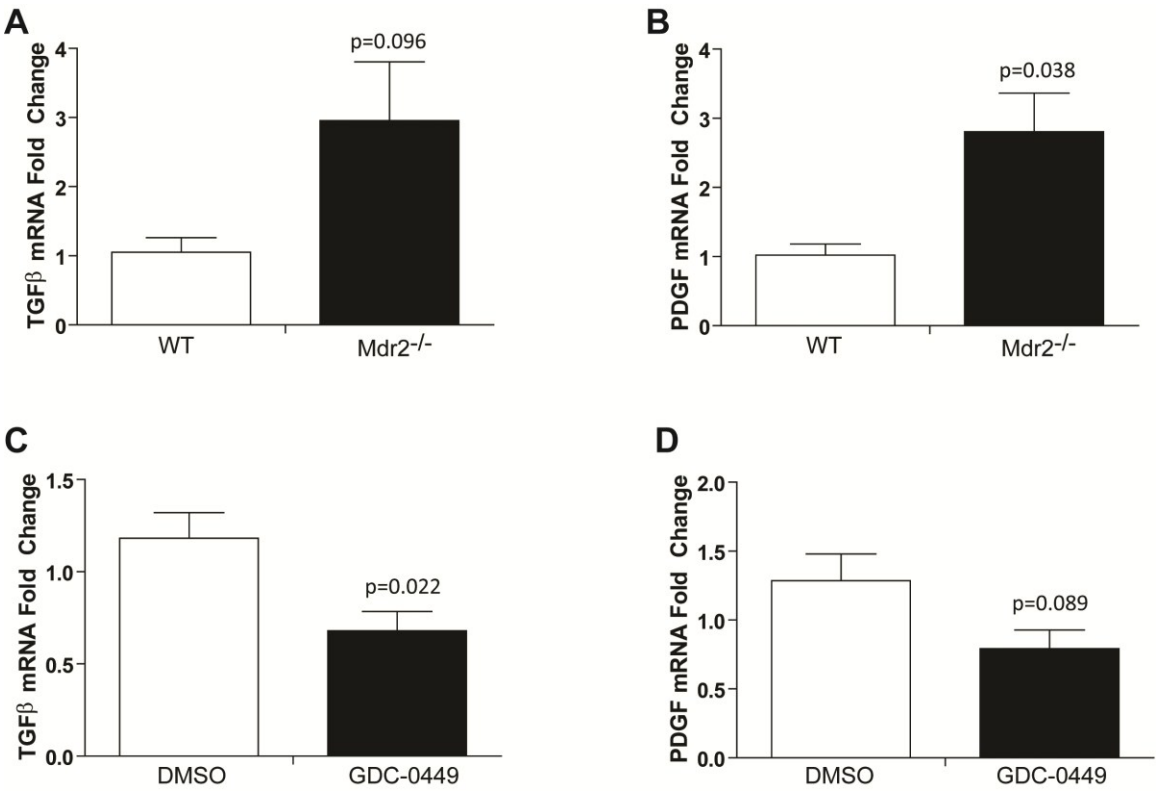

Figure 2.S3. Increased hepatic expression of TGFβ and PDGFβ in Mdr2$^{-/-}$ mice is reversed by GDC-0449 treatment. Whole liver RNA was isolated from Mdr2$^{-/-}$ mice and age/gender-matched wild type controls (WT) (n=3 mice/group) and QRT PCR was done to compare expression of **(A)** TGFβ and **(B)** PDGFβ. Similar approaches were used to assess the effects of a 9 day course of treatment with either the Hh pathway inhibitor GDC-0449 or vehicle (DMSO) on expression of **(C)** TGFβ and **(D)** PDGFβ in Mdr2 $^{-/-}$ mice (n=5 mice/group). Gene expression was normalized to expression of S9 in the same samples; mean +/- SEM values were calculated; values in the experimental groups were graphed relative those in the respective controls. P values are shown.

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CHAPTER III

PARACRINE HEDGEHOG SIGNALING DRIVES METABOLIC CHANGES IN HEPATOCELLULAR CARCINOMA

Summary

Hepatocellular carcinoma (HCC) typically develop in cirrhosis, a condition characterized by Hedgehog (Hh) pathway activation and accumulation of Hh-responsive myofibroblasts (MF). Although Hh signaling generally regulates stromal-epithelial interactions that support epithelial viability, the role of Hh-dependent MF in hepatocarcinogenesis is unknown. Here we used human HCC samples, a mouse HCC model, and hepatoma cell/MF co-cultures to examine the hypothesis that Hh signaling modulates MF metabolism to generate fuels for neighboring malignant hepatocytes. The results identify a novel paracrine mechanism whereby malignant hepatocytes produce HH-ligands to stimulate glycolysis in neighboring MF, resulting in release of MF-derived lactate that the malignant hepatocytes use as an energy source. This discovery reveals new diagnostic and therapeutic targets that might be exploited to improve the outcomes of cirrhotic patients with HCC.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common deadly forms of cancer worldwide (Forner, Llovet et al. 2012). HCCs typically develop in cirrhotic livers (Forner, Llovet et al. 2012). The latter compromises recovery from extensive liver resection, and restricts chemotherapy options and efficacy. Therefore, survival depends mainly upon detection of tumors that are small enough to be safely ablated. Better screening and preventative strategies are needed, however, because the number of HCCs that are already advanced at diagnosis is increasing, and the population at risk for

HCC is growing due to the rising incidence of cirrhosis (Forner, Llovet et al. 2012). Improved understanding of the early events in hepatocarcinogenesis would help to optimize prevention, early diagnosis, and treatment of HCC.

Evidence that HCC occur in 1-5% of cirrhotic patients annually suggests that the cirrhotic microenvironment promotes the outgrowth of malignant hepatocytes (Ye, Takayama et al. 2010). However, the mechanisms involved remain obscure. One possibility is that that stromal-epithelial interactions fuel HCC growth because deregulated, and excessively fibrogenic, repair of liver injury causes cirrhosis itself (Jou and Diehl 2010). The major producers of fibrous matrix during liver injury are myofibroblasts (MF), and cirrhotic livers harbor large numbers of these cells. The role of MF in HCC pathogenesis/progression is unclear, however, despite evidence that MF-derived factors mediate key aspects of the wound healing response including matrix turnover, recruitment of inflammatory cells, vascular remodeling, and outgrowth of liver epithelial progenitors (Friedman 2008). The pivotal importance of MF in cirrhosis pathogenesis justifies evaluating their role in hepatocarcinogenesis.

A key regulator of MF is Hedgehog (Hh), a developmental morphogenic signaling pathway (Omenetti, Choi et al. 2011). Hh pathway activity is barely detectable in healthy livers but becomes robust during all types of liver injury. Injured liver epithelial cells are important drivers of this process because injury stimulates the wounded epithelia to produce and release Sonic hedgehog (Shh) and Indian Hedgehog (Ihh) ligands, as well as other soluble factors, that promote Hh signaling in neighboring Hh-responsive stromal cells (Jung, Witek et al. 2010; Rangwala, Guy et al. 2011). Like other Hh-responsive stromal cells, MF express the Hh ligand transmembrane receptor, Patched (Ptc) (Choi, Omenetti et al. 2009). Interaction of epithelia-derived HH-ligands with Ptc results in the activation of Smoothened, the Hh signaling-competent co-receptor. This leads to accumulation and nuclear localization of Glioma-family proteins (Gli1, Gli2, Gli3) which

regulate the transcription of Hh-responsive genes that control proliferation, viability, and differentiation of the stromal cells. Exchange of paracrine signals between Hh-producing epithelia and Hh-responsive stroma orchestrates organogenesis during development. Similar mechanisms are presumed to modulate some types of carcinogenesis based on findings in mouse models of pancreatic and prostate cancer (Gipp, Gu et al. 2007; Olive, Jacobetz et al. 2009). Although increased Hh signaling has been documented in human HCC (Sicklick, Li et al. 2006), and liver MF are known to be a Hh-responsive cell type, the possibility that HCC growth might be regulated by paracrine Hh signaling between MF and malignant hepatocytes has not, to our knowledge, been examined.

Recently, we demonstrated that treating a mouse model of fibrosis-associated HCC with a Hh signaling inhibitor caused advanced HCCs to regress (Philips, Chan et al. 2011). Tumor involution was accompanied by MF loss and fibrosis improvement. This suggests that the anti-cancer actions of the Smoothened antagonist may have resulted from deletion of Hh-responsive MF, and justifies further work to identify how MF might support the growth of malignant hepatocytes. Here we evaluate the hypothesis that Hh signaling modulates MF metabolism to generate fuels for neighboring malignant hepatocytes. Given that HCCs, like many other epithelial cancers, exhibit enhanced glycolysis (i.e., the Warburg effect) (Kitamura, Hatano et al. 2011), we asked if HCC glycolytic activity was influenced by Hh signaling in tumor-associated MF.

Materials and Methods

Human subjects. The Duke Department of Pathology computer database was searched for cases of HCC arising in nonalcoholic fatty liver disease (NAFLD) patients from 2007 through 2011. Five cases were identified from resections, hepatectomies, and explants. Random tissue blocks containing both tumor and adjacent non-tumor were used.

Mice. Mdr2$^{-/-}$ mice were a gift from D. Schuppan (Beth Israel Deaconess Medical Center, Boston, MA). 10 Mdr2$^{-/-}$ mice (age 51-59 weeks of age) were assigned to treatment with either Vehicle (DMSO, n=5) or 40mg/kg GDC-0449 (n=5) and treated for 9 days as previously described (Philips, Chan et al. 2011).

Immunohistochemistry. Liver specimens fixed in formalin and embedded in paraffin were cut into 4μm sections, dewaxed, hydrated, subsequently incubated in 3% hydrogen peroxide/methanol for 15 min to block endogenous peroxidase. To evaluate tissue architecture, slides were stained with hematoxylin and eosin (H&E) per standard protocol. Antigen retrieval was performed by heating in 10mM sodium citrate buffer or 0.25% pepsin (K19; Invitrogen, Carlsbad, CA) for 10 minutes. Sections were blocked (Dako Envision, Carpinteria, CA) and incubated with primary antibodies overnight at 4^{o}C: Sonic Hedgehog (Epitomics 1843-1; 1:6000), Pyruvate kinase M2 (Cell Signaling; 1:1000), Glioblastoma-2 (Genway 18-732-292462; 1:2000); α smooth muscle actin (Dako; 1:800) (Dako, 1:1000); Mct4 (Santa Cruz; 1:1000), Indian hedgehog (Abcam; 1:750); Polymer-HRP anti-rabbit (K4003; Dako) or anti-mouse (K40011; Dako) were used as secondary antibodies. 3,3'-Diaminobenzidine (DAB) Substrate Chromogen System (K3466; Dako) and/or Vino Green (Biocare) was employed in the detection procedure. Omitting primary antibodies from the reactions eliminated staining which demonstrated staining specificity. Images were acquired on an Olympus IX71 (Tokyo, Japan) inverted microscope using the DP2-BSW (Olympus) image acquisition software system.

Quantitative Immunohistochemical Analysis. Formalin-fixed human and mouse tumor sections were stained for GLI2 and costained for GLI2 and PKM2. A minimum of 10

randomly selected 200x field were evaluated for each mouse by counting the number of cells positive for GLI2 only and co-labeled for both GLI2 and PKM2.

Quantitative Real-Time Reverse Transcription PCR. RNA isolated from cells, whole liver, as well as from resected tumor specimens had standard TriZol extraction as previously described (Choi, Omenetti et al. 2009). 15 ng of cDNAs was used to perform QPCR assay in duplicate with SYBR Green Supermix. Primer sequences listed in Supplemental Table 1. Expression of the examined genes was normalized against internal control gene (rodent β-actin or human β-actin) based on the threshold cycle (C_t) and relative fold change calculated by the $2^{-\Delta\Delta Ct}$ method (Choi, Omenetti et al. 2009).

Morphometry. Formalin-fixed human tumor sections (n=5) were stained for SHH, PKM2 and mouse tumor sections (n=5) as described in the previous section; staining was quantified by morphometric analysis with MetaView software (Universal Imaging, Downington, PA). A minimum of 10 randomly selected 200x fields/section were evaluated for each human and mouse section. Each stain quantified by morphometric analysis was normalized to the non-tumor liver group (n=5 patients/group) or Vehicle-treated group (n=5 mice/group).

