To Hind, the reason I’m here and the reason I carry on
ACKNOWLEDGMENTS

I would like to thank my principle investigator Dr. Bryon Petersen for all that he has given me – a deep understanding of not just the intricacies of scientific research, but also for perspective and the ability to see beyond what’s there and look at the big picture. He was also there for me during the roughest patches of my life, and I will never forget the care he took of me and will be eternally thankful. I would also like to thank the members of my supervisory committee: Drs. Chen Liu, Phyllis LuValle, and Naohiro Terada for their guidance and support in my research and on the doctoral as well as post-doctoral path.

My family and friends have been of the utmost importance in my life, especially for the last five years. Although most of my family lives overseas, an aunt for whom I am named and her son (my brother), have helped give me the guidance and strength to get through. Also my lab mates have been there for me through it all, and I can never really express how much their support has meant to me, in particular Nicole Steiger and Dr. SehHoon Oh. I would also like to thank the friends I have amassed during my time here in Gainesville, as well as those who preceeded them from Polk County.

There is one last person to thank – my mother, who passed away during my fourth year of graduate school. My mother sacrificed many things in life in order for me to have the best schooling, and it seemed that my dream of becoming a scientist was as much hers as it was mine. Were it not for her I would not have made it this far nor would I be the person I am today, and it is with a contented but heavy heart that I move towards graduation, with only the hope that she is watching.
TABLE OF CONTENTS

ACKNOWLEDGMENTS.................................................................................................................... 4

LIST OF TABLES................................................................................................................................ 8

LIST OF FIGURES .............................................................................................................................. 9

LIST OF ABBREVIATIONS ............................................................................................................ 11

CHAPTER

1 INTRODUCTION....................................................................................................................... 15

2 BACKGROUND AND SIGNIFICANCE ................................................................................. 18

  2.1 The Liver ............................................................................................................................... 18
    2.1.1 Gross Anatomy ........................................................................................................... 18
    2.1.2 Hepatic Microarchitecture ......................................................................................... 18
    2.1.3 Cell Types of the Liver .............................................................................................. 19
  2.2 The Functions of the Liver ................................................................................................... 21
    2.2.1 Metabolic Homeostasis .............................................................................................. 21
    2.2.2 Molecular Silo ............................................................................................................ 21
    2.2.3 Detoxification ............................................................................................................. 22
    2.2.4 Production of Bile ...................................................................................................... 22
  2.3 Hepatic Regeneration ............................................................................................................ 23
    2.3.1 General Considerations .............................................................................................. 23
    2.3.2 Models of Regeneration ............................................................................................. 23
  2.4 Liver Stem Cell Biology ....................................................................................................... 24
    2.4.1 The Oval Cell.............................................................................................................. 24
    2.4.2 Canals of Hering: Putative Oval Cell Niche ............................................................. 25
    2.4.3 Oval Cell Plasticity ..................................................................................................... 26
    2.4.4 Oval Cell-Mediated Liver Regeneration ................................................................... 26
    2.4.5 Clinical Applications for the Oval Cell ..................................................................... 28
    2.4.6 Liver Stem Cells and Hepatocellular Carcinoma ..................................................... 29
  2.5 The Notch Signaling Pathway .............................................................................................. 30
    2.5.1 General Considerations .............................................................................................. 30
    2.5.2 A Brief History ........................................................................................................... 31
    2.5.3 Structure of the (Mammalian) Notch Gene .............................................................. 31
    2.5.4 Notch Signal Transduction ......................................................................................... 32
    2.5.5 Effectors of the Notch Cascade ................................................................................. 33
    2.5.6 Hepatic Notch Expression ......................................................................................... 34
    2.5.7 Functions of Notch Signaling .................................................................................... 34
    2.5.8 Notch and Wnt: A Delicate Balance ........................................................................ 35
    2.5.9 Notch-Induced Pathologies......................................................................................... 36
<table>
<thead>
<tr>
<th>Table</th>
<th>page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-1 Cellular markers of the liver</td>
<td>47</td>
</tr>
<tr>
<td>2-2 Components of the Notch signaling cascade</td>
<td>48</td>
</tr>
<tr>
<td>4-1 PCR Primer Sequences</td>
<td>66</td>
</tr>
<tr>
<td>4-2 Antibodies utilized for western blot analysis</td>
<td>67</td>
</tr>
<tr>
<td>4-3 Antibodies utilized for immunohistochemical analysis</td>
<td>67</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-1</td>
<td>Diagram of hepatic architecture</td>
<td>42</td>
</tr>
<tr>
<td>2-2</td>
<td>Compensatory hyperplasia after $\frac{2}{3}$ partial hepatectomy.</td>
<td>43</td>
</tr>
<tr>
<td>2-3</td>
<td>Cellular growth after $\frac{2}{3}$ partial hepatectomy.</td>
<td>44</td>
</tr>
<tr>
<td>2-4</td>
<td>Oval cell response following $\frac{2}{3}$ partial hepatectomy.</td>
<td>45</td>
</tr>
<tr>
<td>2-5</td>
<td>Hepatic oval cell differentiation</td>
<td>46</td>
</tr>
<tr>
<td>4-1</td>
<td>Model for oval cell induction</td>
<td>65</td>
</tr>
<tr>
<td>4-2</td>
<td>Model of oval cell induction with Notch inhibition</td>
<td>65</td>
</tr>
<tr>
<td>5-1</td>
<td>RT-PCR analysis</td>
<td>75</td>
</tr>
<tr>
<td>5-2</td>
<td>Expression of Notch and Jagged via <em>in situ</em> PCR</td>
<td>76</td>
</tr>
<tr>
<td>5-3</td>
<td>Analysis of Notch1 protein expression during 2AAF-PH</td>
<td>77</td>
</tr>
<tr>
<td>5-4</td>
<td>Immunohistochemistry for Notch1 and Jagged1.</td>
<td>78</td>
</tr>
<tr>
<td>5-5</td>
<td>Immunofluorescence for activated Notch1 at day 11 post-2AAF-PH.</td>
<td>79</td>
</tr>
<tr>
<td>5-6</td>
<td>H&amp;E staining of liver from animals on the 2AAF-PH protocol or 2AAF_PH combined with GSIXX</td>
<td>80</td>
</tr>
<tr>
<td>5-7</td>
<td>Real time PCR analysis for expression of Notch pathway members during 2AAF-PH</td>
<td>81</td>
</tr>
<tr>
<td>5-8</td>
<td>Real time PCR analysis for expression of Notch pathway members during 2AAF-PH with GSIXX</td>
<td>82</td>
</tr>
<tr>
<td>5-9</td>
<td>Analysis of Notch1 protein expression during 2AAF-PH with GSIXX.</td>
<td>83</td>
</tr>
<tr>
<td>5-10</td>
<td>OV-6 staining during the 2AAF-PH protocol or 2AAF-PH with GSIXX.</td>
<td>84</td>
</tr>
<tr>
<td>5-11</td>
<td>AFP staining during the 2AAF-PH protocol or 2AAF-PH with GSIXX.</td>
<td>85</td>
</tr>
<tr>
<td>5-12</td>
<td>Analysis of CK19 protein expression during 2AAF-PH or 2AAF-PH with GSIXX</td>
<td>86</td>
</tr>
<tr>
<td>5-13</td>
<td>Analysis of HNF1β protein expression during 2AAF-PH or 2AAF-PH with GSIXX</td>
<td>87</td>
</tr>
<tr>
<td>5-14</td>
<td>Ki67 staining during the 2AAF-PH protocol or 2AAF-PH with GSIXX.</td>
<td>88</td>
</tr>
</tbody>
</table>
5-15 Semi-quantitative analysis of Ki67 staining during the 2AAF-PH protocol or 2AAF-PH with GSIXX...........................................................................................................................89

5-16 Analysis of p21 protein expression during 2AAF-PH or 2AAF-PH with GSIXX...........90

5-17 Analysis of phospho-AKT protein expression during 2AAF-PH or 2AAF-PH with GSIXX.. ..................................................................................................................................91

5-18 Hepatocellular mitoinhibition index for 2AAF-PH alone as well as 2AAF-PH with GSIXX.. ..................................................................................................................................92

5-19 CYP3A2 staining during the 2AAF-PH protocol or 2AAF-PH with GSIXX.. ...............93

