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The Hippo pathway in liver regeneration and tumorigenesis

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THE HIPPO PATHWAY IN LIVER REGENERATION AND TUMORIGENESIS

by

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THE HIPPO PATHWAY IN LIVER REGENERATION AND TUMORIGENESIS

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ABSTRACT

The Hippo signaling pathway has been implicated in both mammalian organ size regulation, as well as tumor suppression. Specifically, the Hippo pathway plays a critical role regulating the activity of transcriptional co-activator, and downstream effector, Yes-associated protein (YAP), which modulates pro-proliferative transcriptional elements. Recent investigations have demonstrated that this pathway is activated in non-regenerating livers and its inhibition leads to liver overgrowth and tumorigenesis. The majority of the existing evidence regarding the role of the Hippo pathway in hepatocyte proliferation is based on in vitro studies and knock-out animal models. However, the role of the Hippo pathway during the natural process of liver regeneration, remains unknown. Here alterations in the Hippo signaling pathway were investigated, namely its interaction with angiomitin-like 2 (AmotL2) and Set7, during liver regeneration using a 70% rat partial hepatectomy (PH) model. Overall, results indicated no significant difference between AmotL2 levels in control and regenerating tissue at various time points during liver regeneration. No significant alterations in YAP methylation during liver regeneration were found compared to control tissue. In the end, results regarding the role of both AmotL2 and Set7 provided inconclusive evidence about their roles during the regenerative process.

Given the role of the Hippo pathway in hepatocyte proliferation, a hypothesis was made that this pathway may play a role in pediatric liver tumors. YAP localization was
evaluated using immunohistochemical analysis in tumor sections from patients with hepatoblastoma or hepatocellular carcinoma. Once again, the results were inconclusive at the time of the preparation of this manuscript due to technical difficulties in achieving satisfactory staining of the specimens.

Further studies will be directed at elucidating the role of the Hippo pathway during liver regeneration as well as developing better conditions for the immunohistochemical staining of human liver specimens.
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LIST OF ABBREVIATIONS

AKT/PKB ...................................................... Protein Kinase B
Amot .......................................................... Angiomotin
Amot L1/L2 .................................................... Angiomotin-like 1/2
CTGF .......................................................... Connective Tissue Growth Factor
EGFR .......................................................... Epidermal Growth Factor Receptor
EMT ............................................................ Epithelial-to-Mesenchymal Transition
ERK1/2 ........................................................ Extracellular Signal-Regulated Kinase 1/2
GAPDH ........................................................ Glyceraldehyde 3-phosphate Dehydrogenase
H3K4 ............................................................ Histone 3 Lysine 4
HB ............................................................... Hepatoblastoma
HCC ............................................................ Hepatocellular Carcinoma
HGF ............................................................ Hepatocyte Growth Factor
HRP ............................................................. Horseradish Peroxidase
IgG ............................................................. Immunoglobulin G
IL-6 ............................................................. Interleukin-6
Lats1/2 ......................................................... Large Tumor Suppressor Kinase 1/2
MAPK ........................................................ Mitogen-Activated Protein Kinase
NF2 ............................................................. Neurofibromin 2
NF-κB ......................................................... Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B cells
PBS ............................................................ Phosphate Buffered Saline
PH ............................................................... Partial Hepatectomy
PI3K………………………………………………………………Phosphoinositide 3-Kinase
SARAH Domain………………………………………………………………..Sav-Rassf-Hpo Domain
Sav1………………………………………………………………………………..Salvador 1
STAT3………………………………..Signal Transducer and Activator of Transcription 3
TAZ…………………………………...Transcription Coactivator with PDZ-binding motif
TGFα/β……………………………………..Transforming Growth Factor α/β
TNFα………………………………………………………..Tumor Necrosis Factor α
YAP……………………………………………………………………Yes-Associated Protein
INTRODUCTION

The liver is a large, solid organ situated in both the right and left upper quadrants of the abdomen. Among its variety of functions, the liver plays a critical role in the maintenance of metabolic homeostasis. The liver is responsible for the synthesis and metabolism of cholesterol and other nutrients, such as carbohydrates and fats, as well as for their storage and redistribution (Hepatology, 2003; Widmaier, 2011). In addition, the liver is involved in the production and the secretion of serum proteins such as albumin, as well as clotting factors and bile salts. One of its most important functions, however, is the detoxification of the body through the removal of foreign substances and various wastes via excretion and conversion. The majority of these functions are carried out by the hepatocyte, which comprises approximately 80% of all the cells in the liver. The remaining 20% of the liver is made up of non-parenchymal cells, which include: lymphocytes, endothelial cells, Kupffer cells, and stellate cells (Taub, 2004; Widmaier, 2011). Lymphocytes, which are located throughout the body and are involved in the innate immune response, help the liver respond to infectious pathogens. The liver endothelial cells make up the lining of capillaries, as well as sinusoids, and act as a barrier between the parenchymal cells and the blood. Kupffer cells reside in the lumen of the sinusoids and represent the hepatic tissue macrophages. Kupffer cells are responsible for phagocytic activity against pathogens and other blood-borne materials circulating through the liver. The stellate cells of the liver have a variety of functions including
vitamin A storage and under pathologic conditions may contribute to the development of hepatic fibrosis (Bouwens et al., 1992; Naito, Hasegawa et al., 2004; Taub, 2004).

The liver carries the unique ability to regenerate and regulate its growth in order to maintain a relatively constant mass relative to the total body mass. This regenerative ability is noteworthy by the fact that adult hepatocytes are normally dormant and do not actively participate in the cell cycle. However, in response to injury or infection that causes a deficit in liver mass, hepatocytes are stimulated to enter the cell cycle and divide. In clinical practice, liver resections are used in humans for living donor liver transplantation or the removal of hepatic tumors. Unlike the limbs of frogs and other amphibians, however, the resected lobes of the liver do not simply grow back. Instead, the remaining liver tissue undergoes hyperplasia, where the majority of the adult cells in the remnant liver replicate; therefore, the word ‘regeneration’ might be a misnomer (Michalopoulos and DeFrances 1997; Fausto 2000; Taub 2004). Experimentally, the liver’s regenerative capacity is illustrated in the 70% partial hepatectomy (PH) rodent model, developed in 1931 by Higgins and Anderson. This model calls for the surgical removal of two-thirds of the liver, while the remaining one-third grows until the mass of the original liver is restored, which takes approximately one week (Higgins & Anderson, 1931).