Cell culture. Clonally derived rat myofibroblastic line (8B cells) were obtained from M. Rojkind (George Washington University, Washington D.C., USA) (Greenwel, Schwartz et al. 1991) were cultured alone (monoculture), in a Transwell co-culture system with HepG2 cells (ATCC, Manassas, VA, USA), Huh 7.5 cells (C. Rice, Rockefeller University), or Panc 10.05 cells (Duke Cell Culture Facility). MF monocultures were also treated with PBS-control or 1000 ng/ml recombinant Shh ligand (StemCell Technologies, Vancouver, Canada) +/- DMSO-vehicle or 3μM GDC-0449 (Selleck Chemicals, Houston,

TX), or conditioned medium from the other cells +/- IgG-control (R&D) or 10ug/ml 5E1 Hh neutralizing antibody (Developmental Studies Hybridoma Bank, Iowa City, IA) for 24 hours. To characterize the effects of myofibroblast-tumor cell interactions *in vitro*, we used a Transwell co-culture system in which liver MF and hepatoma lines were cultured for 24 hours alone or in the co-culture system. To assess the effects of lactate on HepG2 cells, cells were treated with control media and media containing 40μM of lactate (Sigma Aldrich) for 24 hours. Conversely, HepG2 cells were treated for 24 hours simultaneously with 40μM of lactate and either DMSO-vehicle or 10μM of FX11, a LDH inhibitor and gift from C. Dang (Johns Hopkins University, Baltimore, MD), to assess the effects of blocking lactate utilization. All cell lines were plated in triplicate for each experiment and grown as previously described (Choi, Omenetti et al. 2009; Rangwala, Guy et al. 2011). After normalization to respective controls, data from individual cultures were averaged to generate the result of each experiment. All experiments were repeated three times; replicate results were used to calculate final mean +/- SEM values.

Measurement of cellular lipid droplets. PFA fixed slide samples were stained with Oil Red O for 30 min at RT, followed by PBS washing. Oil Red O staining was quantified by morphometric analysis of at least 10 HPF per experiment using 200x magnification.

Measurement of lactate and pyruvate. Cell culture media or whole cell lysate was prepared with Lactate Assay Buffer (Biovision, Mountain View, CA). 250μL of media or lysate was filtered through 10 kd column (BioVision, Mountain View, CA) and stored at -80 ^{0}C for lactate and pyruvate measurement and, when calculating intracellular amounts, for a BCA assay (BioRad, Hercules, CA) to determine protein concentration for normalization. Lactate was measured with the Lactate Assay Kit (BioVision) and pyruvate was measured using the Pyruvate Assay Kit (BioVision) according to the

manufacturer's manual and normalized to the protein concentration.

Measurement of ATP. Whole cell lysates were prepared with ATP Assay Mix (Sigma Aldrich, St. Louis, MO) and ATP was immediately measured using the ATP Bioluminescent Assay Kit (Sigma Aldrich) according to the manufacturer's manual. Results were normalized to protein concentration measured by a BCA assay (BioRad, Hercules, CA).

Luciferase Reporter Assay. The Hh-responsive luciferase reporter assay was performed using Shh-LightII cells (ATCC, Manassas, VA, USA). Cells were treated with conditioned medium from HepG2 cells for two days. Briefly, Shh-LightII cells were stably co-transfected with a Gli-responsive firefly luciferase reporter and a pRL-TK constitutive Renilla luciferase reporter. Firefly and Renilla luciferase activities were determined via a dual luciferase kit (Promega, Madison, WI, USA). In a parallel experiments, Shh-lightII cells were co-cultured with HepG2 cells in a Transwell system. One day after plating, cells were harvested and Firefly and Renilla luminescence were measured.

Results and Discussion

Malignant epithelia produce HH-ligands and stroma is enriched with Hh-responsive, glycolytic MF in human HCC

To investigate Hh ligand expression and localization in HCC, we performed immunohistochemistry for Sonic Hedgehog (SHH) ligand in archived paraffin-embedded tissues from 5 patients with HCC and cirrhosis caused by nonalcoholic fatty liver disease (NAFLD). All of the HCCs demonstrated increased expression of SHH relative to their capsules and adjacent non-tumorous liver tissue (Figure 3.1A). Within tumor nodules,

malignant hepatocytes were a major source of SHH ligand. In contrast, nuclear staining for the Hh-regulated transcription factor, GLI2, was confined to the tumor-associated stroma. Compared to adjacent nontumor liver, HCCs were also significantly enriched with GLI2(+) stromal cells (Figure 3.1B). These findings suggest that malignant hepatocytes produce HH-ligands that promote Hh signaling in adjacent stromal cells.

To assess glycolytic activity in these HCC we stained sections for pyruvate kinase M2 (PKM2), a rate limiting glycolytic enzyme and well-validated marker of glycolysis (Christofk, Vander Heiden et al. 2008). Unexpectedly, we found that PKM2 staining localized to HCC stroma, rather than to the malignant hepatocytes themselves (Figure 3.1C). Dual staining for GLI2 and PKM2 confirmed that the Hh-responsive stromal cells were glycolytic and showed that HCC stroma harbored greater numbers of glycolytic cells than adjacent nontumorous liver (Figure 3.1B, C). To further characterize these glycolytic stromal cells, we co-stained other sections for PKM2 and alpha smooth muscle actin (ASMA), a MF marker (Friedman 2008). Expression of PKM2 co-localized with ASMA, demonstrating that the glycolytic tumor-associated stromal cells were Hh-responsive MF (Figure 3.1D).

Hh inhibitor depletes glycolytic MF from tumor stroma in murine HCC model

Progressive liver injury and fibrosis occur in Mdr2-/- mice due to deficient transport of phosphatidyl choline into bile. Primary hepatocellular carcinomas emerge spontaneously between 50-60 weeks of age, modeling the natural evolution of HCC during fibrogenic repair of various types of chronic liver injury (Katzenellenbogen, Pappo et al. 2006). Therefore, we used immunohistochemistry to characterize the tumor-associated stroma in HCC that were micro-dissected from these mice. As noted in our human HCC cohort (Figure 3.1B-D), tumor-associated stroma in the mouse model was enriched with cells that co-expressed ASMA and PKM2 (Figure 3.2A). Therefore,

glycolytic MF localize within the HCC-associated stroma in both species, and this seems to occur irrespective of the etiology of the underlying liver disease. In human HCC, the glycolytic MF co-stained for GLI2 (Figure 3.1B) and thus, were presumed to be Hh-responsive. To examine the role of Hh signaling in regulating glycolytic activity in the murine tumor stromal cells, we compared expression of Glut1, a glucose transporter, and several key glycolytic enzymes in mRNA isolated from HCCs of Mdr2-deficient mice that had been treated with either vehicle or the Smoothened antagonist, GDC0449, for 9 days prior to sacrifice. Treatment with the Hh inhibitor reduced expression of mRNAs encoding Gli2, Glut1, glycolytic enzymes, and αSma (Figure 3.2B). Immunohistochemistry confirmed that reduced expression of MF- and glycolysis-associated mRNAs was paralleled by depletion of tumor stromal cells that expressed ASMA, PKM2, and monocarboxylate transporter 4 (MCT4), a facilitator of lactate export (Halestrap and Price 1999) (Figure 3.2C).

Paracrine Hh signaling between hepatoma cells and MF stimulates glycolysis in MF

To determine if hepatoma cells generate soluble HH-ligands that might stimulate glycolytic activity in Hedgehog-responsive MF, we compared Gli-luciferase reporter activity in Shh–LightII cells that were exposed to conditioned medium from HepG2 cells, co-cultured with HepG2 cells in a Transwell system , or treated with control medium without or with recombinant SHH (Figure 3.3A). Like recombinant SHH, exposure to HepG2 cell-derived soluble factors significantly increased Hh signaling in the Shh-LightII cells. The stimulatory effect of HepG2 conditioned medium was abrogated by adding 5E1, a Hh neutralizing antibody that blocks HH ligand-Ptc interaction (Berman, Karhadkar et al. 2003) (Figure 3.3A). Moreover, when HepG2 cells were replaced with cells that do not generate HH-ligands (Panc 10.05 cells) (Yauch, Gould et al. 2008) and experiments were repeated, no change in Shh-LightII cell luciferase activity was

observed (Figure 3.S1A). The aggregate data, therefore, indicate that HepG2 cells generate soluble, biologically-active HH-ligands.

To determine if these HepG2-derived HH ligands functioned as inducers of MF glycolysis, we cultured a well-characterized rat liver MF line (8B cells) (Greenwel, Schwartz et al. 1991) alone (monoculture) or in the Transwell system with HepG2 cells, and assessed MF glycolytic activity. Co-culturing MF with HepG2 cells induced MF expression of mRNAs that encode key glycolytic enzymes (Figure 3.3B), and increased their lactate/pyruvate ratio, a measure of glycolytic activity (Figure 3.3C). Treating MF with HepG2 cell-conditioned media had similar effects (Figure 3.3D). The stimulatory effects of HepG2-conditioned medium on MF glycolysis were attenuated by adding 5E1 to block HH ligand-Ptc interactions (Figure 3.3E), suggesting that HH-ligands are the factors that malignant hepatocytes release to induce glycolytic activity in neighboring MF. To verify that activating Hh signaling in MF promotes glycolysis, we treated monocultures of liver MF with recombinant SHH (rSHH) ligand. Compared to vehicle-treated MF, MF treated with rSHH ligand had increased expression of genes encoding glycolytic enzymes (Figure 3.3F) and higher lactate/pyruvate ratios (Figure 3.3G). GDC-0449, a direct antagonist of the Hh signaling intermediate, Smoothened, reversed the effects of rSHH-ligand, confirming that MF glycolysis is Hh-dependent (Figure 3.3G). Treating MF with rSHH also significantly increased their secretion of lactate into the media, while adding GDC-0449 reduced media lactate below basal levels, demonstrating that Hh signaling also regulates MF secretion of lactate (Figure 3.3H).

Lactate generated by glycolytic MF fuels lipogenesis in HepG2 cells

Our aforementioned findings identified a novel Hh-dependent mechanism whereby malignant hepatocytes modulate the metabolic activity of tumor-associated MF. Because tumor stroma is generally believed to support the growth of malignant epithelial

cells, we used the Transwell co-culture system to evaluate the related hypothesis that MF-derived glycolytic end-products (such as lactate) enhance net energy homeostasis of malignant hepatocytes. Compared to mono-cultured HepG2 cells, HepG2 cells that were co-cultured with liver MF demonstrated significant accumulation of Oil Red O-stained lipid droplets (Figure 3.4A). Because lipid accumulation occurs during energy excess, we compared the ATP content of mono- and co-cultured HepG2 cells and found significantly higher ATP content in the co-cultured HepG2 cells (Figure 3.4B). Consistent with increased lipogenesis during co-culture, co-cultured HepG2 cells expressed higher mRNA levels of the lipogenic transcription factor, Pparγ, than mono-cultured HepG2 cells (Figure 3.4C). Co-culture also enhanced HepG2 expression of monocarboxylate transporter 1 (Mct1), which encodes a lactate transporter (Halestrap and Price 1999), but suppressed expression of pyruvate dehydrogenase kinase (Pdk1), which encodes an enzyme that gates entry of pyruvate into the tricarboxcylic acid (TCA) cycle (Figure 3.S3). These findings suggest that malignant hepatocytes import MF-derived lactate and convert it into pyruvate to fuel ATP and lipid biosynthesis. To assess this issue more directly, we treated monocultured HepG2 cells with lactate in the absence or presence of FX11. FX11 inhibits the activity of lactate dehydrogenase, thereby blocking the interconversion of lactate and pyruvate (Le, Cooper et al. 2010). Treating HepG2 cells with lactate significantly increased lipid accumulation and ATP content. Both responses were prevented when cells were pre-treated with FX11 to inhibit intracellular conversion of lactate into pyruvate (Figure 3.4D). Similar results were obtained when another hepatoma cell line, Huh7.5, was co-cultured with liver MF or treated directly with lactate (Figure 3.S4), providing reassurance that the findings were not restricted to a single liver cancer cell line.