5-20 Analysis of Glucose-6-Phosphatase-α protein expression during 2AAF-PH or 2AAF-PH with GSIXX.. ...................................................................................................................94

5-21 Diagram depicting involvement of Notch signaling in the differentiation of stem cells/progenitors.. ...................................................................................................................95
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA</td>
<td>Allyl alcohol</td>
</tr>
<tr>
<td>2AAF</td>
<td>2-acetoaminofluorine</td>
</tr>
<tr>
<td>AFP</td>
<td>α-fetoprotein</td>
</tr>
<tr>
<td>AGS</td>
<td>Alagille Syndrome</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>bHLH</td>
<td>basic helix-loop-helix</td>
</tr>
<tr>
<td>CADASIL</td>
<td>Cerebral dominant arteriopathy with subcortical infarcts and leukoencephalopathy</td>
</tr>
<tr>
<td>CCl₄</td>
<td>Carbon tetrachloride</td>
</tr>
<tr>
<td>CK</td>
<td>Cytokeratin</td>
</tr>
<tr>
<td>CYP</td>
<td>Cytochrome p450</td>
</tr>
<tr>
<td>CCC</td>
<td>Cholangiocarcinoma</td>
</tr>
<tr>
<td>DAB</td>
<td>3’3’-diaminobenzidine</td>
</tr>
<tr>
<td>DEPC</td>
<td>Diethyl pyrocarbonate</td>
</tr>
<tr>
<td>DIG</td>
<td>Digoxigenin</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal Growth Factor</td>
</tr>
<tr>
<td>G6Pase</td>
<td>Glucose-6-phosphatase</td>
</tr>
<tr>
<td>GGT</td>
<td>γ-glutamyl-transpeptidase</td>
</tr>
<tr>
<td>GSI</td>
<td>γ-secretase inhibitor</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Hematoxylin and Eosin</td>
</tr>
<tr>
<td>HCC</td>
<td>Hepatocellular carcinoma</td>
</tr>
<tr>
<td>Hes</td>
<td>Hairy/Enhancer of Split</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>HGF</td>
<td>Hepatic Growth Factor</td>
</tr>
<tr>
<td>HSC</td>
<td>Hematopoietic Stem Cell</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>NICD</td>
<td>Notch Intracellular Cytoplasmic Domain</td>
</tr>
<tr>
<td>NPC</td>
<td>Non-parenchymal cell</td>
</tr>
<tr>
<td>OCT</td>
<td>Optimal cutting temperature</td>
</tr>
<tr>
<td>O/N</td>
<td>Overnight</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PH</td>
<td>Partial Hepatectomy</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative PCR</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNase</td>
<td>Ribonuclease</td>
</tr>
<tr>
<td>RPM</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcription PCR</td>
</tr>
<tr>
<td>SD</td>
<td>Spondylocostal dysostosis</td>
</tr>
<tr>
<td>TEMED</td>
<td>Tetramethylethlenediamine</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
</tbody>
</table>
Abstract of Dissertation Presented to the Graduate School of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

THE ROLE OF NOTCH SIGNALING IN OVAL CELL-MEDIATED LIVER REGENERATION

By

Houda Darwiche

August 2009

Chair: Bryon E. Petersen
Major: Medical Sciences – Molecular Cell Biology

Activation of the oval cell compartment occurs in the liver when hepatocytes are functionally compromised and/or unable to divide. Our goal is to investigate the systemic signals responsible for determining the efficiency of oval cell mediated liver regeneration, focusing on the Notch signaling cascade. The established oval cell induction protocol of 2-Acetylamino fluorine (2AAF) implantation followed by 70% surgical resection of the liver (PH) was employed in a rat model. This oval cell induction model was further combined with injections of GSI XX to examine the effects of Notch inhibition on stem cell-aided regeneration of the liver. Notch signaling was found to be upregulated at the peak of oval cell induction during 2AAF-PH alone. Treatment with GSI XX led to interruption of the Notch signal, as shown by a decrease in expression of Hes1. While there was a robust oval cell response seen at day 11 post-PH, there was a measurable delay in differentiation when Notch was inhibited. This was confirmed morphologically as well as by immunohistochemistry for the oval cell markers AFP, OV-6 and CK19. The hepatocytes seen at day 22 demonstrated an enhanced hepatocellular mitoinhibition index (p21\textsuperscript{Waf1}/Ki67), suggestive of dysregulated proliferation and cell cycle progression. Moreover these hepatocytes exhibited decreased expression of hepatocyte functional markers such as cytochrome p450 and glucose-6-phosphatase-\(\alpha\). Taken together these
results identify the Notch signaling pathway as a potent regulator of differentiation and proliferation in oval cells and is necessary for functional repair of the liver by oval cells.
CHAPTER 1
INTRODUCTION

The signals mediating cellular specification are produced by adjacent or even distant cells, and often these various signals are received by the cell simultaneously; therefore they must be integrated in proper order for the correct specification of cell fate to occur.\(^1\) The Notch family of receptors encompasses a group of proteins that function both as cell surface receptors and as direct regulators of cellular specification, leading to differentiation, cell cycle progression, proliferation or apoptosis.\(^2\)\(^-\)\(^5\) Generally, activation of the Notch pathway inhibits differentiation via transcriptional repression of genes specific to a certain cell lineage. This limits the number of cells that will assume a certain lineage and thereby leaves a small number of uncommitted progenitors that are able to adopt alternative cell fates.\(^5\)

The Notch signaling pathway is said to be activated when the extracellular domain of the Notch receptor is bound by its ligand, preferentially Jagged, on neighboring cells. Following activation the receptor is cleaved by \(\gamma\)-secretase, leading to release and translocation of the Notch Intracellular Cytoplasmic Domain (NICD) from the cytoplasm to the nucleus.\(^6\)\(^-\)\(^7\) This results in the subsequent activation of transcriptional regulators of genes involved in differentiation, proliferation and apoptosis. Inhibition of \(\gamma\)-secretase has been well documented in the literature as a method for inhibition of the Notch signal.\(^8\)\(^-\)\(^10\) Experimental evidence has shown that Notch receptors and ligands are widely distributed, and that the Notch cascade is required for mammalian development and growth in many organ systems, including the liver.\(^11\)

The liver is the only solid organ in the body with the capacity for rapid regeneration in response to injury. If hepatocytes are prevented from initiating the regeneration response, or if the injury is too severe, then the liver stem cell compartment is activated, giving rise to the oval cell population.\(^12\) In normal liver tissue, oval cells numbers are very rare; however, when stem
cell activation occurs it leads to the profuse replication of these cells in the periportal regions of the lobule. Activation of the oval cell compartment is usually achieved via the 2AAF-PH protocol, which employs 2-acetylaminofluorine implantation to prevent proliferation of resident hepatocytes followed by a 70% partial hepatectomy.\textsuperscript{13-14} Approximately 11 days post-PH a large number of oval cells can be seen infiltrating the periportal regions of the liver; these cells further give rise to the hepatocytes and cholangiocytes that restore the liver mass by 22 days after the resection.