Liver mass is governed by the mass of the organism. Following resection or hepatocyte injury, liver regeneration continues until the optimal liver mass to body mass ratio is achieved. In fact, when the mass of the liver surpasses the metabolic needs of the organism, the liver will reduce its mass in order to maintain an optimal ratio between
liver mass and body mass. An example of this occurs upon the termination certain drug therapies, which have induced either hypertrophy or hyperplasia. Removal of growth stimuli leads to apoptosis or programmed cell death of the hepatocytes and a return to optimal mass. Another instance in which the metabolic abilities of the liver exceed the demands of the body occurs when a transplanted liver is “large-for-size.” (Kam et al., 1987; Schulte-Hermann et al., 1995). These examples illustrate how the regenerative process is regulated in such a way to obtain an optimal equilibrium between the metabolic and functional capacity of the liver and the needs of the organism.

**Liver Regeneration: An Overview**

As previously stated, mature hepatocytes are quiescent and normally reside in the G0 phase of the cell cycle. After PH, however, about 95% of the hepatocytes enter the cell cycle (Michalopoulos & DeFrances, 1997). Approximately 12 hours after PH, the rate of hepatocyte DNA synthesis begins to increase in the rat liver, indicating entrance into the S phase. After about 24 hours, the rate of DNA synthesis in rat hepatocytes reaches its peak levels. In contrast, DNA synthesis in the non-parenchymal cells occurs after the hepatocytes – around 48 hours for epithelial cells of the bile ducts and Kupffer cells, and 96 hours for the sinusoidal endothelium. It takes between one and two cycles of replication to completely restore the original mass of a rat liver. In the rat model, the original liver mass is restored one week after PH, with the majority of growth occurring in the first three days (Grisham, 1962; Sigal et al., 1999). In comparison, in a mouse model, DNA synthesis peaks around 40 hours after PH, and in primates, the peak DNA synthesis occurs between 7 and 10 days (Koniaris et al., 2003). Entrance into the S phase
of the cell cycle is coordinated such that it begins in the hepatocytes surrounding the portal triad – which contains the distinct arrangement of a hepatic artery branch, portal vein branch, and a bile duct. Specifically, the onset of the S phase begins with the hepatocytes surrounding the portal vein and then continues in the direction of blood flow toward the central vein region (Taub, 2004; “Lobules of Liver” 2014; “Portal Triad” 2014). With each new round of DNA synthesis, however, the percentage of both polyploid and binucleate hepatocytes increases and limits further regeneration.

Understanding the molecular mechanisms behind liver regeneration has been a process almost a century in the making. Previous studies have focused on biochemical, physiological, and morphological approaches. More recent studies have concentrated on the molecular mechanisms and have illustrated that liver regeneration is a dynamic process that can be divided into several phases. The first phase, which occurs directly after PH and lasts for the first few hours is known as the “priming” phase (Gilgenkrantz & Collin de l’Hortet, 2011). During this time period more than 100 immediate early phase genes are transcribed, even without de novo protein synthesis, as a result of activation by transcription factors that are usually suppressed, but become active after stimulation following hepatocyte loss (Haber et al., 1993). Among the immediate early genes are proto-oncogenes c-fos and c-jun, which encode proteins that complex together to form activating protein-1 (AP-1), as well as c-myc (Halazonetis et al., 1988; Karin et al., 1997). Induction of these genes is dependent on the rapid activation of signal transducer and activator of transcription 3 (STAT3) and nuclear factor-κB (NF-κB), two transcription factors which are turned on only minutes after PH (Taub et al., 1999).
STAT3 and NF-κB require the secretion of two important cytokines released by the action of the innate immune system on Kupffer cells. Interleukin-6 (IL-6), a pro-inflammatory cytokine secreted by the Kupffer cells, acts on the hepatocyte and activates STAT3. The induction of IL-6, however, is dependent on tumor necrosis factor alpha (TNFα), which upregulates NF-κB leading to transcription of IL-6 ("Interleukin 6" 2014; Taub 2004; Gilgenkrantz and Collin de l’Hortet 2011). While the various genes activated during the first phase of regeneration do not share a common function, they work together in order to transition hepatocytes from the G0 phase to the G1 phase of the cell cycle (Figure 1).

As hepatocytes progress through the cell cycle, there is an increased metabolic demand placed on the remnant liver. In fact, the second phase of liver regeneration is marked by numerous metabolic changes including a short-lived period of hypoglycemia which is thought to cause lipolysis, leading to the formation and accumulation of lipid droplets in the hepatocytes (Gazit et al., 2010). While the first phase of the cell cycle relies on transcription factors and immediate early genes, the second, or “metabolic,” phase is dependent on several growth factors and their receptors. Based on studies done in isolated hepatocytes, transforming growth factor alpha (TGFα) and hepatocyte growth factor (HGF) have been shown to be two important growth factors involved in the second phase of regeneration. TGFα is produced by the hepatocyte and can acts as both an autocrine and a juxtacrine factor by binding with the epidermal growth factor receptor (EGFR) on hepatocytes (Fausto et al., 1995; Michalopoulos and DeFrances 1997).
Figure 1: The three steps of liver regeneration. (1) The first phase which involves the secretion of cytokines between the hepatocytes and the Kupffer cells. TNFα is secreted by and acts on the Kupffer cells leading to the activation of NF-κB and upregulation of IL-6. IL-6 acts to activate the STAT3 pathway in hepatocytes, which works to induce the transcription of the immediate early genes: c-myc, c-jun, and c-fos leading to a transition from the G0 phase to the G1 phase of the cell cycle. (2) The second phase, which involves metabolic changes and the activation of growth factors. These metabolic changes, demonstrated by lipid droplet formation, are accompanied by growth factors such as TGFα and HGF that act on the hepatocyte through their respective receptors. This is followed by activation of several intracellular pathways, which leads to progression through the cell cycle from G1 to S. (3) The final step occurs once the remnant liver has achieved its original mass. The TGFβ protein super-family has been shown to act as growth inhibitors with the help of Activin. Recent studies have shown that YAP inactivation may play a role in this step. (Figure adapted from Gilgenkrantz & Collin de l'Hortet, 2011).