The aggregate data, therefore, support a model whereby malignant hepatocytes generate HH-ligands to orchestrate the construction of a Hh-responsive stroma that

nurtures further growth of the malignant epithelia. The cancer-associated process resembles epithelial-stromal interactions that are triggered by injury to nonmalignant liver epithelial cells. When damaged, such cells begin to produce HH-ligands that also act in a paracrine fashion to promote accumulation of Hh-dependent MF (Omenetti, Choi et al. 2011). In nontumorous cirrhotic livers, MF are a major source of fibrous matrix, but also produce various factors that promote the survival of residual liver epithelial cells (Friedman 2008). Here we identify end-products of Hh-dependent changes in MF metabolism as novel trophic factors for malignant hepatocytes by showing that Hh signaling in MF stimulates glycolysis, and demonstrating that malignant hepatocytes use MF-derived lactate to generate ATP and fuel lipogenesis (Figure 3.S5). Evidence that the lactate-induced responses are blocked by treating malignant hepatocytes with an inhibitor of lactate dehydrogenase suggests that the improved epithelial energy balance occurs because malignant hepatocytes convert the assimilated lactate into pyruvate, which is then shunted into the TCA cycle to increase mitochondrial ATP production. In HCCs, therefore, increased aerobic glycolytic activity (i.e., the Warburg effect) is an end result of collaborations between malignant hepatocytes and glycolytic MF in the tumor associated stroma. Via this process, the malignant hepatocytes reap the benefits of the excess lactate generated by glycolysis without becoming glycolytic themselves, thereby fully retaining the capacity for oxidative phosphorylation and efficient ATP synthesis. Although this concept is contrary to conventional dogma which localizes the Warburg effect to the malignant cells themselves (Christofk, Vander Heiden et al. 2008), it is consistent with other recent reports of lactate production by stroma in breast cancer (Bonuccelli, Tsirigos et al. 2010), and raises the intriguing possibility that Hh-mediated switches in stromal cell metabolism also occur in cancers other than HCC. In any case, evidence for increased glycolytic activity in tumor-associated MF has important diagnostic and therapeutic implications. It suggests that positron emission tomography

(PET) scans might be deployed to identify HCC that are particularly enriched with glycolytic stroma. The latter information might facilitate HCC detection, and could also have prognostic significance because highly glycolytic tumors tend to have more aggressive biology (Yeluri, Madhok et al. 2009). Knowing which HCC are most enriched with glycolytic stroma would also justify, and help to refine, novel treatment approaches for HCC, supporting consideration of Hh inhibitors, LDH antagonists, and glycolysis inhibitors, as potential therapies for some patients with this life-threatening disease.

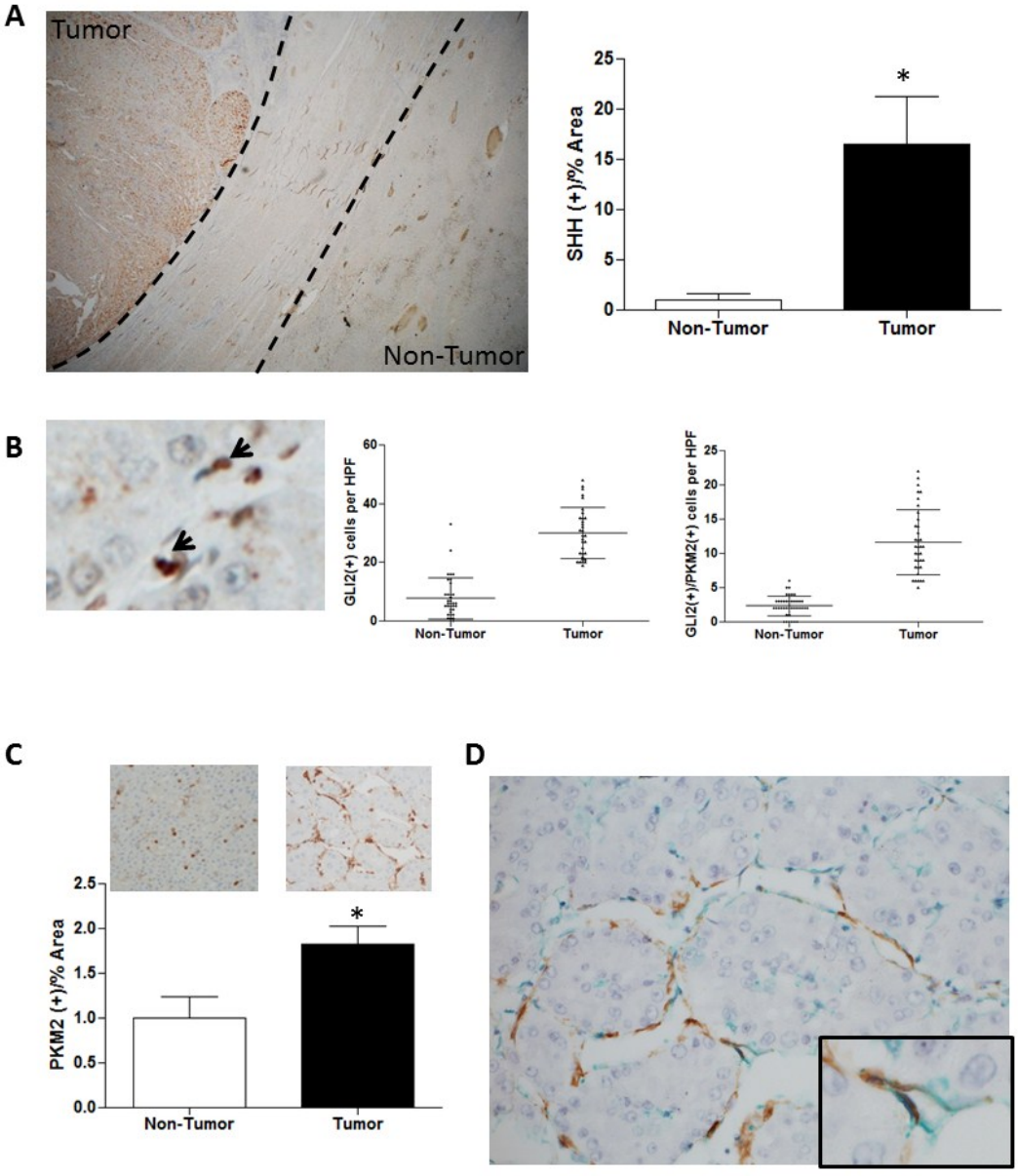

Figure 3.1. Evidence for paracrine Hedgehog signaling between malignant epithelia and tumor stroma in human HCC.

Figure 3.1. Evidence for paracrine Hedgehog signaling between malignant epithelia and tumor stroma in human HCC. Tumor and adjacent nontumorous liver from 5 patients with HCC were evaluated by immunohistochemistry (IHC) and quantitative morphometry. Representative sections are shown and mean +/- SD morphometric data are graphed; *$p<0.05$ for tumor vs nontumorous tissue**. (A)** SHH ligand (brown, 50x). Dotted lines enclose fibrotic capsule. **(B)** GLI2 (arrows, 400x) **(C)** PKM2 (brown) in non-tumor and tumor nodules (400x). **(D)** Co-localization of PKM2 (green) with αSMA (brown) in tumor nodule (400x, 1000x insert).

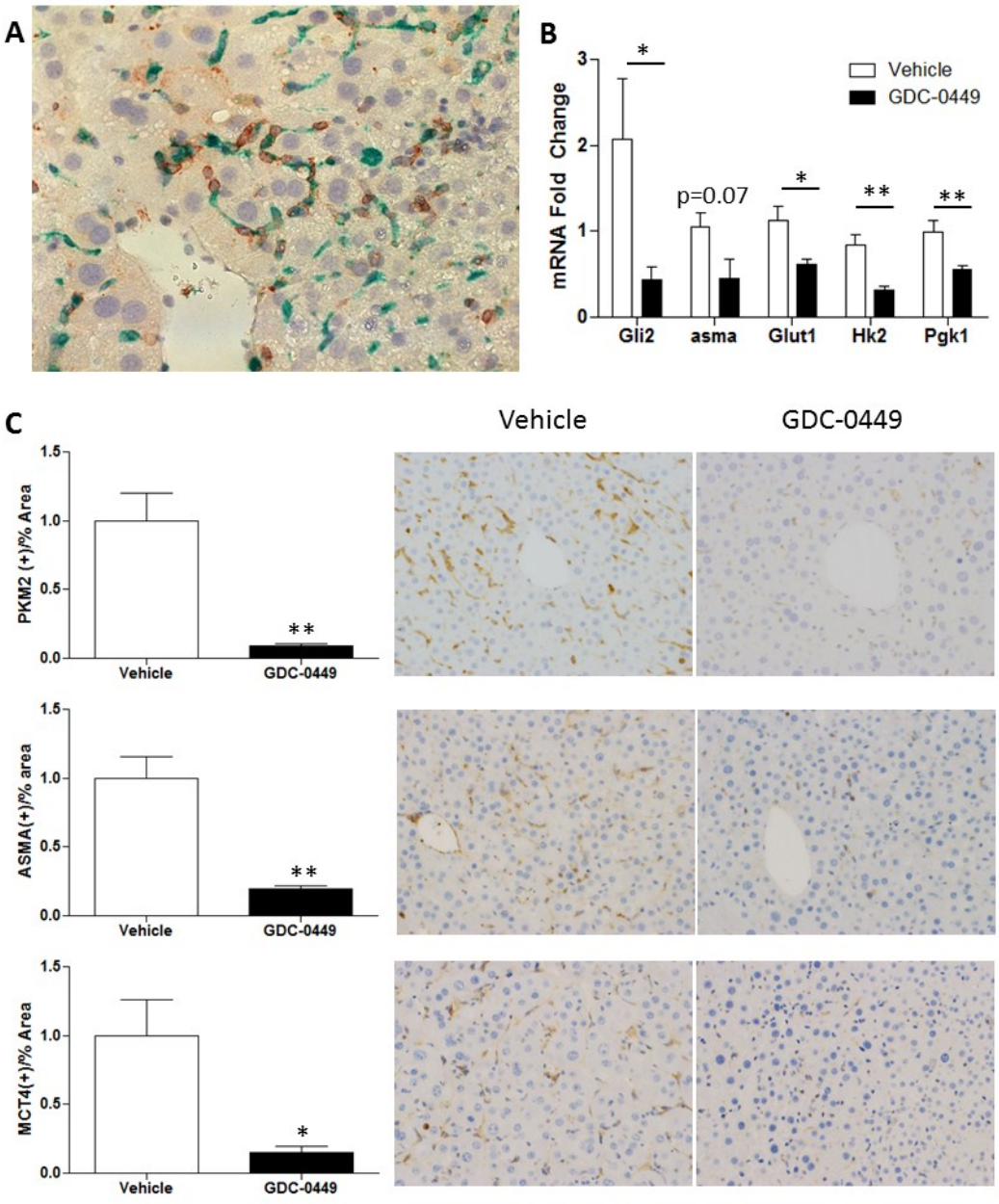

Figure 3.2. Murine HCC stroma is enriched with Hh-dependent glycolytic MF.