Morphologically, oval cells are small in size (approximately 10 µm), with little cytoplasm and an ovoid nucleus that lends them their name. Oval cells possess characteristics similar to ductular cells in their distinct isoenzyme profiles, expressing markers such as OV-6, α-fetoprotein (AFP), and CK19.\textsuperscript{15-16} They are capable of generating both hepatocytes and bile duct cells, thereby qualifying them as bipotential progenitor cells in adult livers.\textsuperscript{17-18}

In the current study we examine the potential role of Notch signaling in the regulation of oval cell-mediated liver regeneration. We have demonstrated induction of oval cells at day 11 post 2AAF-PH as well as a simultaneous increase in Notch1 expression. Combining the 2AAF-PH model with treatments of the γ-secretase inhibitor GSI XX during the peak of the oval cell response terminated the Notch signal, as evidenced by a decrease in expression of downstream effector Hes1. Inactivation of Notch signaling led to a stunted differentiation response by the activated oval cells. This is demonstrated morphologically as well as by aberrant expression levels of such markers as OV-6, AFP, and CK19 at day 22 post-PH. Inhibition of Notch further resulted in an abnormal hepatocellular mitoinhibition index (p21\textsuperscript{Waf1}:Ki67) at day 22 post-PH. The hepatocytes generated at this timepoint appear to be functionally impaired, with decreased levels of CYP3A2 and Glucose-6-Phosphatase-(G6Pase)-α. Taken together these data
demonstrate an important regulatory role for Notch signaling in the proper differentiation and growth of oval cells during 2AAF-PH.
2.1 The Liver

2.1.1 Gross Anatomy

In an untreated “normal” adult rat the liver accounts for approximately 5% of total body weight, or about 7-8 grams, and is comprised of five liver lobes. In humans, the liver is comprised of only four lobes and makes up only 2% of total body weight, or 1400-1600 grams. In order to maintain its highly vascular parenchyma, the blood is supplied with blood from both the portal vein and the hepatic artery.\textsuperscript{18,9} The portal vein supplies the liver with approximately 60% of the incoming blood; although the venous blood is oxygen poor, it is extremely rich in nutrients derived from the direct draining of intestinal epithelia. The remaining 40% of oxygen-rich blood is provided by the hepatic artery. Bile is transported from the liver into the duodenum via the biliary tree, which lies structurally parallel to the blood vessels but flows in the opposite direction. This trifecta of portal vein, hepatic artery and bile duct form the vascular bundle more commonly referred as a portal triad.\textsuperscript{18,19}

2.1.2 Hepatic Microarchitecture

The liver is essentially comprised of hepatic lobules that surround terminal central veins and are outlined by portal triads (Figure 2-1A).\textsuperscript{20} Hexagonal columns of hepatocytes are arranged in cords and radiate from the central vein toward the portal triad.\textsuperscript{21} Each cord of hepatocytes is lined with sinusoids composed of endothelial cells, and it is here that the micro-vascular system of the liver is located. Briefly, blood flows from the portal triads through the parenchyma, coming into direct contact with each hepatocyte contained in that cord and eventually drains into the central vein (Figure 2-1B).\textsuperscript{20} Hepatocytes within the cords produce metabolites which are
excreted via bile canaliculi into the Canal of Hering, a terminal portion of the biliary tree located within the portal triad.\textsuperscript{18,21}

The liver consists of a various group of cell types including hepatocytes, bile ductular epithelial cells, stellate cells, sinusoidal and vascular endothelial cells, and liver specific macrophages known as Kupffer cells.\textsuperscript{19} Hepatocytes encompass 90% of the liver weight and carry out the biochemical functions of the liver as well as the production of bile, arguably the most important function of the liver. Hepatocytes are polygonal in shape, approximately 30-40\textmu m in size, and have a high abundance of smooth and rough endoplasmic reticulum.\textsuperscript{21}

The liver acinus is described as the functional unit of the liver, divided into three zones that are comprised of the hepatocytes that extend from one central vein to the next. Zone 1 includes hepatocytes surrounding the portal triad and receives the greatest concentration of nutrients; Zone 2 is comprised of interzonal hepatocytes, and Zone 3 consists of the poorly oxygenated hepatocytes nearest to the central vein.\textsuperscript{19} Within the liver acinus blood flows through the sinusoids from Zone 1 to Zone 3, while the bile moves from Zone 3 to Zone 1. Interestingly, hepatocytes within Zone 3 have an increased DNA content (4N-16N), predominant bi-nucleation, larger size, and can undergo centrilobular necrosis. Conversely, hepatocytes within Zone 1 are smaller and have normal DNA content (2N).\textsuperscript{21}

\subsection*{2.1.3 Cell Types of the Liver}

The muralium simplex, an arrangement of liver plates, is built up by polygonal parenchymal cells or hepatocytes, which enclose a chicken-wire-like network of bile canaliculi in the central plane, and are flanked by sinusoids on both sides of the muralium. Because the hepatocyte is a highly metabolically active cell it contains large numbers of a variety of organelles: 15\% of the cell volume is for endoplasmic reticulum; it contains over 1,000
mitochondria, about 300 lysosomes, equal numbers of peroxisomes, some 50 Golgi complexes per cell, and a typically organized cytoskeleton.\textsuperscript{18}

Bile duct cells (sometimes referred to as bile duct epithelial cells or BECs) are known to line the system of channels contained in the liver for the drainage of bile, the exocrine secretion of hepatocytes. The most striking differences with hepatocytes include smaller numbers of mitochondria and less extensive endoplasmic reticulum, a more strongly developed network of intermediate cytoskeletal filaments (cytokeratins), and the presence of a basement membrane. BECs are also known to express several markers that are absent from hepatocytes, the most popular of which include cytokeratins 7 and 19 and $\gamma$-glutamyltranspeptidase. BECs modify the composition of bile by secretion of water, proteins, and bicarbonate, and reabsorption of glucose, glutamate, anions, and proteins. BECs have been shown to participate in the immune response by actively secreting certain cytokines, and are also the targets of autoimmune inflammatory destruction.\textsuperscript{18}

Kupffer cells are stellate in shape and attached to the sinusoidal wall, but may also be seen migrating along the lumen. These cells function as “garbage collectors”, storing all kinds of old, unnecessary, damaged, altered or foreign material. When Kupffer cells are activated, say by zymosan or endotoxin uptake, they secrete a variety of factors including proteases and cytokines that influence cells of the parenchymal lining as well as others. The relationship between Kupffer cells and immunologically involved-dendritic cells needs further investigation.\textsuperscript{18}

The term hepatic stellate cells is a consensus term to replace the confusing terminology of the past (para-or perisinusoidal cells, Ito cells, fat storing cells, vitamin A storing cells, lipocytes, etc.). Stellate cells occupy a perisinusoidal location, with cytoplasmic extensions wrapped around the sinusoidal endothelial lining, comparable to pericytes in other locations. Stellate cells
are involved in extracellular matrix (ECM) maintenance via their synthesis and secretion of normal components, as well as their degradation by proteases. Thus hepatic stellate cells have four major functions in a normal liver: (a) production and maintenance of ECM; (b) control of microvascular tone; (c) storage of retinoids; and (d) a role in control of regeneration in the normal liver and in response to necrosis.18

2.2 The Functions of the Liver

2.2.1 Metabolic Homeostasis

The liver performs a plethora of functions designed to maintain homeostasis within the body. For instance, one of the main sites of glycogen storage in the body is the liver. Carbohydrate metabolism, mediated through gluconeogenesis, glycogenolysis and glycogenesis allows the liver to effectively control the concentration of glucose in the blood. However it is not only metabolism of carbohydrates that allows the liver homeostatic control; it plays a major role in protein and lipid metabolism (i.e. cholesterol synthesis/breakdown, lipogenesis) as well. The liver is also responsible for the production of such molecules as albumin (the major osmolar component of the blood), as well as prothrombin, fibrinogen, and non-immune α- and β-globulins.21

2.2.2 Molecular Silo

The liver is responsible for the uptake, storage and utilization of several important vitamins from the bloodstream. For example, the liver stores and converts vitamin D into its circulating form for further modification in the kidney. Vitamin A is stored within the lipid pools of the stellate cells of the liver. Furthermore, vitamin K is utilized by the liver for the production of clotting factors, and decreases in hepatic vitamin K have a legitimate connection to bleeding and/or clotting disorders. The liver also maintains stores of copper and vitamin B12.21
Due to its large size as well as the intricate vasculature found within it, the liver is the largest blood storage organ in the body. An adult human liver can hold approximately 1500mL of blood, which equates to about 25% of cardiac output per minute. On a related note, the liver is also the main site of iron storage in the body and therefore responsible for the homeostasis of blood iron levels in the body.  

2.2.3 Detoxification

Because at any point in time the liver contains such a large percentage of the total blood in the body, this makes it well-suited to function as a detoxification center. Enzymes such as alcohol dehydrogenase (ADH), cytochrome-P (CYP) and uridine glucuronosyltransferase (UGT) allow for the modification of chemical composition of several compounds, such as antibiotics, and their subsequent removal. Furthermore, the ability of the liver to convert nonhydrophyllic drugs to a more water-soluble form aids in their excretion by the kidneys.