EGFR has numerous ligands, and has demonstrated involvement in cell motility, decreased apoptosis, and cell proliferation. Down-regulation of EGFR, which has been studied using RNA interference, results in impairment of hepatocyte proliferation, indicating defective progression from the G1 phase to the S phase (Collin de l’Hortet et al., 2012; Natarajan et al., 2007). In contrast, HGF is synthesized and released by stellate, and other non-parenchymal, cells and acts on hepatocytes via paracrine or endocrine
signaling through the c-Met receptor. HGF binding to its receptor is important for the stimulation of hepatocyte division and for the induction of DNA synthesis after liver injury. Loss of c-Met results in cessation of hepatic proliferation in the G2 phase, indicating that the HGF/c-Met is important for the transition from G2 to mitosis (Factor et al., 2010; Huh et al., 2004). After activation, EGFR and c-Met enlist the help of scaffold proteins and intracellular networks, such as STAT3, extracellular signal-regulated kinase (ERK1/2), phosphoinositide-3-kinase (PI3K) and protein kinase B (AKT), and mitogen-activated protein kinase (MAPK). Together, these pathways provide a redundant mechanisms by which liver regeneration can occur even if one of the pathways is poorly functional (Torre et al., 2011).

The last phase of liver regeneration is the termination phase which is perhaps, the least studied phase of the regenerative process. In both rat and mouse models, after liver mass has reached approximately the same mass as the pre-hepatectomy liver, a number of hepatocytes undergo apoptosis. This suggests a mechanism by which the liver corrects for an unsuitable increase in the number of cells (Sakamoto et al., 1999). Studies have suggested that TGFβ, a growth factor superfamily, and activin are heavily involved in this process. TGFβ1 is known to suppress the proliferation of hepatocytes and is expressed only five hours after PH and remains active until the end of regeneration (Braun et al., 1988). Activin works in collaboration with TGFβ as one of the only cytokines known to have the ability to selectively inhibit hepatocyte regeneration. In fact, the inactivation of TGFβ receptor 1 in hepatocytes is not sufficient to prolong
regeneration unless it is coupled with the hepatocyte-specific inactivation of the activin receptor (Oe et al., 2004).

As outlined above, liver regeneration is a complex process requiring participation of a number of molecular pathways. Despite the significant advancements over the past several decades in our understanding of the molecular mechanisms involved in liver regeneration, the exact mechanisms triggering the initiation and termination of regeneration continues to be poorly understood. Recently, the Hippo/Yes-associated protein (YAP) pathway has emerged as a new pathway which may also play an important role in the regulation of liver mass.

**The Hippo/YAP Pathway**

The Hippo pathway was initially elucidated using mosaic genetic screens in *Drosophila melanogaster* and, due to the dramatic overgrowth that resulted from mutations in its components; it was originally described as a tumor suppressor pathway. Further analysis of the biochemical relationships between certain genes has shaped the existing model of a phosphorylation cascade that regulates transcriptional outputs. Recently the Hippo signaling pathway, also as a regulator of organ size, has been found to be conserved from *Drosophila* to mammals (Barry & Camargo, 2013; Zhao et al., 2010). The majority of the core Hippo pathway components are conserved between the two species, including: Sav1, Lats1/2, Mst1/2, Mob1, NF2 (also known as Merlin), and YAP along with its paralog TAZ. Studies have even shown a functional conservation by illustrating how human Mob1, Lats1, Mst1, and YAP can rescue their counterparts in *Drosophila* mutants (Zhao et al., 2010).
The mechanism by which this pathway is activated is a controversial topic but several possibilities have been proposed, including mechanotransduction, cell-cell contact, and G-protein coupled receptor signaling (Dupont et al., 2011; Yu et al., 2012; Zhao et al., 2007). The Hippo pathway is a kinase cascade, with core kinases Mst1/2 being pro-apoptotic and activated during times of apoptotic stress by caspase-dependent cleavage. There is a known interaction between Sav1 and Mst1/2 through SARAH domains, which are present on the C-terminals of both Sav1 and Mst1/2. Sav1 can also activate Mst1/2, though the mechanism has not been studied extensively (Graves et al., 1998; Zhao et al., 2011). Once activated, Mst1/2 phosphorylates a hydrophobic motif located on Lats1/2 along with Mob1, which is in complex with Lats1/2, leading to its activation. Lats1/2 subsequently phosphorylates and inhibit transcriptional co-activators YAP/TAZ (Chan et al., 2005; Praskova et al., 2008). Phosphorylation of Ser127 on YAP, or the equivalent site on TAZ, results in 14-3-3 binding, cytoplasmic retention and inactivation of YAP/TAZ (Zhao et al., 2007). However, if Lats1/2 phosphorylates YAP at Ser381 it leads to a second phosphorylation by casein kinase 1, and activation of a phosphodegron – a phosphorylation-dependent degradation. In the cytoplasm, SCFβ-TRCP, an E3 ubiquitin ligase, is responsible for the polyubiquitylation and subsequent degradation of YAP (Figure 2).
Figure 2: Model of the mammalian Hippo pathway. It is unclear what initiates pathway activity, however it is known that activation leads to a kinase cascade which affects apoptosis and cell proliferation. Core kinase Mst1/2 phosphorylates and activates Sav1, Mob1, and Lats1/2. Lats1/2 can then activate the downstream effector proteins YAP and TAZ at multiple locations. If YAP/TAZ is phosphorylated at Ser127 it is sequestered and retained in the cytoplasm via 14-3-3 binding. Phosphorylation of Ser381 results in the polyubiquitylation and cytoplasmic degradation of YAP. A mutation in one or both of those binding sites, or the ablation of one of the core kinases leads to the nuclear translocation of YAP/TAZ. Once in the nucleus, YAP/TAZ is free to bind to its target transcriptional effector TEAD and activate genes involved in cell proliferation (Figure adapted from Zhao et al., 2010).