Figure 3.2. Murine HCC stroma is enriched with Hh-dependent glycolytic MF. Mdr2-deficient mice with HCC were treated with the Hh-inhibitor, GDC-0449, or vehicle (N=5 mice/group); effects on stroma of microdissected tumor nodules were evaluated by morphometry of immunostained sections and QRT-PCR. Representative sections and mean +/- SEM data are displayed (*p<0.05, **p<0.01 GDC-0449- vs. vehicle-treated groups). **(A)** Co-localization of PKM2 (green) with αSMA (brown) in tumor from vehicle-treated mouse (400x). **(B)** QRT-PCR for Gli2, αSma, Glut1, Hk2, Pgk1 in liver tumor RNA. **(C)** Tumor sections from Vehicle- and GDC-0449 treated mice demonstrate (*Top*) PKM2, (*Middle*) αSMA, and (*Bottom*) MCT4 (brown, 200x)

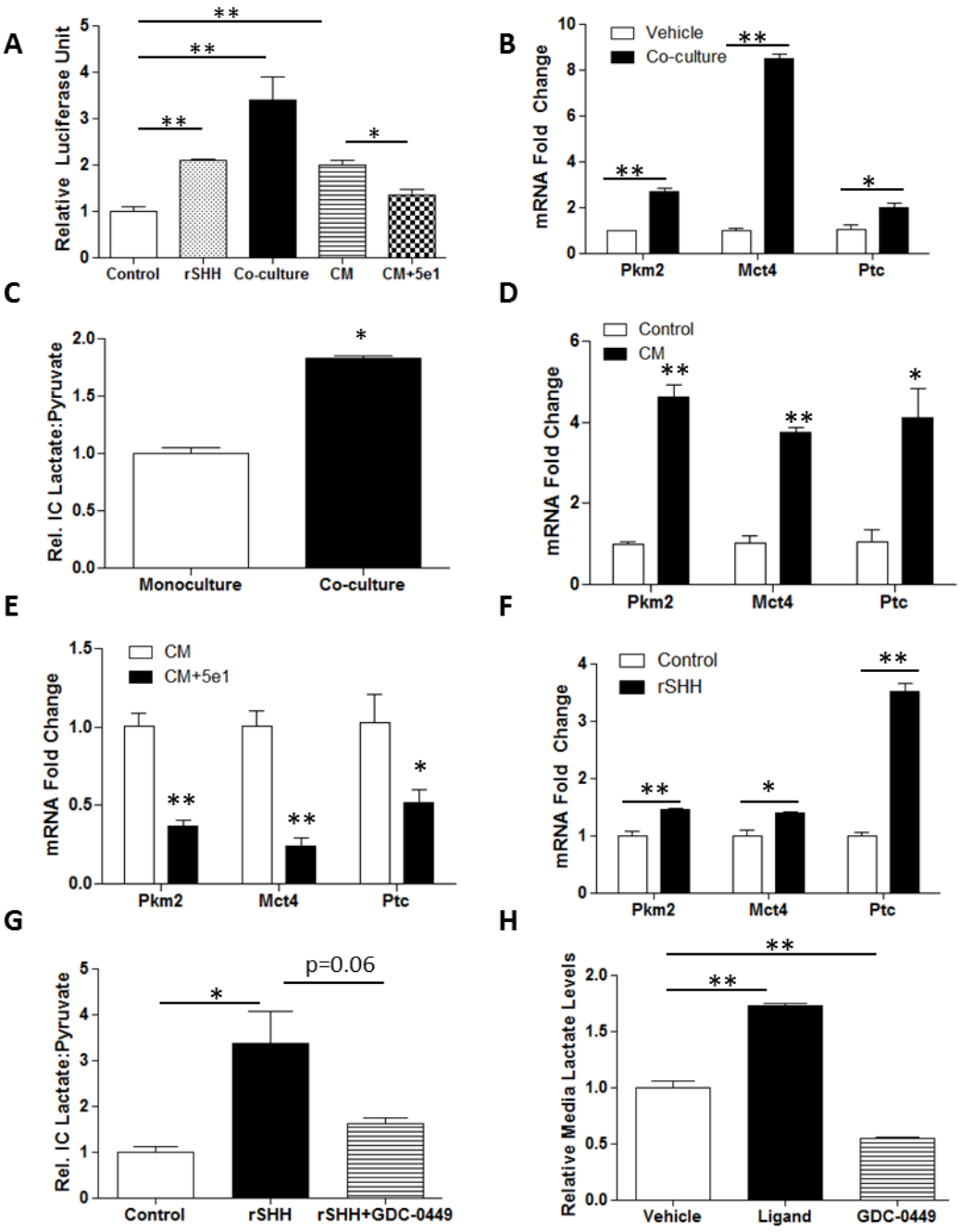

Figure 3.3. Paracrine Hh signaling stimulates MF glycolysis.

Figure 3.3. Paracrine Hh signaling stimulates MF glycolysis. (A) Gli-luciferase reporter activity in Shh–LightII cells incubated with control medium, 1000ng/mL recombinant SHH ligand, co-cultured with HepG2 in a Transwell system, incubated with HepG2-conditioned media without or with 5E1 neutralizing antibody; (*p<0.05, **p<0.01). **(B)** Pkm2 and Mct4 RNA levels in MFs grown in monoculture or co-cultured with HepG2s in Transwells. **(C)** Intracellular (I.C.) lactate/pyruvate ratio in MFs mono-cultured or co-cultured with HepG2 cells. **(D)** Pkm2, Mct4, and Patched RNA levels in MFs grown in control-media or with HepG2-conditioned media (CM). **(E)** Pkm2, Mct4, and Patched RNA levels in MFs grown in HepG2-conditioned media (CM) without or with 5E1 neutralizing antibody **(F)** Pkm2 and Mct4 mRNA levels in MFs treated with Vehicle or recombinant SHH ligand (rSHH-L). **(G)** Intracellular (I.C.) lactate/pyruvate ratio in MFs after treatment with rSHH-L. **(H)** Lactate exported into media by MFs treated with control media, rSHH-L or rSHH-L+GDC-0449. Mean +/- SEM data from triplicate experiments are graphed; *p<0.05, **p<0.01 vs. respective controls.

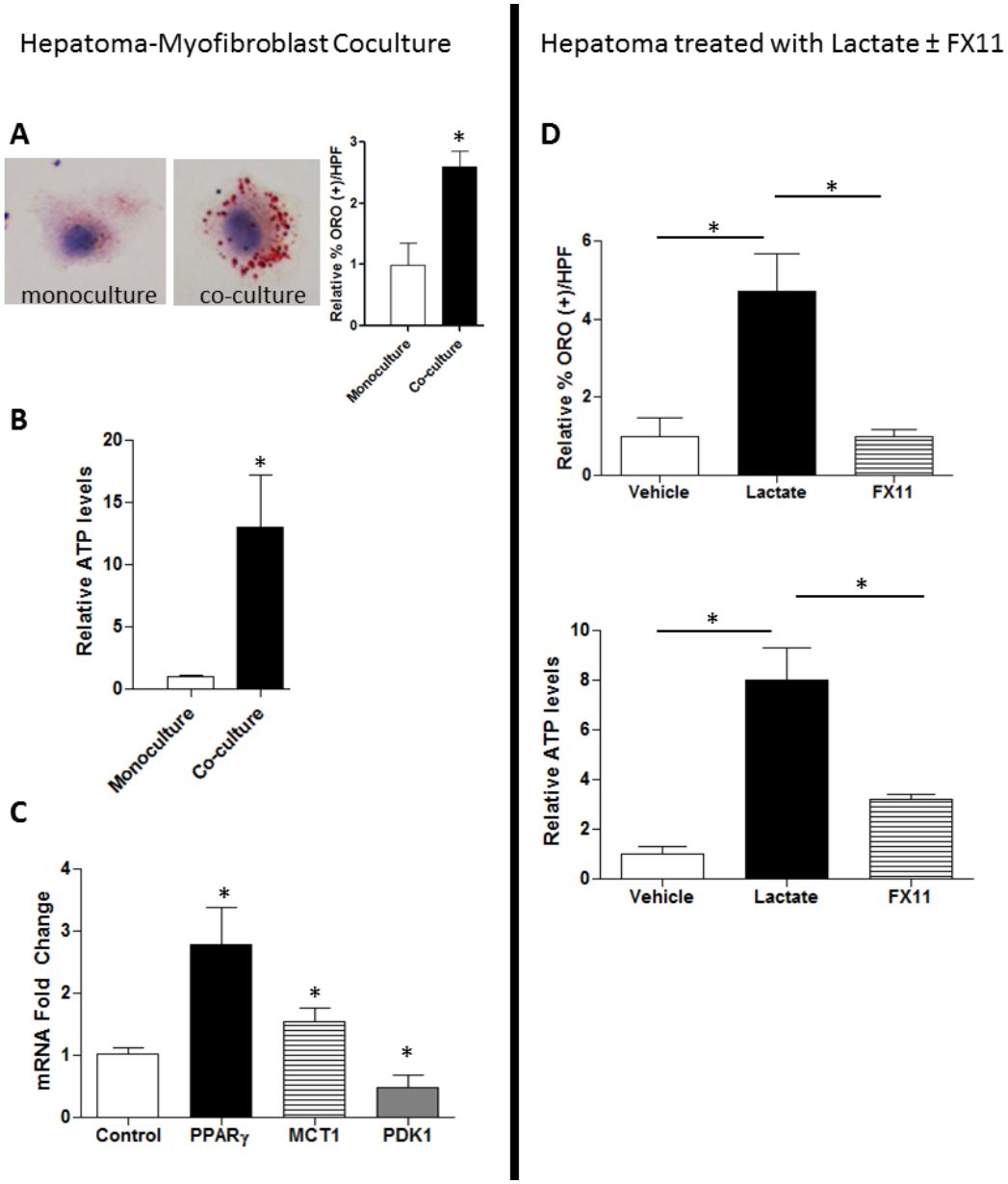

Figure 3.4. Lactate generated by glycolytic MF fuels lipogenesis in HepG2 cells.

Figure 3.4. Lactate generated by glycolytic MF fuels lipogenesis in HepG2 cells. **(A)** Oil Red O staining of HepG2 cells grown alone or co-cultured in Transwells with MFs and quantified by morphometry. **(B)** Intracellular ATP and **(C)** MCT1, PPARγ, PDK1 mRNA levels in HepG2 cells cultured alone or in Transwells with MFs. **(D)** Change in Oil Red O staining and ATP in HepG2s after treatment with 40mM lactate or FX11. Mean +/- SEM data from triplicate experiments are graphed; *$p<0.05$, **$p<0.01$ vs. respective control

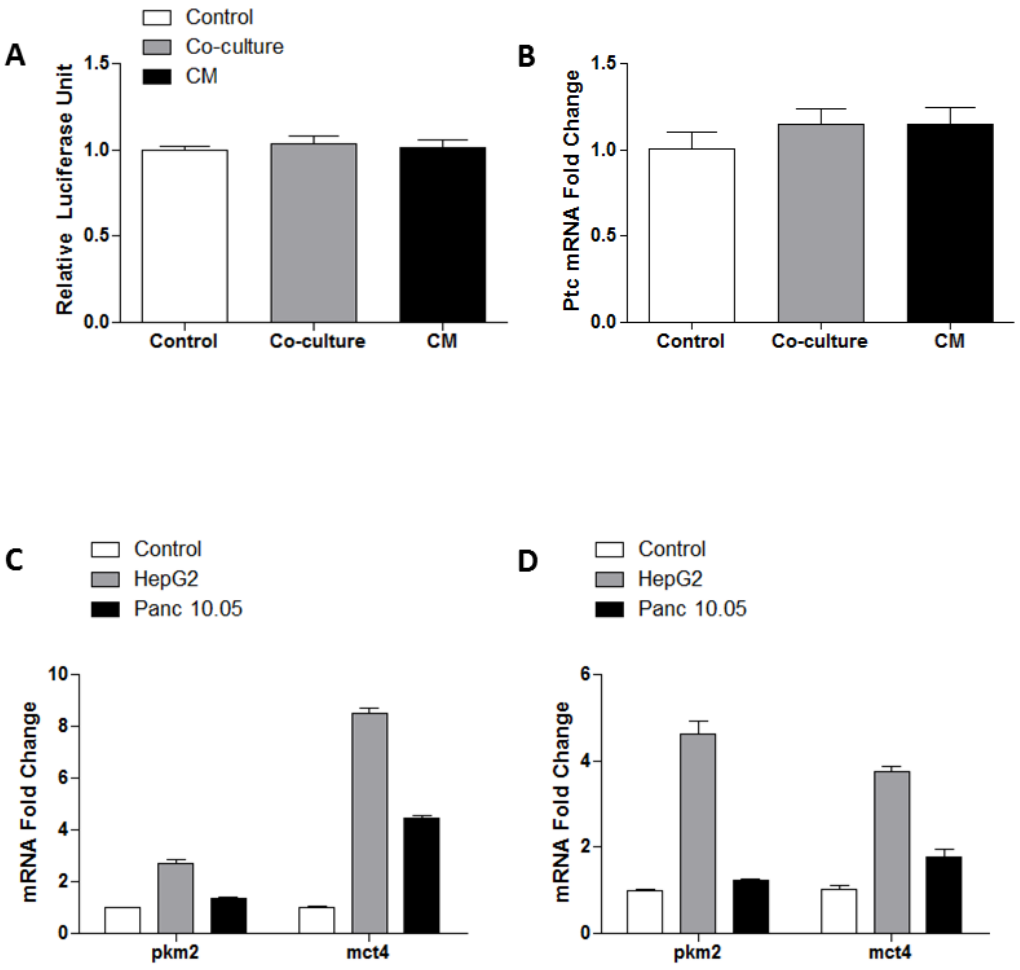

Figure 3.S1. Panc 10.05 cells do not generate functional Hh ligands (A) Gli-luciferase reporter activity in Shh–LightII cells incubated with control medium (white bar), grown in co-culture with Panc 10.05 cells (gray bar), or incubated with Panc 10.05-conditioned media (black bar). **(B)** Ptc RNA levels in 8B MFs grown in monoculture (white bar), grown in co-culture with Panc 10.05 cells (gray bar), or incubated with Panc 10.05-conditioned media (black bar). **(C)** Pkm2 and Mct4 RNA levels in 8B MFs grown in monoculture (white bar) or in co-culture with HepG2 (grey bar) (*p<0.05, **p<0.01).