2.2.4 Production of Bile

Arguably the most important function of the liver is the production of bile. Bile is mostly comprised of conjugated bilirubin, bile salts, phospholipids, cholesterol, bicarbonate and water. Bile salts mix with ingested fats to promote absorption of nutrients from the gastrointestinal tract. After its formation in the liver, bile flows into the hepatic duct on through to the common bile duct, which then enters the duodenum. When food in the gastrointestinal canal enters the duodenum, it stimulates contraction of the gall bladder and further release of the bile into the small intestine.
2.3 Hepatic Regeneration

2.3.1 General Considerations

The liver is one of the few organs that are known to be capable of rapid regeneration. This process usually takes place in response to liver injury resulting from surgical resection or exposure to destructive agents. Although this ability is referred to as regeneration, what is actually occurring is a process of compensatory growth or hyperplasia. Instead of the resected liver tissue growing back, the portions of the liver remaining after (e.g.) a two-thirds or partial hepatectomy (PH) increase in size to make up for the loss of tissue. This expansion continues until the mass of the “regenerated” liver reaches within 10% of the original liver mass, give or take. Completion of this process usually takes approximately 2 weeks in rodents, but can take up to 2 months in humans. While the original mass of the liver may be restored, the original anatomical form of the liver is not, indicating that regenerative growth after PH is a closely regulated process synergistic with the bodily demand for liver functionality independent of its anatomical form. The process of compensatory hyperplasia is demonstrated graphically in Figure 2-2.

2.3.2 Models of Regeneration

At any given time, under normal conditions, only 1 in every 20,000 hepatocytes is undergoing mitotic division; however, during conditions of regeneration hepatocytes are the driving force behind the compensatory hyperplasia exhibited. Figure 2-3 demonstrates graphically the number of resident hepatic cells within the cell cycle during the time following partial hepatectomy. In the rat, hepatocytes first move from G0 resting phase of the cell cycle into G1, as mediated by cyclin D signaling within the first 15 hours after partial hepatectomy. Periportal hepatocytes are the first to undergo DNA synthesis, and eventually the hepatocytes proximal to the central vein begin to also proliferate. A large peak of DNA synthesis occurs
24 hours after PH with another smaller peak occurring 48 hours post-PH, reflecting the synthesis of DNA by non-parenchymal cells and pericentral hepatocytes. Unlike hepatocytes, whose proliferative capacity post PH spreads from periportal to pericentral, NPCs across the hepatic lobule exhibit simultaneous DNA synthesis.\textsuperscript{25}

Several animal models incorporating the insult to the liver via chemical have been developed to study mechanisms regarding the proliferative response to liver injury. Among the most extensively utilized agents are carbon tetrachloride (CCl\textsubscript{4}), which causes necrosis of the centrilobular regions of the liver, and allyl alcohol (AA), which causes periportal necrosis.\textsuperscript{27-29} In both models, regeneration of the necrotic area is mediated by hepatocyte proliferation elsewhere in the lobule. In addition to the hepatocyte capacity for regeneration of the liver lobule, there is also a stem cell compartment that acts as a backup regeneration system. Activation of this compartment only occurs when the hepatocytes are functionally compromised and/or unable to divide. In this model of liver regeneration, a transit-amplifying population of stem cells known as oval cells proliferates within the compartment to reconstitute the liver mass. If hepatocytes are not compromised, the oval cell contribution to the regeneration process is negligible. Although oval cells have been widely studied, their origins are still controversial and several key questions regarding activation of this cell population as well as direct lineage specification remain.

\textbf{2.4 Liver Stem Cell Biology}

\textbf{2.4.1 The Oval Cell}

In normal liver tissue, oval cells numbers are so small that they are almost beyond detection; however, stem cell activation leads to the profuse replication of these cells in the periportal regions of the liver. Morphologically, oval cells are small in size (approximately 10 \textmu m), with a large nuclear to cytoplasmic ratio and an ovoid nucleus, thereby giving them their
name. Oval cells possess characteristics similar to ductular cells in their distinct isoenzyme profiles, expressing markers such as cytokeratin 19 (CK-19) and γ-glutamyl transpeptidase (GGT), and also have been shown to express α-fetoprotein (AFP).\textsuperscript{12,15} They are capable of generating both hepatocytes and bile duct cells, thus qualifying them as bipotential progenitor cells in adult livers.\textsuperscript{17,30} Characterization of these progenitors can be achieved via the utilization of monoclonal antibodies such as OV-6 and Thy-1.\textsuperscript{31} Figure 2-4 demonstrates immunohistochemical analysis of the oval cell response at day 11 of the 2AAF-PH protocol.

2.4.2 Canals of Hering: Putative Oval Cell Niche

In tissues that continuously proliferate (i.e. skin, intestinal epithelia, etc), the stem cell population tends to produce progeny ad infinitum; as these cells begin to mature toward a specific lineage, they begin to lose the ability to constantly replicate. Among organs, the liver is unique in that there are two pools of cells from which progeny can be produced: the intrahepatic stem cells, which actually act as a secondary mechanism for proliferation, and a pool of highly differentiated cells that act as the primary source for proliferating cells.\textsuperscript{12} The prevailing model localizes the hepatic stem cell niche to the canals of Hering (terminal bile ductules). These cells are components of a functional segment of the biliary system\textsuperscript{32,33} and not part of a separate compartment of proliferative cells such as basal cells in the skin or the gastro-intestinal tract, which continuously generate lineage.

Interestingly, it has been shown that a small percentage of oval cells may in fact arise from within the bone marrow and may traverse to the liver upon migratory signals.\textsuperscript{15} Furthermore, several other investigators have confirmed that bone marrow derived cells possess the capability to produce both functioning hepatocytes and bile duct cells. While there are studies that
demonstrate the existence of oval cells within the hepatic architecture adjacent to bile duct epithelium, the precise oval cell niche has yet to be elucidated.

2.4.3 Oval Cell Plasticity

One of the key factors in determining the developmental potential of a stem cell is its environment. It has been demonstrated in a rat model that HSCs can give rise to oval cells, which retain hematopoietic markers such as Thy-1 but also gain expression of liver-specific markers such as AFP. Scientists have found that HSCs obtained from adult peripheral blood retain a tremendous developmental plasticity. Taken together this indicates that HSCs and oval cells may share a common developmental origin or may even arise directly from the same cell. Figure 2-5 is a schematic diagram of the potential endpoints of oval cell differentiation according to a recent review by Lowes et al. Regardless of origin, all stem cells execute their developmental programs by regulating gene expression. Determining which signals are responsible for such processes as induction of differentiation, self-renewal and/or maintenance of pluripotentiality will lead to a broader and much better understanding of the biology of oval cells and their role in the liver.

2.4.4 Oval Cell-Mediated Liver Regeneration

There have been many studies looking into the possibility that hepatic progenitor cells can modulate the regeneration process; however, no experimental evidence exists to confirm this hypothesis. In fact, based on data that has been published over the last 100 years or so, hepatocytes irrefutably are the cells responsible for regeneration after partial hepatectomy. However, in the last 10 years a clear distinction has been made between regeneration after PH alone and regeneration after PH combined with a chemical injury. Though oval cells do not normally participate in the regenerative response to PH or chemical insult, they can be induced to do so by suppressing mature hepatocyte proliferation. Administration of 2-acetylaminofluorine...
in addition to injury via PH or CCl₄ blocks hepatocyte proliferation by interfering with their ability to divide properly.

Oval cell replication is important in many models of liver injury including carcinogenesis induced by azo-dyes and choline deficient/ethionine-containing diets (CDE diet), injury caused by d-galactosamine, and injury produced by 2-aceylaminofluorene (2AAF), dipin or CCl₄ treatment in combination with PH.³⁶,³⁷ In fact, oval cells may constitute more than 50% of the liver during regeneration caused by administration of 2AAF/PD; it is thought that the cells form a transit amplifying compartment that includes undifferentiated progenitors, medially-differentiated transit cells, and newly differentiated hepatocytes.¹²

It has been shown that growth factors (i.e. TGF-α, EGF, HGF, and SDF) can stimulate oval cell growth³⁸, and in fact, oval cells can produce and respond to several of their own cytokines. It has also been shown that non-parenchymal cells (NPCs), such as stellate cells, can enhance oval cell growth and differentiation via secretion of growth factors, and also by direct cell-cell interactions.³² Oval cells are similar to hepatocytes in that both require growth factors for cell cycle progression, and both cell types also require a ‘priming’ process in order to respond to these stimuli³⁹; however, global gene expression patterns, as well as expression of specific markers, tend to differ between the two cell types. For example, expression of genes related to the interferon-gamma signaling network is greatly increased in oval cells when they are mediating liver regeneration, but these genes are not activated in the regenerating liver after PH alone³⁹. Table 2-1 shows the variable expression of protein markers across the hepatic cell lines.
2.4.5 Clinical Applications for the Oval Cell

The existence of a hepatic stem cell compartment gives rise to expectations regarding its clinical applications. With the increasing interest in characterizing oval cells with respect to their origins, questions arise regarding the mechanism of their recruitment and their differentiation potential. Understanding and identification/isolation of these cells in humans may hold promise for new therapeutic treatments to a wide range of liver pathological conditions ranging from congenital metabolic diseases, end-stage liver cirrhosis, and hepatocarcinogenesis.