Ablation of Mst1/2 or mutation of either Ser127 or Ser381 leads to activation and translocation of YAP/TAZ into the nucleus (Zhao et al., 2010). YAP does not have a DNA-binding domain, however, it does contain (from N-terminal to C-terminal): a proline-rich region, a TEAD-binding domain, two WW regions, an SH3-binding motif, a coiled-coil, a transcription-activating region, and a PDZ-binding domain. TAZ lacks a
few of these domains, but, overall, YAP and TAZ have similar structures. Because both lack a DNA-binding region, YAP/TAZ functions through TEAD – target transcription factors, which have the ability to bind directly to DNA (Zhang et al., 2009; Zhao et al., 2008). Numerous targets of the YAP/TAZ-TEAD complex have been identified in mammals, such as CTGF and Cyr61. CTGF, or connective tissue growth factor, is involved in anchorage-independent growth and YAP-induced cell proliferation. Disruption of the YAP-TEAD complex or knockdown of the TEAD family of target transcription factors will abolish the epithelial-to-mesenchymal transition (EMT), oncogenic transformation, and YAP-induced cell-proliferation. Therefore, regulation of the YAP/TAZ-TEAD interaction is crucial for the maintenance of normal cell physiology (Lai et al., 2011; Zhang et al., 2009; Zhao et al., 2008).

**Hippo Pathway Interactions**

Recently it has been shown that components of the Hippo pathway interact with other proteins, and that these protein-protein interactions influence the translocation of YAP from the cytoplasm to the nucleus, or vice versa. The first of these interactions is with the Motin family, comprised of angiomotin (Amot), angiomotin-like 1, and angiomotin-like 2 (AmotL1 and AmotL2, respectively). As a group of scaffold proteins, the Motin family interacts with various other proteins that contain WW, coiled-coil, and PDZ domains (Yi et al., 2013). Amot exists as two isoforms, p80 and p130, both of which are localized to tight junctions, which were initially identified in endothelial cells as an angioatin-binding protein. In addition to its role in angiogenesis, which is beyond the scope of this manuscript, Amot has been shown to interact with the Hippo/YAP
pathway. Both the p80 and p130 isoforms of Amot bind to Merlin through coiled-coil domains; however, Amot-p80 lacks the same glutamine-rich N-terminal domain that is present on Amot-p130, AmotL1, and AmotL2 and, therefore, cannot interact with YAP (Wang et al., 2011; Yi et al., 2011). Yi et al. have shown that overexpression of the p130 isoform leads to inhibition of Lats1/2 phosphorylation of YAP at Ser127, which resulted in a subsequent increase in nuclear YAP. Conversely, the knockdown of Amot-p130 results in an increased cytoplasmic YAP and a reduced nuclear YAP. The results observed by Yi et al. imply that Amot-p130 plays an important role in maintaining the steady-state concentration of active, nuclear YAP by regulating the interaction between Lats1/2 and YAP.

Another interaction of the Hippo pathway involves the SET-domain containing lysine methyltransferase known as Set7 (or Setd7). Lysine methyltransferases are known to add a methyl group to specific lysine residues on histones, thereby modifying the structure of the chromatin and either activating or repressing the genes at that locus. Recently, however, it has been shown that methylation of lysine residues on nonhistone proteins can serve as a regulatory mechanism to control protein stability (Huang & Berger, 2008; Jenuwein & Allis, 2001; Oudhoff et al., 2013). Set7 was originally thought to mono-methylate histone 3 at its fourth lysine residue (H3K4). It is now known, however, that Set7 cannot add methyl groups to H3K4 and it is more likely that methylation of nonhistone proteins is the primary function of Set7 (Pradhan et al., 2009; Wang et al., 2001). It was observed by Oudhoff et al. that in high-density cultures of Setd7+/- cells YAP remained in the cytoplasm, but when Setd7 was knocked out in those
same high-density cultures YAP was localized primarily to the nucleus. It was also determined that, because Set7 lacks a signal sequence that would allow it to enter the nucleus, Set7 interacts with YAP in the cytoplasm (Donlin et al., 2012; Oudhoff et al., 2013). Oudhoff et al. also demonstrated that monomethylation of YAP at lysine 494 by the action of Set7 is a requirement for the cytoplasmic retention of YAP.

**The Hippo Pathway and Liver Cancer**

In *Drosophila* the Hippo signaling pathway was originally described as a tumor suppressor pathway, based on the tremendous overgrowth that occurred in tissues harboring Hippo pathway mutations (Harvey et al., 2013). In humans, the most well-known tumor suppressor gene from this pathway is NF2 (Merlin). Approximately one in 25,000 people have a mutation in NF2 – the only Hippo pathway gene currently known to be mutated and inactivated in cancer – which can lead to the development of lesions on the skin, eyes, and in the nervous system (Asthagiri et al., 2009). Though poorly studied, mutations in other components of the Hippo pathway in human cancers have provided convincing evidence in support of its role in tumorigenesis. Most importantly, recent genetic studies have provided unequivocal evidence regarding the role of Mst1/2 as tumor suppressors. In particular, hepatocellular carcinoma (HCC) has been shown to develop in both *Mst1*<sup>−/−</sup> *Mst2*<sup>+/−</sup> mice and liver tissue where either *Mst1* or *Mst2* has been ablated (Lu et al., 2010; Song et al., 2010; Zhou et al., 2009). One study demonstrated markedly decreased Mst1/2 in 70% of the human HCC samples and, in all but three of these samples, the attenuated Mst1/2 activity was coupled with a decreased YAP phosphorylation (Zhou et al., 2009).
The downstream effector of the Hippo signaling pathway, YAP functions as an oncogene – a gene that could potentially induce cancer. Abnormal nuclear localization and overexpression is seen in almost half of human HCC cases, and two studies recently have shown that YAP is an important oncogene in HCC (Dong et al., 2007; Zender et al., 2006). When pairs of matched HCC and normal tissue, along with the donors’ complete medical records, were examined, it was found that YAP can be considered an independent marker for not only overall survival, but also for disease-free survival in patients with HCC (Xu et al., 2009). As for the underlying mechanism of YAP activation in human HCC, reports have shown that approximately 30% of HCC tumors have decreased Ser127 phosphorylation on YAP. A similar proportion of tumors have shown that activated Mst1/2 is either severely reduced or absent, which leads to a greatly reduced Mob1 phosphorylation. Thus, what may actually account for YAP activation in human HCC is the loss of Mst1/2 – a common feature in such tumors (Praskova et al., 2008; Zhou et al., 2009). However, whether YAP activation and dysregulation is a consequence of tissue transformation or a prominent feature of the preceding hyperproliferative states of HCC is unknown.