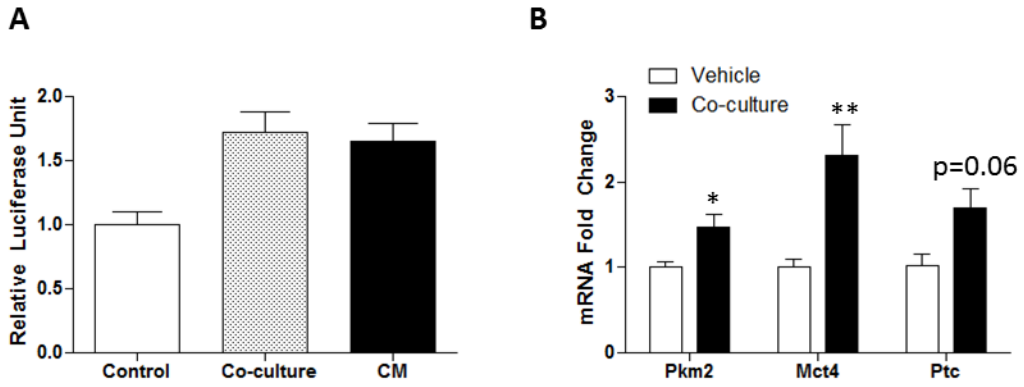

Figure 3.S2. Other malignant hepatoma lines can increase MF Hh and glycolytic activity. (A) Gli-luciferase reporter activity in Shh–LightII cells incubated with control medium (white bar), grown in co-culture with Huh7.5 cells (gray bar), or incubated with Huh7.5-conditioned media (black bar). **(B)** Pkm2, Mct4, and Ptc RNA levels in 8B MFs grown in monoculture (white bar) or grown in co-culture with Huh7.5 cells (black bar).

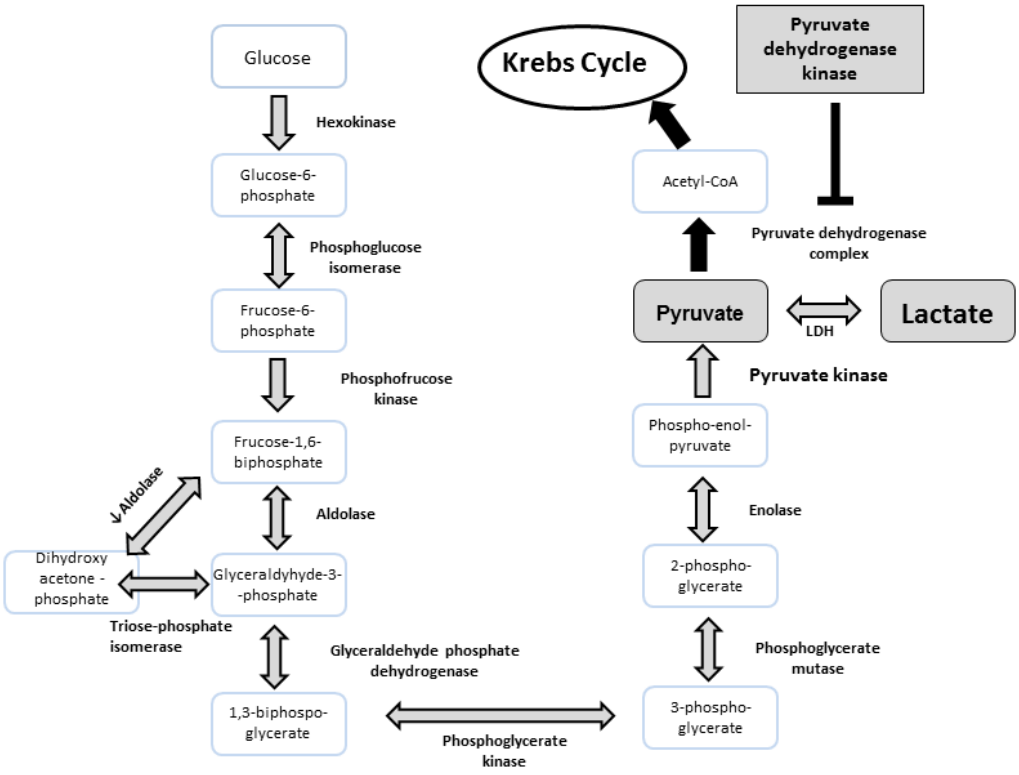

Figure 3.S3. Summary of glycolytic metabolism. During glycolysis, glucose is converted to pyruvate, which can either be shunted to lactate via the enzyme lactate dehydrogenase (LDH) or taken into the tricarboxcylic acid (TCA) cycle after conversion into acetyl Co-A. Entry into the TCA cycle is regulated by the enzyme pyruvate dehydrogenase kinase.

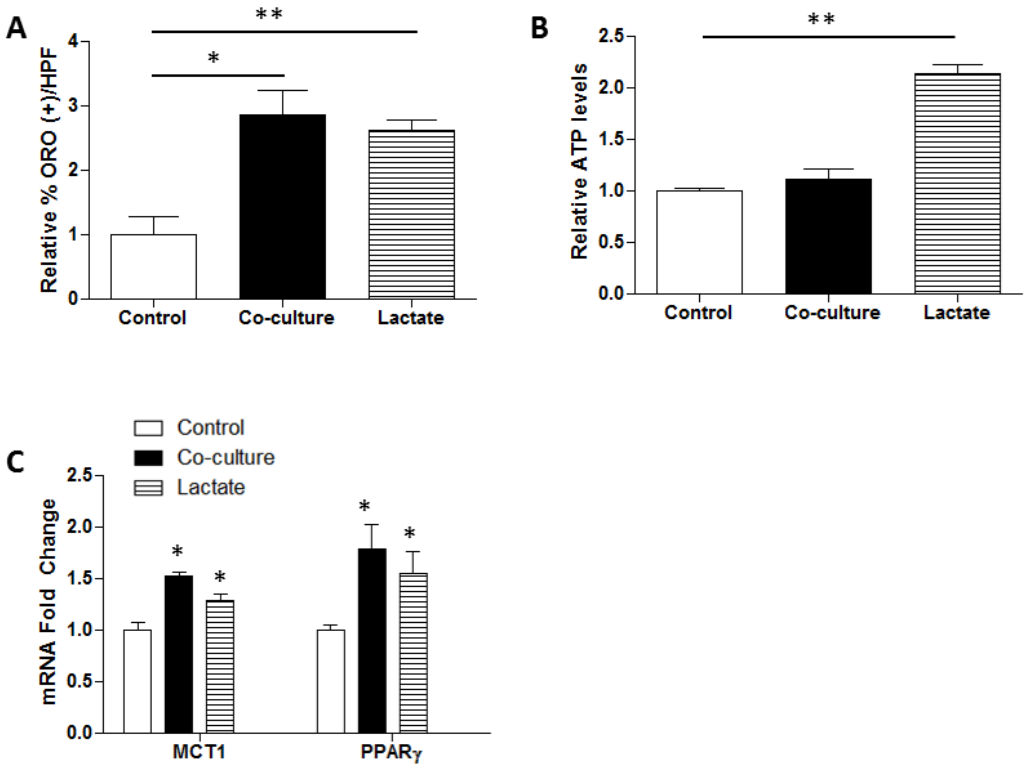

Figure 3.S4. MF-derived lactate provides energy source for lipogenesis in other malignant hepatoma lines. (A) Oil Red O staining quantified by morphometric analysis (*p<0.05, **p<0.01), **(B)** Intracellular ATP and **(C)** MCT1, PPARγ mRNA levels in Huh7.5 cells cultured alone (white bar), co-cultured with 8B MFs (black bar), or cultured alone and treated with 40mM of lactate (striped bar).

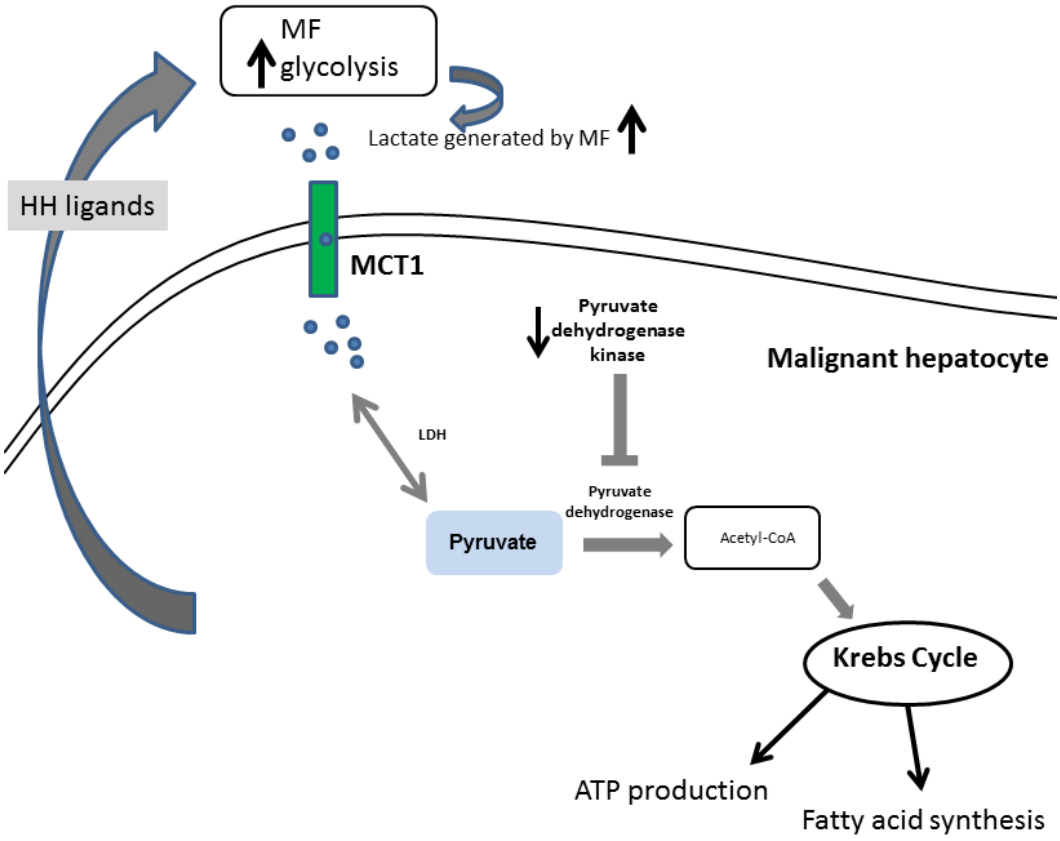

Figure 3.S5. Schematic of alterations in hepatoma cells due to release of metabolic end products by glycolytic MF. Hepatoma cells take up MF-derived lactate (denoted by blue circles) across the cellular membrane via the MCT1 lactate importer. Lactate is then converted to pyruvate via lactate dehydrogenase (LDH) and enters the TCA cycle, via a process that is gated by pyruvate dehydryogenase kinase (PDK1).

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CHAPTER IV

CONCLUSIONS AND FUTURE DIRECTIONS

4.1. Summary

Hepatocellular carcinoma (HCC) is a common and deadly cancer with limited treatment options, creating a patient-driven need for more effective therapies. Because the major risk factor for HCC is fibrosis and cirrhosis, primary liver cancer arises in the context of an impaired physiologic response to injury. Patients with compensated liver cirrhosis have as much as a 5% annual risk for developing HCC, and HCC commonly recurs in cirrhotic livers after tumor ablation. These observations suggest that the cirrhotic microenvironment promotes the outgrowth of malignant hepatocytes, but the mechanisms involved remain obscure.