Two distinct obstacles must be overcome for oval cells to be considered for clinical application. Maintenance of oval cells in an undifferentiated state in culture has surfaced as the first major hurdle. Developing this technique is critical, because any therapeutic use of these cells will require the expansion of a small population of cells \textit{ex vivo} prior to transplantation. The second impediment has been selectively directing the differentiation of oval cells down a hepatocyte or cholangiocyte committed pathway as needed. It is anticipated that the signals mediating these differentiation processes will be sufficiently complex as to disallow their exact replication \textit{in vitro}. Factors governing oval cell differentiation may include contact-dependence, ECM contact, or exposure to soluble signaling proteins in the serum. To circumvent the need for overcoming this second hurdle, cells could be transplanted in their precursor form and the natural microenvironment of the liver and used to dictate their differentiation, but this theory has yet to be validated.

An avenue for the clinical use of stem cells presents itself with the advent of bioengineered organs and/or tissues. The development of tissue scaffolds for the seeding of stem cells has immense potential, but until recently the clinical applications of these scaffolds have been limited. The most clinically relevant engineered tissue has been cartilage.\textsuperscript{40} The injection of tissue engineered cartilage into osteoarthritic as well as nonarthritic knees and other joints were
reported to have greatly improved joint stability and motion; however, further long-term studies must be made to determine the stability and long-term effects of these grafts.\textsuperscript{41} The growth of autologous cells on decellularized human heart valves and subsequent implantation of these valves has also been clinically worthwhile.\textsuperscript{42} Another engineered tissue that had been evaluated in a clinical study was the bladder. Here, patients received bladders engineered with autologous urothelial and muscle cells; these patients demonstrated clinical benefits from the implanted tissue for up to five years post-implantation.\textsuperscript{43} The successes seen with bladders, heart valves and cartilage demonstrate the endless possibilities for the clinical use of stem cells.

\textbf{2.4.6 Liver Stem Cells and Hepatocellular Carcinoma}

It has been shown that human hepatic stem cells may be able to give rise to hepatocellular carcinoma (HCC) as well as cholangiocarcinomas (CCC).\textsuperscript{33,44} In these models, a periportal population of small “primitive” oval epithelial cells proliferate either in association with or before hepatocyte multiplication. Multiple studies have demonstrated that a substantial number of HCCs contain a progenitor cell population. Furthermore, detailed immunophenotyping of HCCs indicated that 28\% to 50\% of HCCs express markers of progenitor cells such as CK7 and CK19. These tumors also consist of cells that have an intermediate phenotype between progenitors and mature hepatocytes. In fact, HCCs that express hepatocyte and biliary cell markers such as albumin, CK7 and CK19 carry a significantly poorer prognosis and higher recurrence after surgical resection.\textsuperscript{45}

One of the goals in this type of research is the identification of markers specific to cancer stem cells in the liver as well as other organs. However, the problem with this lies with the ability to isolate these cells from normal liver or from HCC samples, and the subsequent functional and molecular characterization. Strict double and triple immunohistochemical and confocal labeling is necessary to identify marker-positive stem cells. Such rigorous labeling,
however, has not been consistently adhered to. Furthermore, definitive experiments showing serial transplantability of marker-positive-cells has yet to be demonstrated. Moreover, it is difficult to identify gene products that are specifically associated with putative LPCs or with HCC. The challenge lies in defining the markers specific to these cells at varying stages of differentiation, in HCC, and the elusive liver cancer stem cell.  

2.5 The Notch Signaling Pathway

2.5.1 General Considerations

Cellular differentiation during development is mediated via signals that are produced by adjacent tissues. Often these various signals are received simultaneously by the cell simultaneously, and therefore they must be integrated in order for the correct specification of cell fate to occur. The Notch family of proteins consists of an evolutionarily conserved group of proteins that serve two functions, the first being that of cell-surface receptors, and the second being as direct transcriptional regulators.1 This family of proteins thereby represents a distinct channel for signal transduction from the cell surface to the nucleus, and also allows for cells to directly influence gene expression in those adjacent to them.

Usually, activation of the Notch pathway inhibits differentiation via transcriptional repression of genes specific to a certain cell lineage, thereby limiting the number of cells that will assume a certain lineage and leaving a small number of uncommitted progenitors that are able to adopt alternative cell fates. During development activation of the Notch cascade is essential, and global expression of Notch genes is continuously altered, allowing for Notch influence on cell fate decisions in tissues throughout the body.4
2.5.2 A Brief History

Much of what is now known and understood about the Notch gene and its signaling cascade could not have been determined without studies performed in the *Drosophila melanogaster* model. The Notch gene was “discovered” during genetic experiments in which several different phenotypic variations were noted, one of which exhibited a “notch” in the wing of flies. These “notched” flies carried a haploinsufficiency for that particular gene. The gene was first successfully identified and cloned in the early 1980s by Artavanis-Tsakonas {Artavanis-Tsakonas, 1983}.47

Throughout years of study in *Drosophila*, Notch was found to encode a developmentally conserved transmembrane receptor that is expressed in both embryonic and adult cells. This transmembrane receptor and its ligands are part of a signaling cascade in which a family of basic helix-loop-helix (bHLH) transcription factors regulates the expression of a myriad of other genes.48

2.5.3 Structure of the (Mammalian) Notch Gene

The human Notch receptor family is comprised of four members (Notch1-4), each of which shares a high degree of structural homology with the rest. This homology extends across species as diverse as *C elegans* and *D melanogaster* as well as *Mus and Rattus*.

In general, the extracellular domain of Notch-1 and Notch-2 is comprised of 36 EGF-like repeats and 3 membrane proximal Lin-12/Notch/Glp-1 (LNG) repeats. Notch-3 and Notch-4 have 34 and 29 EGF-like repeats, respectively. The intracellular domain of the Notch receptor, or NICD, consists of a RAM domain, 6 ankyrin (ANK) repeats, 2 nuclear localization sequences (NLS), a transcriptional activator domain (TAD) and a praline-glutamine-serine-threonine (PEST)-rich domain, with the exception of Notch-4. Notch-4 has a shortened NICD due to the lack of NLS. The RAM domain is the primary binding site for RBPjκ, a homolog of *Drosophila*
Su(H). The ANK repeat domain serves as a binding site for regulators of Notch expression such as Deltex, Mastermind, and Numb.\textsuperscript{4}

### 2.5.4 Notch Signal Transduction

The Notch signaling pathway is said to be activated when the extracellular domain of the Notch receptor is bound by its ligand, preferentially Jagged (mammalian homolog to Drosophila Serrate), from neighboring cells. However on the molecular level it has been demonstrated that Notch activation and processing requires three separate cleavage events: the first proteolytic cleavage produces a functional Notch receptor via modification of the EGF-like modules of the Notch extracellular domain in the Golgi, and is performed by a glycosyltransferase known to be a product of the Fringe gene. The resulting receptor unit within the membrane is a non-covalent heteroduplex consisting of a 180kD fragment (the majority of the extracellular domain) and a 120kD fragment (the NICD). This cleavage alters the ability of the receptor to bind certain ligands, thereby effectively regulating ligand specificity.\textsuperscript{4}

The second proteolytic cleavage occurs following ligand binding to the extracellular domain of the receptor, and is dependent on a member of the ADAM metalloprotease family, usually ADAM17.\textsuperscript{6,7} It is possible that this cleavage event is mediated by more than one enzyme, and inspection of protein sequences indicates that this cleavage mechanism may not be tightly conserved between members of the Notch family.