Liver cancer is the seventh most prevalent cancer among women and the fifth most prevalent among men, with almost 20,000 new cases of HCC in the United States and more than 500,000 new cases worldwide diagnosed each year. The highest incidence rates of HCC occur in the developing countries of Sub-Saharan Africa and Southeast Asia – areas where the hepatitis B virus is endemic (El-Serag, 2011). In fact, 50% of worldwide adult HCC cases and the majority of pediatric cases are caused by chronic
hepatitis B infection. Among those patients with hepatitis B, however, the risk of HCC is greater in those that are male and elderly (Chen et al., 2009). Another risk factor for the development of HCC is the hepatitis C virus. Those infected with hepatitis C have a 20-fold greater risk of HCC compared to non-infected, with an even greater risk extended to those with cirrhosis or advanced liver fibrosis (Donato et al., 2002). In the United States, the fastest-rising cause of cancer-related deaths is due to HCC as a result of infection with the hepatitis C virus. As the five-year survival rate of HCC continues to be below 12%, the incidence of HCC in the United States has seen a three-fold increase over the past 20 years (El-Serag, 2011).

Hepatoblastoma (HB) is recognized as distinct from HCC. HB is a rare malignant tumor, which responsible for over two-thirds of liver malignancies in children – making it the most prevalent primary hepatic malignancy in pediatric patients (Miller et al., 1995; Raney, 1997). As with HCC, the male population is twice as susceptible to HB when compared to females (Zheng et al., 2009). Close to 90% of HB cases occur in patients under the age of five, with almost 70% occurring in the first two years of life (Lack et al., 1982). If HB is diagnosed in adolescents or young adults, though extremely rare, the prognosis tends to be worse. HB is extremely uncommon in adults as most of the tumors are embryonic and arise in utero (Ahn et al., 1997; Remes-Troche et al., 2006). The exact etiology of HB has eluded researchers, however some recent investigations have shown the loss of heterozygosity in certain chromosomal loci as a potential genetic basis for its development (Nagata et al., 2005).
Hypotheses and Specific Aims

The aim of this thesis is two-fold. The first was to examine the role of two Hippo pathway interactions – AmotL2 and Set7 during liver regeneration. AmotL2 has been shown to have glutamine-rich N-terminal domain known to interact with YAP and prevent phosphorylation by Lats1/2. Therefore, it was hypothesized that levels of AmotL2 protein will be higher in regenerating liver tissue compared to quiescent liver tissue. Studies of Set7 in mouse liver have illustrated that this enzyme methylates YAP and retains it in the cytoplasm. Given this, it is hypothesized that YAP methylation will be decreased in the regenerating liver tissue compared to quiescent liver tissue. In the second, the role of YAP in pediatric liver cancer, specifically hepatocellular carcinoma and hepatoblastoma will be examined. Based on its function as an oncogene, and the fact that it has been found to play a role in adult liver tumors, is can be predicted that there is YAP nuclear localization in a subset of pediatric liver tumors.
METHODS

Animals

Male Lewis rats (*Rattus norvegicus*) weighing 300-350 g were obtained from Charles River Laboratories (Wilmington, MA). The rats were housed individually, with their own food and water supplies, and maintained at Boston Children’s Hospital’s animal facility. All animal experiments were approved by Boston Children’s Hospital Institutional Animal Care and Use Committee.

Partial Hepatectomy

Rat partial hepatectomies were performed at Boston Children’s Hospital by a member of the research team. Briefly, rats were subjected to a 70% partial hepatectomy similar to that described in the Higgins and Anderson model with a few minor modifications (Higgins & Anderson, 1931). The lobes that were resected represent non-regenerating tissue and were used as matched control for each animal. Following partial hepatectomy, animals were euthanized at the following time points: 1, 3, 7, 10, 14, 21, and 30 days. At the time of sacrifice, both the remnant liver weight, as well as the body weight, were measured and the liver-to-body weight ratio for each animal was calculated. Sham animals underwent laparotomy with manipulation of the liver without liver resection.

Western Blot Analysis

For Western blot analysis, approximately 0.05g of fresh, frozen rat liver tissue from both control and regenerating samples was homogenized in 200µl of lysis buffer.
(0.5g SDS, 0.3g Tris Base, 500µl 100mM PMSF, 100µl 0.5M EDTA, 100µl 0.5M EGTA, a Protease Inhibitor tablet dissolved in 1.5ml dH2O, filled with dH2O to 50ml) at room temperature (RT). After sonication of each sample, the protein levels were determined using a Bradford assay (Bio-Rad Laboratories, Hercules, CA). Protein samples (50-75µg) were resuspended and boiled in the presence of 5X sample buffer for five minutes at 95° before being separated on a 4-20% glycine gel (Invitrogen, Carlsbad, CA) for 90min at 130V. Proteins were then transferred to a PVDF membrane and blocked for 60min with either 5% BSA or a 1:1 ratio of 5% BSA and 5% milk (both prepared in Tris buffered saline with Tween 20, or TBST). The membranes were then incubated overnight at 4°C with varying dilutions of the following primary antibodies: anti-AmotL2 (Cell Signaling Technology, Beverly, MA) and GAPDH (Millipore, Billerica, MA). Primary antibody incubation was followed by at least 3 washes – 15-30min, each at RT – in TBST before the membrane was placed in secondary antibody for 60min (HRP-conjugated goat anti-rabbit IgG (Cell Signaling Technology) and HRP-conjugated goat anti-mouse IgG for AmotL2 and GAPDH, respectively). Incubation in secondary antibody was succeeded by at least 3 more washes in TBST before proteins were visualized using the ECL detection system (NEN, Boston, MA). Coomassie staining was used to check for even protein loading.