Cirrhosis results from dysregulated repair of liver injury, leading to the progressive accumulation of excessive fibrous matrix. Our lab discovered that injury activates the normally dormant Hedgehog (Hh) signaling pathway in the liver. The Hh pathway is an evolutionarily conserved signaling pathway that is activated when HH ligands bind to the Patched (PTC) receptor which is expressed on the surface of Hh-responsive cells, resulting in eventual Hh signaling activity mediated by the GLI family transcription factors. Liver injury dramatically increases local production of Hh ligands by multiple cell types, including PDGF/TGF beta-mediated induction of Hh ligand synthesis by hepatic stromal cells, as well as de novo synthesis of HH ligands by injured liver epithelial cells, (e.g. hepatocytes). The release of HH ligands activates Hh pathway activity in responsive cells found in progenitor and stromal compartments, mobilizing them to repopulate and repair the liver. In particular, Hh pathway activity regulates the transcription of factors that enhance the growth, viability and fibrogenic activity of liver myofibroblasts (MF). Thus, injury-related activation of Hh signaling creates a Hh-rich

microenvironment that promotes hepatic MF accumulation, liver fibrosis, and HCC.

As reviewed extensively in Chapter 1, dysregulated Hedgehog signaling can result in development of various cancers, either through ligand-independent mechanisms (i.e. Smoothened or Patched mutations), or ligand-dependent mechanisms (i.e. paracrine signaling between stromal cells and malignant epithelia). In HCC, high levels of Hh pathway activity correlate with worse patient outcomes. There is evidence for both dysregulated ligand-independent and ligand-dependent Hh signaling. Sicklick et al. first discovered that Smoothened (Smo) hyperactivity in HCC tissue, possibly caused by a point mutation in Smo, correlates with tumor size and pharmacologic targeting of Hh signaling reduces proliferation of HCC cell lines. However, Pereira et al. show PTC positive stromal cells surrounding the tumor, suggesting that high Hh signaling activity does not occur in malignant hepatocytes *in vivo*, but possibly contributes in a paracrine fashion. Based on these pre-clinical and clinical observations, we postulated that sustained Hh signaling contributes to liver fibrosis and fibrosis-associated HCC. The studies we report in Chapters 2 and 3 provide direct evidence that dysregulated paracrine Hh signaling results in HCCarcinogenesis.

4.1.1 – Mdr2$^{-/-}$ mice as a model of HCC

To test our hypothesis that sustained Hh signaling contributes to liver fibrosis and fibrosis-associated HCC, we treated a mouse model of chronic liver injury and HCC with a Hh pathway inhibitor. In mice, Mdr2 encodes for P-glycoprotein, which is expressed by hepatocytes and permits the transport of phosphatidylcholine across the cannicular membrane into bile (Smit, Schinkel et al. 1993). The absence of phospholipids in the bile fosters liver injury that manifests as early as two weeks of age. These mice demonstrate consistently higher serum aminotransferase levels at every age, consistent with other evidence that Mdr2 deficiency provokes chronic hepatocyte injury. As liver

injury continues, Mdr2$^{-/-}$ mice develop progressive hepatic fibrosis and between 50 to 60 weeks of age, HCCs spontaneously arise, making Mdr2 knockout mice a good model for fibrosis-induced HCC. Similar to acute and chronic models of liver injury, Hh signaling is activated in Mdr2$^{-/-}$ mice and increases with time. Therefore, aged Mdr2$^{-/-}$ mice with HCC mirrors the clinical presentation of HCC in older patients with a history of chronic liver disease, fibrosis, and cirrhosis.

4.1.2 – Inhibition of Hedgehog signaling in aged Mdr2$^{-/-}$ mice

High levels of Hh signaling in aged Mdr2$^{-/-}$ mice led us to hypothesize that treatment with a direct antagonist of Smoothened, GDC-0449 (reviewed in Chapter 1.6), would provide substantial disease improvement. We validated GDC-0449 treatment by examining its effect on Hh signaling. Using markers for Hh signaling activity (Gli2, Gli1, Pparγ), we noticed a significant decrease in Hh activity after treatment both in non-tumor liver parenchyma and within the tumor nodule. To evaluate the impact of Hh signaling inhibition, pre- and post-treatment tumor volumes were analyzed by magnetic resonance imaging (MRI). After 9 days of treatment, vehicle treated animals evidenced persistent tumor growth, while mice that received GDC-0449 treatment demonstrated decreased tumor volume (22.7% ± 9.1% versus -6.7% ± 11.7%). These data were consistent with necropsy findings: 80% of vehicle treated mice had visible liver tumors compared with only 56% of GDC-0449 treated mice. Histological analysis of tumor nodules also revealed decreased rates of hemorrhagic infarcts, acidophilic necrosis and degenerative cytoplasmic changes, and microvesicular steatosis. All three methods used to analyze tumor burden in Mdr2 KO mice following treatment consistently demonstrated significant tumor regression. Furthermore, both MRI and necropsies showed a decreased number of metastatic lesions in GDC-0449 mice, compared to vehicle treated mice. Thus, the prohibitive effect on hepatic tumor growth *in vivo* pertains to both intra-hepatic HCC and

distant metastasis. Excitingly, this is the first documented *in vivo* evidence that Hh signaling inhibition has potential therapeutic benefit against HCC. Since our publication, another group has shown that knockdown of Hh signaling through inhibition of GLI1 *in vivo* reduces tumor burden in subcutaneous and orthotopic xenograft HCC models, providing additional evidence for targeting Hh signaling as a viable strategy to treat HCC (Xu, Chenna et al. 2012).

4.1.3 – Impact of Hh signaling inhibition on progenitor populations of aged Mdr2$^{-/-}$ mice

Since liver injury causes Hh-responsive liver progenitors to proliferate and contribute to carcinogenesis, we asked whether these populations would be affected by Hh signaling inhibition. Hh-responsive progenitors, marked by CK-19 and AFP, showed significant decreases after treatment, suggesting that Hh signaling is required to maintain progenitor populations even in mice with advanced HCC. The tumor environment is also thought to be populated with CD44 positive cells, a receptor for the stem cell growth factor and immunomodulator osteopontin (OPN). Treatment with GDC-0449 significantly decreased OPN expression within the primary tumors and a similar decrease in CD44 positive cells were also noted, suggesting that Hh signaling may regulate liver progenitor cells by modulating availability of OPN. In this regard, Xu et al. also observe, following *in vivo* inhibition of Hh signaling in their models of HCC, significant decreases in liver progenitors (Xu, Chenna et al. 2012).

4.1.4 – Loss of tumor volume in GDC-0449 treated Mdr2$^{-/-}$ mice is accompanied by a reduction in fibrosis

Unlike the xenograft models used by Xu et al., Mdr2$^{-/-}$ mice progressively accumulated liver MF and developed liver fibrosis, allowing us to study a model that parallels the natural evolution of HCC on a background of chronic inflammation, liver

injury and fibrosis. Hh inhibition resulted in significantly reduced hepatic expression of alpha smooth muscle actin (a marker for liver MF) and fibrosis, suggesting that sustained Hh signaling is also required to maintain and expand populations of liver MF and sustain fibrogenic repair. Reductions in the expression of other pro-fibrogenic and MF growth factors such as Tgf-β and Pdgf-β reinforce the effects of canonical Hh signaling on fibrogenic repair. Osteopontin can also act as a pro-fibrogenic, factor and promotes MF accumulation (Syn, Choi et al. 2011). Thus, loss of tumor volume was accompanied by reduction in fibrosis and cells that participate in fibrogenic repair. While these results, discussed in Chapter 2, do not directly imply that fibrosis causes HCC, the observations form the basis of our hypothesis for our studies in Chapter 3, that the tumorigenicity of malignant hepatic epithelial cells is affected by factors generated by Hh-responsive liver MF.

4.1.5 – Paracrine Hedgehog signaling in human HCC

In injured livers, wounded liver epithelial cells are a major source of Hh ligands. In a paracrine fashion, these ligands then activate Hh signaling in nearby Hh-responsive cells, which include liver MF. Because HCCs develop out of cirrhotic livers, we hypothesized that a similar paracrine mechanism between malignant hepatocytes and liver MF is possible. To localize Hh ligand expression in HCC, we performed immunohistochemistry for Sonic Hedgehog (SHH) in archived paraffin-embedded tissues from 5 patients with HCC and cirrhosis caused by nonalcoholic fatty liver disease (NAFLD). All of the HCCs demonstrated increased expression of SHH ligands relative to adjacent capsular tissue and non-tumorous liver. Furthermore, within the tumor nodule, as in injured livers, the main source of SHH ligands are from the hepatocytes. In contrast, nuclear staining for the Hh-regulated transcription factor, GLI2, was confined to the tumor-associated stroma, which was significantly enriched with GLI2(+) stromal cells

compared to adjacent non-tumorous liver. These results demonstrate that malignant epithelia in the liver are not Hh responsive but produce HH ligands that activate Hh signaling in Hh-responsive stromal cells. This system of ligand-dependent paracrine Hh signaling is congruent with observations in other cancers, most notably in pancreatic cancer, and has been shown *in vitro* (Yauch, Gould et al. 2008) and *in vivo* (Bailey, Mohr et al. 2009).

4.1.6 – Human HCC harbors glycolytic liver myofibroblasts

Observations of paracrine signaling between malignant hepatocytes and Hh-responsive stromal cells in HCC led us to question the benefits of a collaborative relationship between these two cell types. Again we turned to injured livers to form the framework for our hypothesis. Other work in our lab indicates that Hh-responsive MF in fibrotic livers express pyruvate kinase M2 (PKM2), a well-established marker of glycolytic activity (Chen, Choi et al. 2012). During liver fibrogenesis, most MF are derived from resident hepatic stellate cells (HSC). Our lab has recently shown that paracrine Hh signaling can induce HSC to become proliferative and myofibroblastic (Rangwala, Guy et al. 2011) and this process is mediated by glycolysis (Chen, Choi et al. 2012). Given that localization of glycolytic cells in the pre-neoplastic liver is limited to the stromal compartment, we wanted to access glycolytic activity in HCC. We found that PKM2 staining also localized to cells within HCC stroma (rather than to the malignant hepatocytes themselves) in human samples and showed that human HCC stroma harbored greater numbers of glycolytic cells than adjacent nontumorous liver. Dual staining for PKM2 and GLI2 confirmed that these glycolytic stromal cells are Hh-responsive. Finally, expression of PKM2 co-localized with ASMA in human HCC, demonstrating that glycolytic tumor-associated stromal cells are Hh-responsive MF. Over 80 years ago, Otto Warburg noted that the growth of many cancers and other

highly proliferative cells relies upon glycolysis, even when sufficient oxygen is available for oxidative phosphorylation, a phenomenon dubbed the Warburg Effect (Warburg 1956). Our findings in a small cohort of NAFLD-associated HCC introduce novel evidence that this phenomenon occurs in HCC-associated MF, rather than the malignant hepatocytes.

4.1.7 – Hh inhibition in aged Mdr2$^{-/-}$ mice reduces glycolytic activity in HCC tumor nodules

In the Mdr2$^{-/-}$ mouse model of HCC, the stromal compartment is also enriched with glycolytic MF that are Hh responsive. To examine the role of Hh signaling in regulating glycolytic activity in the HCC-associated MF, we compared expression of Glut1, a glucose transporter, and several key glycolytic enzymes in mRNA isolated from HCCs of Mdr2-deficient mice treated with either vehicle or GDC-0449. Treatment with the Hh inhibitor reduced expression of mRNAs encoding Glut1 and other glycolytic enzymes (Figure 3.2B). Reduced expression of MF- and glycolysis-associated mRNAs was paralleled by depletion of tumor stromal cells that expressed ASMA and PKM2. These results suggested to us that Hh signaling mediates the glycolytic potential of stromal MF in the tumor microenvironment.