The final cleavage event takes place prior to the signal-generating step in the Notch cascade in the transmembrane portion of the receptor, and releases a soluble NICD to translocate to the nucleus. Presenilin proteins, specifically 1 and 2, associate with the $\gamma$-secretase enzyme responsible for the cleavage, and are also thought to be required for the event to occur.
However, it should be noted that results obtained by several studies have suggested that nuclear translocation may not be required for Notch function in all systems.\textsuperscript{49,50}

Upon entry to the nucleus the NICD plays a role in transcriptional regulation of its target genes which include basic helix-loop-helix transcription factors. This is done largely through the binding of the NICD transcriptional regulator C promoter binding factor (CBF)-1/recombination signal sequence binding protein (RBP)-J\kappa, which allows for transcription to take place.

### 2.5.5 Effectors of the Notch Cascade

Other than the Notch receptors, the main constituents of the signaling cascade in mammals include extracellular ligands such as Jagged, Delta, and the Delta-like family of proteins (\textit{Dll1}, \textit{Dll3}), and also intracellular effectors such as CBF-1/RBP-J\kappa and the HES (Hairy/Enhancer of split) proteins.\textsuperscript{4} Other effectors, targets, and modulators of Notch have also been evolutionarily conserved. Generally, extracellular ligands are referred to as the DSL ligands, while the intracellular effectors are referred to as the CSL ligands.

In the dominant model of Notch signaling, activation of the receptor via binding of DSL ligands results in a proteolytic cleavage event with subsequent release and nuclear translocation of the NICD. Interaction of the NICD with CSL effectors induces transcriptional activation of the HES genes, which function as negative regulators of lineage-specific gene expression.\textsuperscript{4} In the nucleus, the NICD can interact with both cytoplasmic and nuclear proteins, allowing for signal transduction through either the CSL ligands or independently of them. CSL-independent signaling via molecules such as Deltex also leads to transcriptional regulation of distinct target genes.\textsuperscript{4}

Other modulators of the pathway include Deltex, a cytoplasmic Notch binding protein that is associated with CBF-1-independent signaling. Downregulation of the Notch pathway can be
mediated by such enzymes as ubiquitin ligase via its stimulation of phosphorylation-dependent-ubiquitinazation and subsequent degradation of the NICD by the proteosome. Other molecules such as the transcriptional activator Mastermind can regulate phosphorylation and turnover of the NICD as well.51

2.5.6 Hepatic Notch Expression

Transcripts for all of the Notch receptors as well as their ligands Jagged1 and Delta1 can be detected in normal liver, as well as during several models of regeneration. In normal adult rat liver, Notch1, Notch2, Notch3 and Jagged1 expression can be seen on bile ductular cells whereas Notch2, Notch3 and Delta1 can be found on normal adult rat hepatocytes.52

Expression of pathway members has also been examined during regeneration using the standard model of ⅔ partial hepatectomy (without 2AAF).53 Baseline expression of Notch and Jagged in the different liver cell types reveals widespread expression, with higher levels in the hepatocytes and biliary epithelium, but also expression of Notch1 on endothelial cells of the sinusoids and small vessels. These findings are comparable to what has been described in human liver.54,55

2.5.7 Functions of Notch Signaling

When it comes to cellular specification, there are three choices available: differentiation, cell cycle progression and apoptosis, and Notch signaling is capable of regulating cell fate decisions for all three processes.56 It has been shown that targeted disruption of Notch-1, Notch-2, Jagged-1 or Jagged-2 genes in mice leads to severe developmental defects or embryonic lethality.2 Expression of constitutively active forms of Notch receptors inhibits or delays terminal differentiation in vitro in many cellular models.2-4

On the other hand, it has been illustrated that Notch-1 is needed for differentiation in a number of cell types such as adipocytes,57 erythroid cells,58 myeloid cells,4 T-cells,59 murine60
and human keratinocytes and many others. In summary, studies by and large have demonstrated that in many circumstances, signaling via the Notch pathway inhibits cellular differentiation toward primary cell fates while generally permitting cells to respond to secondary cellular specification.

2.5.8 Notch and Wnt: A Delicate Balance

Cellular responses to internal and external stimuli are regulated by numerous positive and negative feedback loops, both within individual signaling cascades and also between them. Accordingly, pathway crosstalk is a very complex yet highly utilized feature required for the molecular regulation of cell homeostasis and adaptation, where modulation of one pathway can affect multiple regulatory circuitries. Considering that precise signaling strength is important for productive cell responses, it is not surprising that change in the major biochemical pathways and in pathway crosstalk manifests within many diseases that typically accompany human aging.

Notch-Wnt interactions are implicated in several developmental stages, such as somitogenesis and hematopoiesis (Wnt activates Notch signals for self-renewal in hematopoietic stem cells), as well as adult intestinal and skin regeneration. Additionally, one of the major components of Wnt pathway, GSK3β, is also a crosstalk integrator of multiple signal transduction networks. For example, recently published work suggests that a balance between Notch and Wnt signaling controls cellular homeostasis during the regeneration of adult skeletal muscle. Namely, Notch promotes the proliferation of myogenic progenitor cells, and inhibits their precocious terminal differentiation by inhibiting Wnt via GSK3β activation. Conversely, during later stages of muscle repair and regeneration, Wnt inactivation of GSK3β thereby promotes myoblast and myotube differentiation.
2.5.9 Notch-Induced Pathologies

2.5.9.1 Cerebral Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy (CADASIL)

CADASIL is an inherited degenerative vascular disorder that is caused by mutation in the Notch3 gene. It affects vascular smooth muscle cells, and is the most common genetic form of stroke and vascular dementia. Affected individuals exhibit a variety of symptoms, including migraines, mood disorders, recurrent subcortical ischemic strokes, progressive cognitive decline, dementia and premature death. One of the most distinguishing features of the disease is the accumulation of granular osmophillic material (GOM) within the smooth muscle cell basement membrane, but the disease is further characterized by progressive degeneration of vascular smooth muscle cell. 68

All Notch3 mutations associated with CADASIL result in a gain of loss of a cysteine reside in one of the 34 EGF-like repeats in the extracellular domain of the Notch3 receptor. The characteristic nature of these mutations, in addition to the absence of any examples of in CADASIL patients of mutations or deletions of the Notch3 gene that are obviously inactivating, strongly suggesting that mutations that cause CADASIL are not Notch3-null alleles. In CADASIL patients the ectodomain of the Notch3 protein accumulates in the cerebral microvasculature at the cytoplasmic membrane of vascular smooth muscle cells. 69

No specific treatment for the disorder is available; however anti-platelet agents such as aspirin or Plavix may slow down the disease and help prevent strokes. Given the propensity for cardiovascular and cerebrovascular complications, minimizing vascular risk factors and implementing therapy for primary or secondary prevention of stroke and myocardial infarction seems prudent. 69
2.5.9.2 Spondylocostal Dysostosis

Two of the five known human Notch ligands have been shown to develop mutations that lead to developmental disorders in man. Lesions in the human Delta-like-3 gene give rise to spondylocostal dysostosis (SD), a group of vertebral malsegmentations syndromes resulting from axial skeletal defects. SD is a disorder with autosomal-dominant and autosomal-recessive modes of inheritance and is characterized by multiple hemivertebrae, rib fusions, and deletions with a nonprogressive lateral curving of the spine. In patients with SD, two mutations in the human Delta-3 gene were identified within the conserved extracellular domain and are predicted to give rise to truncated proteins. A third missense mutation present in a highly conserved glycine residue of the fifth epidermal growth factor-like repeat highlights the functional importance of this domain.  

Notch signaling plays a major role in regulating somite formation and in partitioning somites into anterior and posterior compartments. Notch pathway components appear to regulate, or constitute essential components of, a cell autonomous oscillator functioning in the presomitic mesoderm. This oscillator has been termed the somite clock. Analysis of marker gene expression in Dll3 mutant mice indicates that progression of the somite clock is disrupted in these mutations. A similar mechanism probably underlies the vertebral defects observed in patients with SD.  