**Co-Immunoprecipitation (Co-IP)**

Tissue was processed and homogenized as previously stated. For the IP samples: 100µg of protein was incubated with 2.5µl anti-YAP antibody (Cell Signaling) and GAL4 immunoprecipitation buffer was added to bring the volume to 150µl. The samples were
placed on a shaker at 4˚ C for 3.5 hours. Magnetic beads were prepared by washing them twice with phosphate buffered saline (PBS) and then resuspending them in a volume of PBS equating to 20µl/sample. After the 3.5-hour incubation period, 20µl of magnetic protein A-beads were added to each sample and then left spinning overnight at 4˚ C. Samples were placed on a magnetic rack to facilitate removal of the supernatant. This process was repeated three times using GAL4 to wash the samples, removing the supernatant each time. 2X sample buffer was added to the samples in a 1:1 ratio with the beads and then the samples were boiled for 5 minutes at 95˚ C.

For the input samples, 50µg of protein was resuspended in 10µl 2X sample buffer and boiled at 95˚ C for 5min. The samples were then loaded onto a 4-20% glycerine gel and the protocol for a Western blot was followed using the following primary antibodies: anti- Methyl-Lysine (Cell Signaling Technology) and GAPDH (Millipore). HRP-conjugated goat anti-rabbit IgG (Cell Signaling Technology) and HRP-conjugated goat anti-mouse IgG were used as the secondary antibodies for methyl-lysine and GAPDH, respectively.

**Human Tumor Immunohistochemistry (IHC)**

Formalin-fixed, paraffin-embedded human liver samples that included both tumor and non-tumor tissue were obtained from 9 (only 8 were used for analysis) patients at Boston Children’s Hospital who had previously undergone liver resection or liver transplantation (Table 1). This protocol was approved by the Institutional Review Board of Boston Children’s Hospital.
**Table 1:** Pediatric patient characteristics. Including: given patient number, age in years, surgical intervention performed, tumor pathology (HCC or HB), whether there was presence of vascular invasion, and their current status. (NED = no evidence of disease).

<table>
<thead>
<tr>
<th>#</th>
<th>Age (yr)</th>
<th>Surgery</th>
<th>Tumor pathology</th>
<th>Histologic vascular invasion</th>
<th>Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.33</td>
<td>Hepatic resection</td>
<td>Hepatoblastoma</td>
<td>N</td>
<td>Alive, NED</td>
</tr>
<tr>
<td>2</td>
<td>2.00</td>
<td>Liver transplant</td>
<td>Hepatoblastoma (fetal and embryonal)</td>
<td>Y</td>
<td>Alive, NED</td>
</tr>
<tr>
<td>3</td>
<td>13.00</td>
<td>Hepatic resection</td>
<td>Hepatocellular carcinoma, moderately diff</td>
<td>Y</td>
<td>Alive, NED</td>
</tr>
<tr>
<td>4</td>
<td>3.00</td>
<td>Resection + Liver transplant</td>
<td>Hepatoblastoma (fetal and embryonal)</td>
<td>N</td>
<td>Alive, NED</td>
</tr>
<tr>
<td>5</td>
<td>0.92</td>
<td>Liver transplant</td>
<td>Hepatoblastoma (macrotrabecular and fetal)</td>
<td>Y</td>
<td>Alive, NED</td>
</tr>
<tr>
<td>6</td>
<td>13.00</td>
<td>Multivisceral transplant</td>
<td>Hepatocellular carcinoma</td>
<td>Y</td>
<td>Alive, NED</td>
</tr>
<tr>
<td>7</td>
<td>0.67</td>
<td>Liver transplant</td>
<td>Hepatoblastoma (w/ embryonal features)</td>
<td>N</td>
<td>Alive, NED</td>
</tr>
<tr>
<td>8</td>
<td>11.00</td>
<td>Hepatic resection</td>
<td>Hepatoblastoma (fetal and embryonal)</td>
<td>Y</td>
<td>Alive with tumor recurrence</td>
</tr>
<tr>
<td>9</td>
<td>1.50</td>
<td>Hepatic resection</td>
<td>Hepatocellular carcinoma</td>
<td>Y</td>
<td>Died with tumor recurrence</td>
</tr>
</tbody>
</table>
Tissue sections were de-paraffinized and re-hydrated using the following process: two 10min washes in xylene, two 10min washes in 100% ethanol, one 5min wash in 95% ethanol, one 5 minute wash in 75% ethanol, one 5min wash in 50% ethanol, 2min in dH$_2$O, and 5min in PBS. Tissue then sat in 0.5% Triton for 30min at RT before being placed in blocking solution (1% goat serum in PBS) for 60min at RT. Sections were washed once in PBS for 5 minutes and then incubated overnight at 4˚ C in two primary antibodies: a 1:50 dilution of anti-YAP (Cell Signaling Technology) and a 1:50 dilution of anti-Ki67 (Abcam, Cambridge, England). The tissue was then washed three times in PBS and incubated in the following secondary antibodies: Alexa Fluor 488 goat anti-rabbit for YAP and Cy3 goat anti-mouse for Ki67, for 1.5 hours. Sections were washed for a total of 15min in PBS and then incubated in DAPI for 20min. The tissue was washed three more times with PBS before being mounted in Vectashield (Vector Laboratories, Burlingame, CA) and imaged using fluorescent microscopy. Open source software from the National Institutes of Health (Image J) was used for data analysis.
RESULTS