4.1.8 – Malignant hepatocytes secrete HH ligands which enhance glycolytic activity in liver MF

Given these observations, we hypothesized that liver MF glycolysis might have been induced in a paracrine fashion by Hh ligands generated by the malignant hepatic epithelial cells. To test this hypothesis, we first needed to determine if malignant hepatocytes could generate HH ligands *in vitro*. To this end, we exposed Shh-LightII cells, a clonal 3T3 cell line stably incorporating a Gli-responsive firefly luciferase

reporter, to soluble factors from malignant hepatocytes (HepG2 cells). Similar to their exposure to recombinant SHH (rSHH) ligand, exposure to HepG2 cell-derived soluble factors significantly increased Hh signaling in the Shh-LightII cells. The stimulatory effect of HepG2 conditioned medium was abrogated by adding 5E1, a Hh neutralizing antibody that blocks HH ligand-PTC interaction, further suggesting that paracrine interaction involving malignant hepatocyte-produced HH ligands drive Hh signaling in neighboring Hh responsive cells. To determine if these HepG2-derived factors directly functioned as inducers of MF glycolysis, we exposed a well-characterized rat liver MF line (8B cells) to HepG2 cells products, either directly in co-culture or indirectly with HepG2 conditioned media, and assessed MF glycolytic activity. Compared to growth in monoculture, exposure of MF to HepG2-derived factors increased several markers of MF glycolytic activity, including increasing the lactate to pyruvate ratio. The stimulatory effects of HepG2-conditioned medium on MF glycolysis were attenuated by adding 5E1 antibody, suggesting that HH-ligands are the factors that malignant hepatocytes release to induce glycolytic activity in neighboring MF. To verify that paracrine activation of Hh signaling in MF promotes glycolysis, we treated liver MF with rSHH ligand and found similar increases in glycolytic activity. GDC-0449 reversed the effects of rSHH-ligand, confirming that MF glycolysis is Hh-dependent.

4.1.9 – Liver MF secrete glycolytic end products which promote lipogenesis in malignant hepatocytes

Several groups studying breast cancer and ovarian cancer have suggested that resident stroma in the tumor microenvironment release metabolic end-products that influence the growth and invasion of malignant epithelia. As we discussed in Chapter 1, lactate is one such metabolite that promotes tumorigenicity. Immunohistochemistry in $Mdr2^{-/-}$ mice for monocarboxylate transporter 4 (MCT4), a lactate exporter, shows the

same stromal pattern as ASMA and PKM2 staining, suggesting that stromal MF in HCC export lactate. Both *in vivo* and *in vitro* data demonstrate that Hh signaling regulates MF secretion of lactate: 1) Treatment of Mdr2-/- mice with the Hh inhibitor reduced MCT4 expression, and 2) Adding rSHH to liver MF media significantly increased their secretion of lactate into the media, while including GDC-0449 decreased media lactate below basal levels. Because of these results, we hypothesized that the tumorigenicity of malignant hepatic epithelial cells is supported by liver MF-generated end-products of glycolytic metabolism, specifically lactate.

In vitro and *in vivo* models of lactate treatment demonstrate that lactate promotes lipogenesis (Palacin, Lasuncion et al. 1988; Tabernero, Vicario et al. 1996; Sanchez-Abarca, Tabernero et al. 2001). In multiple cancers including breast (Zhang, Tai et al. 2005), prostate (Dhanasekaran, Barrette et al. 2001; Rossi, Graner et al. 2003), lung (Visca, Sebastiani et al. 2004; Migita, Narita et al. 2008), ovarian (Gansler, Hardman et al. 1997; Alo, Visca et al. 2000), and liver (Calvisi, Wang et al. 2011), a lipogenic phenotype is associated with poorer prognosis. To evaluate the related hypothesis that MF-derived glycolytic end-products enhance net energy homeostasis of malignant hepatocytes, we co-cultured malignant hepatocytes with liver MF. Compared to monocultured HepG2 cells, HepG2 cells in co-culture demonstrated significant accumulation of Oil Red O-stained lipid droplets and expressed higher mRNA levels of the lipogenic transcription factor, Pparγ, suggesting that MF factors increased lipogenesis. Because lipid accumulation occurs during energy excess, we compared the ATP content of mono- and co-cultured HepG2 cells and found significantly higher ATP content in the co-cultured HepG2 cells. Co-culture enhanced the HepG2 expression of monocarboxylate transporter 1 (Mct1), which encodes a lactate importer, but suppressed expression of pyruvate dehydrogenase kinase (Pdk1), an enzyme that gates the entry of pyruvate into the tricarboxcylic acid (TCA) cycle. These findings suggest that malignant

hepatocytes import MF-derived lactate to fuel ATP and lipid biosynthesis. To address this question more directly, we treated HepG2 cells with lactate in the absence or presence of a lactate dehydrogenase (LDH) inhibitor. Treating HepG2 cells with lactate significantly increased lipid accumulation and ATP content and both responses were prevented when cells were pre-treated with the LDH inhibitor.

4.1.10 – Paracrine Hedgehog signaling within the HCC tumor could explain clinical observations about HCC progression

Our results demonstrate that crosstalk between stromal liver MF in the tumor microenvironment and malignant hepatic epithelial cells are regulated by paracrine Hedgehog signaling, which ultimately can alter the metabolic profile of the tumor and drive lipogenesis. These results are fascinating in light of clinically relevant observations that HCCs, and specifically malignant hepatocytes, undergo a fatty metamorphosis. In the earliest documentation of this phenomenon, needle biopsy specimens were taken from a patient with HCC and over the course of 18 months, the authors observed progressive fatty metamorphosis in well-differentiated tumor cells (Chan and Ma 1975). Since then, using a variety of imaging techniques and a larger number of patients, there have been several clinical reports confirming fatty metamorphosis in HCC throughout the progression of the disease (Isomura and Nakashima 1980; Yoshikawa, Matsui et al. 1988; Tsunetomi, Ohto et al. 1989; Martin, Sentis et al. 1995). In a bioinformatics analysis of tumors from $Mdr2^{-/-}$ mice, compared to normal mouse livers, genes regulating lipogenesis and fatty acid synthesis such as fatty acid synthase (fasn) and fatty acid binding protein 4 (fabp4) were upregulated 2.6 and 13.64 fold, respectively, which suggests that increased lipogenic activity in HCC tumors is not species-specific (Katzenellenbogen, Mizrahi et al. 2007). These same lipogenesis-associated genes are elevated in human HCC and associated with worse clinical outcomes (Yamashita,

Honda et al. 2009). Furthermore, the functional connection between lipogenesis and increased proliferation and survival in HCC cells has been established (Calvisi, Wang et al. 2011), and that suppression of lipogenesis reduces these malignant phenotypes (Bhalla, Hwang et al. 2012). Our study provides the first evidence that liver MF within the tumor microenvironment can contribute to these fatty changes.

4.2. Future Directions

As with any scientific inquiry, new answers raise new questions. Based on our understanding of dysregulated Hedgehog signaling in injured livers, we postulated that Hedgehog signaling contributes to the pathogenesis of hepatocellular carcinomas. Paracrine Hedgehog signaling between HH ligand producing malignant hepatocytes and Hh-responsive liver MF alters the cellular metabolism of both cell types. But one immediate question that arises: What mechanisms regulate malignant hepatocyte production of HH ligands?

Answers to this question have broad clinical implications. If malignant hepatocytes use HH ligands to communicate with their stromal neighbors, localizing the stimulus for the production of HH ligands may provide new therapeutic avenues. Despite not expressing much Hh associated activity, malignant hepatocytes secrete high levels of HH ligands, suggesting that HH ligand production is not always regulated by intracellular Hh signaling. In Chapter 3, we focused on one aspect of the reciprocal relationship between liver MF and malignant hepatocytes, and in particular, how malignant hepatocytes stimulate liver MF to release tumorigenic metabolites. Crosstalk, however, can occur in both directions. When cultured with conditioned media from malignant hepatocytes, Shh-LightII cells express less Hh signaling activity than when co-cultured with malignant hepatocytes (Figure 3.3A). This piece of data suggests that fibroblast-derived factors can stimulate malignant hepatocytes to increase secretion of

HH ligands. Thus, a reasonable hypothesis would predict that products of liver MF stimulate malignant hepatocytes to release HH ligands. In Chapter 2, after treating Mdr2$^{-/-}$ mice with a Hh inhibitor, we observed decreased tumor burden and fibrosis. Because liver MF are the main producers of fibrous matrix in the injured liver, one area that remains open for investigation is how malignant hepatocytes respond to extracellular matrix (ECM) remodeling by liver MF.

4.2.1 – The role of extracellular matrix in HCC

The influence of ECM on HCCarcinogenesis is a nascent and recent area of investigation. ECM structural integrity and stiffness is maintained, in part, by collagen fibers secreted by liver MF (de Leeuw, McCarthy et al. 1984; Kolacna, Bakesova et al. 2007). Since the early 2000s, clinical evidence for a relationship between ECM and HCC has emerged. The first study analyzed levels of ECM remodeling in patients, determined by collagen I and metalloproteinase activity, and correlated increased ECM remodeling with greater tumor recurrence (Theret, Musso et al. 2001). Similarly, a later study of 106 HCC patient samples found that greater ECM mechanical stiffness correlates with a more invasive and metastatic HCC phenotype (Zhao, Cui et al. 2010). A prospective study involving 866 patients demonstrated that increased ECM stiffness, as measured by transient ≥ 10 kPa; 95% CI, 9.75-212.3; $P < 0.001$) for developing HCC (Masuzaki, Tateishi et al. 2009). At an *in vitro* level, increasing ECM stiffness increases the proliferative and cisplatin-resistant capacity of malignant hepatocyte cell lines (Schrader, Gordon-Walker et al. 2011).

4.2.2 – Integrins: How cells experience the world

The ability for cells to sense and process environmental cues, such as changes in the ECM, is mediated by a family of cell surface receptors called integrins (Miranti and

Brugge 2002). Integrins are essential for both cell-cell and cell-matrix interactions (Hynes 1987). Stimulation of integrins and subsequent signal transduction relies on binding of integrin receptors to ECM proteins (Hanks, Calalb et al. 1992). This action recruits the tyrosine kinase, focal adhesion kinase (FAK), which itself is auto-phosphorylated to become FAK^{PY397}, and further propagates integrin signal transduction. Given early observations that tumors result from faulty wound healing (i.e. dysregulated ECM deposition), it is no surprise that integrin signaling has been implicated in cancer development. Breast cancer cells that form invasive colonies with disrupted polarity and exhibit uninhibited growth all have enhanced integrin signaling (Paszek, Zahir et al. 2005). Furthermore, inhibition of integrin signaling restores tissue polairity and downregulates malignant behavior. Intriguingly, the Pasek et al. saw that ECM stiffness also returns to baseline with integrin signaling inhibition, suggesting malignant epithelia and ECM reciprocally act on each other. Additional links between fibroblasts, ECM, and malignancy have been developed. Mouse mammary fat pads preconditioned with fibroblasts programmed to produce more collagen crosslinks (i.e. increasing ECM stiffness) permit the growth of more invasive and proliferative tumors (Levental, Yu et al. 2009). These authors further demonstrate that integrin signaling in malignant epithelial cells requires an appropriate level of ECM stiffness to promote malignant behaviors and this signaling is reliant on PI3K-AKT activity. Previous studies have also shown that integrin signaling can regulate PI3K activity in non-malignant settings (Chen, Appeddu et al. 1996).

4.2.3 – Integrins and HCC

In the study by Levental, breast cancer progression from a non-malignant to malignant environment relied on fibroblast-mediated ECM remodeling and integrin-driven PI3K activity in malignant epithelia. Given the interplay between dysregulated

wound healing, liver MF production of ECM components (e.g. collagen), and HCC development, these results raise the compelling possibility that a similar mechanism and model could exist in HCC. Since type I and IV collagen are the major ECM components of fibrotic and cirrhotic liver tissue, it is no surprise that α1 and α2 subunits of β1 integrin, subunits that bind collagen, are highly elevated in malignant hepatocytes (Yang, Zeisberg et al. 2003) and are required for migration through and adhesion to the ECM (Torimura, Ueno et al. 2001). Two studies also observed high expression of β1 integrin within HCC tumor nodule in human patients, specifically within malignant hepatocytes and not sinusoidal cells (Zhao, Cui et al. 2010; Schrader, Gordon-Walker et al. 2011). In the study by Schrader et al., HCC cells grown on stiffer matrices not only exhibited increased proliferation and epithelial disruption, but also showed increased expression of phospho-AKT. *In vitro* pharmacologic targeting of FAK^{PY397}, a key mediator of integrin signaling, diminished the proliferative advantage of malignant hepatocytes growing on stiff matrices, providing further evidence these phenotypic changes are facilitated by integrin signaling. These results are supported by a complementary study demonstrating that increased ECM stiffness *in vitro* directly results in increased β1 integrin mRNA in HCC cell lines (Zhao, Cui et al. 2010).