2.5.9.3 Alagille Syndrome

Mutations in the second human Notch ligand, Jagged1, lead to Alagille Syndrome, or AGS. Also referred to as arteriohepatic dysplasia, AGS is an autosomal dominant developmental disorder that mainly affects structures in the liver but also heart, skeleton, eyes, face, kidney and other organs. It occurs with a minimal estimated frequency of 1:70,000 live
births when ascertained by the occurrence of neonatal jaundice, but the incidence of the disease is likely higher. The syndrome has traditionally been defined by a paucity of intrahepatic bile ducts in association with five main clinical abnormalities: cholestasis, cardiac disease, skeletal abnormalities, ocular abnormalities, and a characteristic facial phenotype. 69

As a result of the paucity in bile ducts the patients will usually develop cholestasis, and 30% of patients will require a liver transplant. The pathogenesis of bile duct paucity in AGS is unknown. Intralobular bile duct development and number is normal or even increased around the time of birth, even in some patients who go on to develop profound paucity later in infancy. The bile ducts that are present are histologically and immunohistochemically normal. Furthermore, several groups have reported a reduction in the number of portal tracts in patients with AGS, suggesting that the development of the portal tract or ductal plate may be abnormal as well. The Notch-Jagged interactions may be involved in the development of the portal tract itself along with the bile ducts within it. 73

Analyses of many patients with cytogenetic deletions or rearrangements have mapped the gene to chromosome 20p12. The initial association of deletions with a multisystem disorder led to the belief that AGS may be a contiguous gene deletion syndrome; however the frequency of observable deletions is actually low, suggesting that the disorder may result from mutations in a single gene with pleiotropic effects. 74

The prognosis for Alagille syndrome is variable, and depends on the severity of liver, cardiac, or renal disease. Up to 50% of children may regain normal liver function by adolescence whereas others may develop renal or liver failure or manifest significant cardiologic or neurological disease. Liver transplantation is indicated for the development of
cirrhosis and decompensated portal hypertension. Liver transplantation may be contraindicated because of severe inoperable cardiac disease.\textsuperscript{74}

2.5.10 Notch and cancer

Although it has been known for more than a decade that Notch can function as an oncogene, the mechanisms by which this occurs and the subsequent effects have not been very well defined. Nonetheless, the consequences of aberrant Notch signaling that have been shown to date are very interesting indeed. A few of these include the phosphorylation of PI3K (phosphatidylinositol 3-kinase), activation and consequent induction of ERB2, and induction of NF-kB2 expression.\textsuperscript{60,75,76} These target proteins may confer: resistance to anoikis and p53-mediated apoptosis, stimulation of proliferation and growth of cells (ERB2), and induction of genes that encode anti-apoptotic proteins (NF-kB2).\textsuperscript{77-79}

While Notch signaling alone certainly contributes to tumorigenesis, actually causing cancer requires cooperation with another oncogene, usually one that is able to override the G1/S checkpoint. For instance, simultaneous expression of Notch with proteins such as adenovirus E1A, papillomavirus E6 and E7, SV40T, Ras and Myc has been shown to induce transformation \textit{in vitro}.\textsuperscript{80-82} The feature that all of these proteins have in common is their ability to override the G1/S checkpoint. Since Notch does not possess this capability, it is likely that Notch signaling functions in another oncogenic capacity, such as transferring resistance to apoptosis, anoikis or differentiation.

As Notch can exert tumor-suppressive and differentiation-promoting activities, it could be suggested that tumor progression might select for cells that are refractive to Notch signaling in tissues in which absence of this pathway promotes growth, enhances survival and/or inhibits differentiation. It is possible that in such tissues, late-stage tumor cells might counter-select
against expression of Notch receptors or ligands to escape from differentiation and cell-cycle
arrest.\textsuperscript{83}

\textbf{2.5.10.1 Notch as an oncogene}

One of the most clearly defined pro-transforming roles for Notch is in T cell acute
ymphoblastic leukemia/lymphoma (T-ALL). A small fraction of human T-ALL is associated
with a t(7:9) translocation that breaks the Notch1 gene and fuses its 3’ end to enhancer sequences
of the T cell antigen receptor β subunit (TCR-β), which drives the expression of a series of
truncated mRNAs. These mRNAs encode several constitutively active t(7:9)-specific Notch1
polypeptides.\textsuperscript{84}

Notch4 was discovered in DNA isolated from a murine mammary tumor adjacent to the
site of a murine mammary tumor virus (MMTV) proviral insertion that truncates that Notch4
gene and drives the expression of constitutively active forms of Notch4. These transcripts are
similar to Notch1-IC proteins that are expressed in T cell leukemia. In transgenic mice
expressing activated Notch4, breast cancer development is preceded by perturbed ductal
development in non-pregnant mice and alveolar/lobular development in lactating animals.\textsuperscript{85}

Further work has shown that Notch4-associated breast cancer cell lines depend on Ras for
anchorage-independent growth. At present, there are no definite data supporting a role for
Notch4 (or other Notch variants) in human breast cancer.\textsuperscript{81} However, it has been shown that
Notch1, Notch2 and certain Notch ligands are highly expressed in human colon
adenocarcinomas.\textsuperscript{86} As Notch signaling can maintain cells in proliferative and undifferentiated
state, it is thought that its role in cancer is to prevent neoplastic cells from responding to
differentiation cues in their immediate environment.\textsuperscript{87}
2.5.10.2. Notch as a tumor suppressor

The largest amount of data pointing to Notch as a tumor suppressor has been obtained from skin. Experiments performed both *in vivo* and *in vitro* indicate that Notch1 signaling induces differentiation in mammalian skin. Notch1 signaling in murine keratinocytes stimulates expression of early differentiation markers such as keratin1 and involucrin. In addition, Notch1 signaling increases expression of WAF1, which causes cell cycle arrest in basal cells, to allow the onset of terminal differentiation. Jagged1-mediated Notch signaling in keratinocytes also results in activation of NFκB and the onset of terminal differentiation. Also, activated Notch2 can decrease AP1 activity via repression of c-Fos expression and a concomitant increase in FRA1, resulting in inhibition of cellular proliferation. So the tumor-suppressive activity of Notch1 might be mediated by several routes, to induce cell cycle arrest and differentiation.