AmotL2 levels during Liver Regeneration

Previous studies have demonstrated that the p130 isoform of angiomotin interacts with YAP, and that overexpression of Amot-p130 leads to increased YAP nuclear localization (Yi et al., 2011). The role of another Motin family member, AmotL2, has not been as thoroughly studied. This study was designed to determine if the levels of AmotL2 protein differed between control and regenerating liver tissue. Using Western blot analysis, levels of AmotL2 were assessed in regenerating livers at 2 hours and 1, 3, and 7 days post-PH and compared to matched control tissue. At 2 hours post-PH, AmotL2 was present in both the regenerating liver, as well as the control tissue (Figure 3a). The amount of AmotL2 protein was normalized against GAPDH, with the average percent of GAPDH for regenerating tissue being 17.78%, compared to 21.94% for control tissue. Figure 3b represents these values along with the standard errors of 7.74 and 2.07 for control and regenerating liver, respectively. No statistically significant difference in AmotL2 protein levels was found between control and regenerating tissue. AmotL2 was present in all 1-day control tissue, but was missing from one of the regenerating liver samples (Figure 3c). Protein levels were once again normalized using GAPDH and graphed along with the standard errors for both sets of tissue. At 1-day post-PH the average percent of GAPDH was 8.62% for control tissue, standard error of 1.38, and 7.96% for regenerating liver, standard error of 1.49 (Figure 3d).
Figure 3: AmotL2 protein levels in regenerating versus control liver tissue normalized to GAPDH. (A) 2-hours post-partial hepatectomy. Graph illustrates average percent of GAPDH for control (21.94%) and regenerating (17.78%) liver tissue, along with standard error bars for each. (B) 1-day post-partial hepatectomy. Average percent GAPDH for control (8.62%) and regenerating (7.96%), as well as the error bars, are shown in the bar graph. (C) 3-days post-partial hepatectomy. The average percent of GAPDH are 16.79% and 27.79% for control and experimental tissue, respectively. The graph also illustrates the standard error bars for each tissue type. (D) 7-day post-partial hepatectomy. Graph illustrates average percent GAPDH for control (36.09%) and regenerating (45.62%) along with the standard error of the mean for each set of samples.
The differences in AmotL2 levels between control and regenerating tissue were not found to be statistically significant. In the 3-day post-PH liver, the amount of AmotL2 was unable to be quantified in two of the regenerating samples but was quantifiable in all control samples (Figure 3e). Even so, the average percent of GAPDH in regenerating tissue was 27.79% compared to 16.79% in control tissue. The standard error for 3-day post-PH tissue was found to be 5.23 and 10.33 for control and regenerating liver, respectively, and no significant difference in AmotL2 levels was demonstrated (Figure 3f). Figure 3g illustrates that AmotL2 was present in all tissue samples at 7-days post-PH. When quantified, the average percent of GAPDH for control tissue was 36.09%, with a standard error of 6.14, and for regenerating tissue was 45.62%, with a standard error of 5.70 (Figure 3h). Once again, no statistically significant difference was found in AmotL2 between the control and regenerating tissues. Overall, there was no statistically significant difference in the amount of AmotL2 levels during liver regeneration at the examined time points.

**YAP Methylation in the Regenerating Liver**

Recently studies in mice have shown that Set7 monomethylates YAP at a specific lysine residue, and that this action is required for the cytoplasmic retention of YAP (Oudhoff et al., 2013). This study was designed to determine whether YAP methylation by Set7 also occurred in rat liver tissue during liver regeneration. Using co-immunoprecipitation analysis, the amount of methylated YAP was measured in rat liver tissue at 1, 7, and 21 days post-PH. At 1 day post-PH, methylated YAP was present in both control and regenerating liver tissue (Figure 4a). The bands were too close to be
quantified and, therefore, no statistical analysis could be done on the 1-day tissue. At 7
days post-PH, methylated YAP was no longer present in either control or regenerating
tissue, and the same results were seen at 21 days post-PH (Figure 4b and 4c). This was
most likely secondary to technical problems given the lack of any methylated YAP even
in the control tissue. Because of this, no conclusive evidence could be obtained as to the
level of methylated YAP in control versus regenerating rat liver.

Figure 4: Levels of methylated-YAP in control versus regenerating rat liver at
different time points. (A) Results of a co-immunoprecipitation assay (CoIP) with 1-
day post-PH liver tissue to see if methylated-lysine and YAP are in complex
together. (B) 7-day post-PH CoIP comparing methylated-YAP in control and
regenerating liver tissue. (C) CoIP using 21-day post-PH rat liver tissue to compare
methylated-YAP levels in control and regenerating tissue. (C = control, R =
regenerating, In = input sample, IP = immunoprecipitation sample).
**YAP Nuclear Localization in Liver Tumors**

As an oncogene, and the downstream effector of the Hippo pathway, YAP has shown nuclear localization in close to 50% of adult, human HCC samples. In other recent studies it has been suggested that YAP may act as an independent prognostic marker for adult HCC patients (Xu et al., 2009; Zender et al., 2006). The role YAP plays in pediatric HCC tumors and HB tumors have not been previously reported. To evaluate the role of YAP in pediatric HCC and HB, we performed immunohistochemical analysis to determine the subcellular localization in tumor cells compared to the cells of adjacent non-tumor tissue. In the HCC tissue, DAPI staining, which identifies the nuclei of individual cells, was present in all 3 tumor samples (Figure 5b). When staining for YAP, however, none of the tumor or control liver tissue demonstrated any nuclear or cytoplasmic staining (Figure 5c). The merging of both DAPI and YAP staining best illustrates the lack of either cytoplasmic or nuclear YAP staining (Figure 5d). Overall, there was no conclusive staining evidence that suggested YAP staining in the nucleus. Also, because of the heavy background staining and artifact staining it was impossible to tell whether cytoplasmic YAP staining was present.
The same HCC tissue was stained for Ki67, a marker of cell proliferation, to determine whether cells were undergoing mitosis. Once again all 3 HCC samples stained with DAPI (Figure 6a). It was thought that Ki67 would be a good marker given the proliferative nature of tumors, however there was no presence of Ki67 staining in the tumor cells of...
the HCC samples (Figure 6b). The lack of Ki67 nuclear staining is clearly seen in the merged data with DAPI staining (Figure 6c).