4.2.4 – A conceivable role of extracellular matrix in HCC

Taken together with the clinical observations, the *in vitro* experiments support a hypothesis that malignant hepatocytes sense and respond to changes in ECM through integrin signaling. Additional evidence is needed to establish whether: a) liver MF remodeling of ECM can induce tumorgenic changes in malignant hepatocytes, and b) the activation of integrin signaling in response to ECM changes is essential for HCC progression *in vivo*. The link between integrin signaling and PI3K-AKT activity is especially intriguing because of the relationship between PI3K-AKT signaling and the

production of HH ligands. In a study by Androutsellis-Theotokis et al., the authors showed that SHH ligand production was downstream of PI3K-AKT activation and regulated by mTOR/STAT3 signaling (Androutsellis-Theotokis, Leker et al. 2006). Furthermore, two groups have shown that pharmacologic treatment of HH-ligand expressing cells with an AKT inhibitor resulted in diminished production (Yang, Wang et al. 2008; Ramirez, Singh et al. 2012). These cumulative studies help us refine our initial hypothesis that the secretion of HH ligands by malignant hepatocytes is in response to liver MF signaling. By activating integrin signaling in response to ECM remodeling by liver MF, we can hypothesize that malignant hepatocytes stimulate a PI3K-AKT signaling cascade leading to increased production of HH ligands. The net result would create a HH-rich microenvironment that promotes further fibrosis and secretion of tumorigenic molecules (e.g. cytokines, ligands, and growth factors). If the hypothesis holds true, the results would add more evidence in support of anti-fibrotic agents as potential therapies for patients with HCC as well as inhibitors targeting the PI3K-AKT pathway. Co-culture of liver MF with HepG2 cells results in increased β1 integrin expression in HepG2 cells, suggesting that malignant hepatocytes utilize integrins in response to factors produced by liver MF (Figure 4.1).

4.2.3 – Other potential reasons for increased HH ligand production

Other possibilities for increased production of HH ligands by malignant hepatocytes could simply be due to dysregulated Hh signaling. SHH ligand is a downstream target of Hh signaling in some models of cancer (Clement, Sanchez et al. 2007). Both HCC specimens and HCC cell lines have been found to have elevated levels of Hh signaling (Huang, He et al. 2006; Sicklick, Li et al. 2006). Furthermore, levels of Smoothened (Smo) expression showed a positive correlation with tumor size and a novel point mutation of Smo was identified in one patient, suggesting that

persistent Smo activity can lead to dysregulated tumor growth (Sicklick, Li et al. 2006). However, both studies analyzed whole HCC tissue, which does not account for the heterogeneity of cells within the HCC tissue. The lack of positive GLI2 staining in malignant hepatocytes from patients with NAFLD-induced cirrhosis and HCC suggests, along with observations that several HCC cell lines are unresponsive to Hh signaling inhibition, that not all malignant hepatocytes have dysregulated Hh signaling despite their production of HH ligands. Although observations of ligand-independent and ligand-dependent modes of Hh signaling seem to differ in HCC, this discrepancy is not unique to liver cancer; studies in prostate and pancreas cancers also have divergent results (Thayer, di Magliano et al. 2003; Karhadkar, Bova et al. 2004; Tian, Callahan et al. 2009). These differences merely emphasize the need for additional research to understand the circumstances when Hh signaling is intrinsically dysregulated in malignant hepatocytes and whether this dysregulation can lead to increased HH ligand production. Finally, as mentioned above, PI3K signaling can regulate HH ligand production and dysregulation of this signaling pathway in malignant hepatocytes (Lee, Soung et al. 2005) could be the primary cause of HH ligand overproduction.

4.3 – A student's perspective on the "War on Cancer"

By signing the National Cancer Act into law on December 23, 1971, President Richard Nixon made finding a cure for cancer a national effort and cultivated hope that this disease was as mortal as any human. This goal of finding a cure was christened by media outlets as a national declaration of "war" against cancer. And many people, scientists and non-scientists alike, were optimistic for a quick cure: In 2003, Andrew von Eschenbach, then the director of the NCI, vowed that we would "eliminate [the] suffering and death" caused by cancer by 2015. All it would take to eradicate cancer was the right amount of organization and resources and the focus of our country's top scientists.

These efforts have not been hindered by a lack of funding: Since 1971, we have invested $105 billion to understand what makes the enemy tick and how to stop the ticking.

Beginning in the 19th century, when Rudolf Virchow first advanced cellular derivation as the source of cancers, we have dedicated much of our attention on the individual tumor cell. And after the discovery of DNA, we could fully appreciate that the dysregulation of certain genes and their pathways lead to the oncogenic behavior of tumor cells. It is undeniable that chemotherapies based on this understanding led us to formulate new and better treatments for patients. We have moved from using nitrogen mustard (Goodman, Wintrobe et al. 1946) and folic acid antagonists (Farber and Diamond 1948) as potential treatments to targeted therapies such as imatinib (Druker, Guilhot et al. 2006). Yet despite the progress the field has made, cancer has overtaken cardiovascular disease as the leading cause of death worldwide (World Health Organization 2012). After adjusting for age and size of the population, from 1950 to 2005 the death rate for cancer has dropped 5%, whereas during this same period, the death rate for cardiovascular diseases has dropped 64% (Kolata 2009). In fact, in 2011, the 5-year mortality trend for pancreatic and liver cancer has even increased (Marshall 2011). These numbers have led several to question whether the war could be won at all (Bailar and Gornik 1997), whether we should focus on cancer prevention and detection (Lyman 2009), whether we need to develop more efficient ways to tailor chemotherapies (Chan and Ginsburg 2011), or whether our approach to drug development (Rubin and Gilliland 2012) and preclinical models (Ocana, Pandiella et al. 2011) is flawed.

Strikingly, only 5% of agents identified as potential anticancer compounds in the preclinical setting make it past phase III trials (Kola and Landis 2004). Worse, compounds that are approved for clinical care often face high recurrence rates and offer medium effectiveness at improving survival (Begley and Ellis 2012). While there is no

doubt that cancer care has enormously improved, mortality statistics contends that more can be done. The first of several steps must be to improve preclinical models used to validate potential anticancer compounds. Our *in vitro* models are limited to poorly annotated and sometimes highly passaged malignant epithelial cell lines (e.g. NCI-60 lines). Even primary cell lines developed from patients do not necessarily provide an accurate representation of the molecular heterogeneity of human tumors, nor do they fully recapitulate how malignant epithelia exist *in situ*. These models lack the dimension, matrix support, and cell-to-cell interactions that cells normally experience *in vivo*. Nor do they account for environmental cues like exposure to environmental stressors and hypoxia. Unfortunately, animal models such as tumor xenografts are still tumor cell driven and are limited as well; the stromal component comprised entirely of host cells and these animals are often immunocompromised, making it difficult to assess the impact of the immune system on tumor development and treatment. For example, Sorafenib, despite its ability to inhibit growth and angiogenesis in xenograft models of HCC and induce cellular apoptosis in HCC cell lines (Liu, Cao et al. 2006), was only able to extend average survival by three months in human patients. While this was a significant breakthrough in treatment of HCC patients, the need for better treatments remains.

Stromal cells and extracellular matrix components provide growth and survival signals to the tumor cell in a capacity that we are only beginning to discover. Our results contribute to a burgeoning body of literature that chronicles how the network of stromal cells and tumor cells promote malignant phenotypes and spawn an environment that nurtures tumorigenesis. The data suggest that it is time to move beyond a tumor cell-based approach to cancer research. Instead of reducing and eliminating complexities in our model, we need to take a holistic, systems biology approach to understanding how tumors work. Models therefore ought to be 1) sensitive, 2) specific, 3) reproducible, 4)

tractable, 5) scalable, and 6) affordable. Currently, although many of these models are highly sensitive (low false negative rates), they are not very specific (high false positive rates) nor are they always reproducible at the clinical level. Genetically engineered mouse models are effective and adaptable systems to assess transformation events and microenvironment contributions to cancer. They are immune competent, so they retain a crucial component of tumor response. Yet they are expensive and the molecular alterations that often generate tumors are limited and artificial (as compared to the spontaneous emergence of human tumors and their heterogeneous compositions), making them still imperfect models (Olive and Tuveson 2006). Our analysis of these models also requires broadening: Bioinformatics should be harnessed to account for intercellular communications instead of limiting analysis to intracellular genomic information (Gentles and Gallahan 2011). Antitumor compounds need to be evaluated in *in vitro* and *in vivo* models that accurately represent how tumors propagate and persist in humans. Damage signals from injured stroma have been shown to actually promote tumor drug resistance and survival, suggesting that cytotoxic chemotherapy may have unanticipated effects (Sun, Campisi et al. 2012). Failure to account for stromal cells during drug development might be a cause of why we observe drug resistance and a combinational approach may be required to target both stromal and tumor compartments (Olive, Jacobetz et al. 2009).

Traditionally, funding for riskier ideas has not always been available. Economic incentive needs to be shifted to spur scientists towards inventing truly novel models that addresses the limitations of our current ones. But while it is easy to remain critical at the perceived infancy of effective clinical treatments against different cancers (Bailar and Gornik 1997), it is also important to remember that scientific progress, especially those that lead to effective therapies, results from decades of incremental research. As Thomas Kuhn states in *The Structure of Scientific Revolutions*, “The success of a

paradigm…is at the start largely a promise of success discoverable in selected and still incomplete examples." Changes in scientific thought can occur only through what, on the surface, must seem to be disjointed examples and slow moving progress. Bold scientific thought takes a great deal of time and investment to rigorously test and an even greater commitment to develop clinical strategies based on these ideas. A reminder of this fact is the 30-year story of the chemotherapy paclitaxel, which began in 1962 with the collection of tree bark, entered phase I clinical trials in 1984, and was finally approved for clinical use in 1992 (Goodman and Walsh 2001). More work is required and superior models need to be established, but we are steadily traversing into a new paradigm where our understanding of cancers will be expanded to include all the cells within the tumor. Stephen Paget, the distinguished English surgeon credited for proposing the seed and soil theory of cancer, observed over a century ago that cancers require both the tumor cell and a suitable microenvironment for growth. His advice, that "properties of the soil may also be useful", still holds true 123 years later.

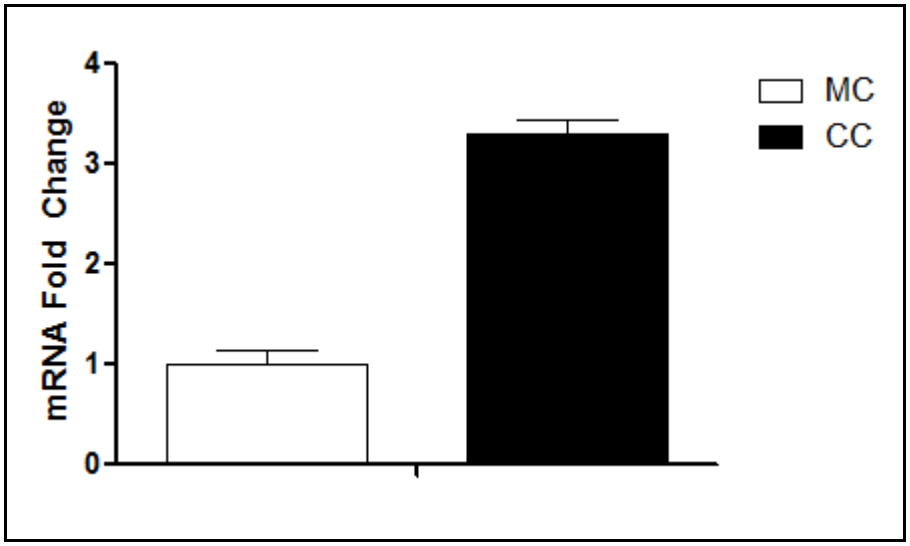

Figure 4.1. Coculture with liver MF increases β1-integrin expression in HepG2 cells. As compared to HepG2 cells in monoculture (MC), HepG2 cells in co-culture with liver MF (CC) express a 3-fold increase in β1-integrin expression.

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