Figure 6: Immunofluorescence staining of hepatocellular carcinoma tissue with DAPI and Ki67. (A) DAPI nuclear staining of the HCC tumor tissue. (B) Ki67 staining of the HCC tumor tissue using Cy3 secondary antibody. (C) Merged data with both DAPI and Ki67 staining, which shows no Ki67 staining in the tumor cells of HCC sample tissue.
In pediatric hepatoblastoma samples, DAPI nuclear staining was present in 4 of the 5 samples (Figure 7b). When the tissue was stained for YAP, the background was heavily stained and the presence of artifact staining was noted (Figure 7c). When DAPI staining and YAP staining were merged, it was clear that there was no YAP staining within the nucleus of either the tumor tissue or the adjacent non-tumor tissue (Figure 7d). In the end, the HB tissue showed no nuclear YAP staining nor did it show cytoplasmic YAP staining.

The same hepatoblastoma samples were again stained for Ki67 to determine whether cells were undergoing proliferation. All 4 of the HB samples had successful DAPI nuclear staining (Figure 8a). Though it was thought that Ki67 would be a good marker given the proliferative nature of tumors, however there was no presence of Ki67 staining in the tumor cells of the HCC samples (Figure 8b). The lack of Ki67 nuclear staining is clearly seen in the merged data with DAPI staining (Figure 8c).
Figure 7: Immunofluorescence staining of pediatric hepatoblastoma tissue with DAPI and YAP. (A) Hematoxylin and Eosin staining of matched hepatoblastoma tissue to illustrate hepatic tumor morphology. (B) DAPI nuclear staining of pediatric hepatoblastoma tissue. (C) YAP staining of pediatric HB tissue. (D) Merged staining of both DAPI and YAP illustrating that YAP nuclear staining is not present in the pediatric hepatoblastoma samples.
Overall, there was no conclusive evidence of YAP staining in either pediatric hepatocellular carcinoma or hepatoblastoma tissue to determine the localization of YAP within each tumor type. There was also no Ki67 staining in either tumor type and, therefore, it is unknown whether this can be used as a marker for cell proliferation in
pediatric tumor samples. In the end, these experiments should be repeated as no conclusions can be drawn from them.
DISCUSSION

It has been demonstrated that the Hippo signaling pathway plays a role in liver size regulation and inhibition of its signaling results in massive liver overgrowth. In addition, prolonged inactivation of the Hippo pathway resulting YAP overexpression has been shown to lead to tumorigenesis. Importantly, when Hippo signaling is restored liver overgrowth is reversed and the organ is able to return to near normal liver-to-body weight ratio (Dong et al., 2007; Lu et al., 2010; Zhou et al., 2009). Despite numerous studies demonstrating the role of the Hippo pathway in modulating hepatocyte proliferation and apoptosis, the physiologic role of the Hippo pathway, along with its numerous interactions, during liver regeneration and tumorigenesis has not been elucidated. This study aimed to investigate possible alterations in Hippo pathway interactions during liver regeneration, as well as the role this signaling pathway plays in hepatocellular carcinoma and hepatoblastoma. Briefly, it was found that AmotL2 was present in regenerating tissue at multiple time points during the regeneration process but was also present in matched control tissue. It was also found that YAP was methylated in liver tissue 1-day post-partial hepatectomy, but not at any other time point examined. As for the role of Hippo signaling in liver tumorigenesis, inconclusive evidence was obtained in both hepatoblastomas and hepatocellular carcinomas.

In order to study the Hippo pathway interactions during liver regeneration, the well-established 70% partial hepatectomy model in rats was used. Previous use of this model has allowed researchers to monitor the time course and rate of liver growth
following partial hepatectomy. These reports have found that the liver-to-body weight ratio is restored to pre-hepatectomy levels 7 days after 70% hepatectomy. Notably, almost 30% of the pre-hepatectomy liver weight has been regained after 24 hours and 70% has been regained by 3 days post-partial hepatectomy – indicating that the greatest amount of liver growth occurs within the first three days after 70% hepatectomy (Higgins & Anderson, 1931).

Given this information, the majority of this study on Hippo pathway interactions was focused on the first seven days after partial hepatectomy, with the exception of a 21-day study done to evaluate YAP methylation. The results obtained indicate that AmotL2 is present in regenerating liver tissue at 2 hours, as well as at 1, 3, and 7 days post-70% hepatectomy. The problem, however, is that AmotL2 was also found in control tissue at these time points and there was not a statistically significant difference between the levels of protein between control and regenerating tissue (Figure 3a-h). While previous studies have indicated that other members of the Motin family, namely the p130 isoform of angiomitn, are present in increased amounts after partial hepatectomy it was not possible to duplicate these results in regards to AmotL2 (Yi et al., 2013). In regards to YAP methylation, the results were less conclusive. Methylated-YAP appeared to be present in 1-day post-PH liver tissue, however, the presence of multiple bands in close proximity to each other made the levels of methylated-YAP impossible to quantify (Figure 4a-c). At 7 and 21 days post-hepatectomy, methylated-YAP was not present in the results of our CoIP assay. The presence of methylation – which would keep YAP sequestered in the cytoplasm – at 1-day post-PH is counter-intuitive considering the
previously mentioned timeline of liver growth after hepatectomy. Ultimately no conclusive evidence as to the role Hippo pathway interactions play in liver regeneration was obtained.

The second part of this study aimed to evaluate whether YAP was activated and localized in the nucleus of pediatric tumor cells. Previous studies have reported that YAP is activated in adult hepatocellular carcinoma cells, but no such studies have been done using pediatric HCC tumor samples or hepatoblastoma samples (Xu et al., 2009; Zender et al., 2006). In the pediatric HCC tissue samples, along with the matched non-tumor tissue, YAP staining appeared to be present in the cytoplasm of many cells and, possibly, in the nucleus of a few tumor cells. However, due to the inconsistent nature of the staining and the presence of artifacts, no conclusions could be made about whether YAP is activated in pediatric HCC tumor cells. The same or similar staining was also seen in hepatoblastoma tissue and it’s adjacent non-tumor tissue. Therefore, no conclusions could be drawn about the role of YAP in pediatric liver tumors.

In summary, the results of these studies provided no definitive conclusions as to the role of Hippo pathway interactions in liver regeneration nor did they provide any evidence as to the role of Hippo signaling, in particular YAP activation, in two types of liver cancer. Because of this, the questions posed throughout this thesis require further investigation.
REFERENCES