Furthermore, mice with Notch1-deficient epithelia develop spontaneous basal-cell-carcinoma-like tumors over time. Moreover, Notch1 deficiency in the skin facilitates chemically-induced carcinogenesis. Interestingly, the absence of Notch1 in this tissue is concomitant with downregulation of Waf1, which might explain the increased sensitivity to chemically-induced carcinogenesis, as Waf1-deficient mice are also more susceptible to chemically-induced tumorigenesis. In mice and humans, this tumor type is frequently associated with aberrant Sonic-hedgehog (Shh) signaling, and absence of Notch1 in the mouse epidermis leads to aberrant expression of Gli2, a downstream component of the Shh pathway.
Figure 2-1. Diagram of hepatic architecture. A. Diagram of a “classic” hepatic lobule. B. Hepatic microarchitecture.
Figure 2-2. Compensatory hyperplasia after $\frac{2}{3}$ partial hepatectomy.\textsuperscript{23}
Figure 2-3. Cellular growth after ⅔ partial hepatectomy.²⁴
Figure 2-4. Oval cell response following partial hepatectomy. A. Hematoxylin and eosin staining of rat liver from day 11 of the 2AAF-PH protocol. The small oval cells (arrows) are situated between the largest hepatocytes surrounding the portal triad. B. Immunohistochemistry for oval cell marker Thy-1 at day 11, showing positivity in the periportal regions of the section. C. Dual immunofluorescence staining from day 11 of the 2AAF-PH protocol. Yellow indicates co-expression of the markers Thy-1 and OV-6, showing oval cells surrounding the portal triad. Magnification 40x.
Figure 2-5. Hepatic oval cell differentiation.
Table 2-1. Cellular markers of the liver. Expression of various protein markers in different hepatic cell lines including oval cells, hepatocytes, bile duct cells and endothelial cells.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Oval Cells</th>
<th>Hepatocytes</th>
<th>Bile Duct Cells</th>
<th>Endothelial Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>CK8</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>_</td>
</tr>
<tr>
<td>CK18</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>_</td>
</tr>
<tr>
<td>CK19</td>
<td>+</td>
<td>_</td>
<td>+</td>
<td>_</td>
</tr>
<tr>
<td>ALB</td>
<td>+</td>
<td>+</td>
<td>_</td>
<td>_</td>
</tr>
<tr>
<td>AFP</td>
<td>+</td>
<td>_</td>
<td>_</td>
<td>_</td>
</tr>
<tr>
<td>GGT</td>
<td>+</td>
<td>_</td>
<td>+</td>
<td>_</td>
</tr>
<tr>
<td>OV-6</td>
<td>+</td>
<td>_</td>
<td>+</td>
<td>_</td>
</tr>
<tr>
<td>OV-1</td>
<td>+</td>
<td>_</td>
<td>+</td>
<td>_</td>
</tr>
<tr>
<td>BDS7</td>
<td>+</td>
<td>_</td>
<td>+</td>
<td>_</td>
</tr>
<tr>
<td>OC.2</td>
<td>+</td>
<td>_</td>
<td>+</td>
<td>_</td>
</tr>
<tr>
<td>OC.3</td>
<td>+</td>
<td>_</td>
<td>+</td>
<td>_</td>
</tr>
<tr>
<td>A6</td>
<td>+</td>
<td>_</td>
<td>+</td>
<td>_</td>
</tr>
<tr>
<td></td>
<td>Mammals</td>
<td>D melanogaster</td>
<td>C elegans</td>
<td></td>
</tr>
<tr>
<td>--------------------------------</td>
<td>-------------</td>
<td>----------------</td>
<td>-----------</td>
<td></td>
</tr>
<tr>
<td><strong>Notch Receptors</strong></td>
<td>Notch1</td>
<td>Notch</td>
<td>Lin-12</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Notch2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Notch3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Notch4</td>
<td></td>
<td>Glp-1</td>
<td></td>
</tr>
<tr>
<td><strong>Extracellular Ligands</strong></td>
<td>Delta1</td>
<td></td>
<td>Lag-2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Delta-like1 (Dll1)</td>
<td></td>
<td>Apx-1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Delta-like3 (Dll3)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Jagged1 (Serrate1)</td>
<td></td>
<td>Serrate</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Jagged2 (Serrate2)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Intracellular Effectors</strong></td>
<td>CBF-1/RBP-Jκ</td>
<td>Suppressor of Hairless [Su(H)]</td>
<td>Lag-1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Deltex</td>
<td>Deltex</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Target Genes</strong></td>
<td>Enhancer of Split [E(spl)]</td>
<td>HES</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>bHLH</td>
<td>bHLH</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Groucho</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Processing Molecules</strong></td>
<td>Kuzbanian</td>
<td></td>
<td>SUP-17</td>
<td></td>
</tr>
<tr>
<td><strong>γ-secretase Complex</strong></td>
<td>Presenelin1</td>
<td>Presenelin</td>
<td>Se112</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Presenelin2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Nicastrin</td>
<td></td>
<td>Aph1,2</td>
<td></td>
</tr>
</tbody>
</table>
CHAPTER 3
SPECIFIC AIMS

Cellular differentiation during development is mediated via signals that are produced by
adjacent or even distal cells. Often these various signals are received by the cell simultaneously,
and therefore they must be integrated in order for the correct specification of cell fate to occur.
The Notch family of receptors and its ligands, Jagged in particular, are believed to be involved in
regulating the delicate balance between terminal cell differentiation and stem cell proliferation.\(^2\)
Notch-4 is thought to be expressed primarily in endothelial cells during development and adult
life.\(^{93}\)

In mice, it has been shown that Notch-1 expression is fairly stable during gestation as well
as after birth, whereas Notch-2 expression peaks at embryonic day 15, at the time when bile duct
differentiation starts.\(^{94}\) Notch-3 and Notch-4 expression are low during embryogenesis, and
usually peak postnatally by 12 weeks of age. It has also been established that Jagged-1 is
expressed during embryogenesis in the ductal plates and after birth in bile ducts, endothelial
cells, and hepatocytes.\(^{94}\)

Although it is clear that the Notch signaling pathway is essential for developmental,
growth, and regenerative processes in the liver, the role of this signaling system in oval cell-
mediated liver regeneration has not been well studied. The studies proposed in this application
have been designed to test the hypothesis that the Notch signaling is critical for the proper
response with regards to proliferation and differentiation during the oval cell-mediated
regenerative response. The specific aims are as follows:
• **Specific Aim I:** To determine if and which isoforms of Notch and Jagged are predominantly expressed during oval cell-mediated liver regeneration.

• **Specific Aim II:** To examine the effects on oval cell-mediated liver regeneration when Notch signaling is downregulated.

### 3.1 Specific Aim I

Although many resident cell types in the liver are known to be activated in response to injury, it has been shown that only certain types of injuries will induce the activation of oval cells. As mentioned previously, while it is suspected that Notch signaling is required for development, growth, and regeneration, the exact Notch receptor-ligand interactions that regulate these processes have yet to be defined in the oval cell. It has been shown that this pathway is activated in the adult liver during regeneration and that it may be essential for the formation of new, differentiated tissue. The well-established protocol of oval cell induction via partial hepatectomy (PH) combined with chemical insult, in this case 2-acetylaminofluorene (2AAF) to inhibit resident hepatocyte proliferation, was employed in a rat model to examine the expression of Notch receptors 1-4 and of Jagged-1 and -2 during the oval cell-mediated regenerative response.

Briefly, animals received an implant of 28-day time release 2AAF pellet. Seven days after 2AAF implantation, animals underwent a 2/3 partial hepatectomy. The peak of the oval cell response to injury is approximately 9-11 days post-PH; around day 13 the oval cells begin to differentiate into small, basophilic hepatocytes, and by 21 days post-PH the liver has resumed its normal architecture and little evidence of the oval cell infiltrate remains. RT-PCR was performed to determine if and which of the 4 Notch and 2 Jagged isoforms were expressed during the oval cell response, and it was found that only Notch-1 and 2 as well as Jagged-1
showed a change in expression as compared to normal liver, while Notch-3, Notch-4, and Jagged-2 did not appear to be expressed. Western blot using an antibody specific to cleaved Notch-1 demonstrated increased activation of the receptor on days 7-9 of the oval cell response, with expression leveling back down to normal by day 21 post-PH. Immunohistochemistry demonstrated evidence for nuclear translocation of the NICD in the oval cells, while the ligand Jagged was localized to the peripheral hepatocytes.

3.2 Specific Aim II

Upon determination of the requirement of Notch signaling for properly mediated regeneration, the next step will be to explore the effects that dysfunction of the pathway would have on the regeneration process. A chemical inhibitor of the Notch pathway was employed in conjunction with the oval cell induction protocol mentioned above to inhibit the signaling cascade and thereby determine their effect on the activation, proliferation or differentiation capacity of oval cells during the regenerative response.

Briefly, the 2AAF-PH oval cell induction protocol mentioned above was also applied here but concomitant with a chemical inhibitor designed to halt transduction of the Notch signal. GSI XX, or dibenzazepine (DBZ), is a potent γ-secretase inhibitor now being tested in the treatment of Alzheimer’s Disease. γ-secretase, along with its partner β-secretase, produces the beta amyloid peptide via the cleavage of its amyloid precursor protein, and inhibition of these enzymes may reduce the burden of beta amyloid buildup in the brain, potentially slowing the progression of the disease. With no functional γ-secretase, the NICD remains tethered to the membrane and can no longer translocate to the nucleus to modulate expression of its target genes.
GSI XX, in a solution of 0.05% Methocel E4M and 0.01% Tween80, was delivered via tail vein injection to male Fisher-344 rats on the 2AAF/PH protocol for one week, covering days 7-14 of the oval cell response as well as the peak of Notch expression. 3 animals per timepoint were sacked on days 8, 11, 14, and 21. Northern blot demonstrated reduced mRNA expression of Hes1, a downstream target of the NICD in the nucleus, thereby indicating termination of Notch signal transduction. To further confirm muting of the signal by the inhibitor, western blot using an antibody specific to cleaved Notch-1 demonstrated a reduction in activated receptor. Further examination demonstrated an increase in p21 expression when Notch signaling is inhibited, indicative of cells that are trapped in the cell cycle and can no longer progress forward. These cells were also positive for the proliferative marker Ki67 through day 22 post-PH when Notch was inhibited, an observation not seen during 2AAF-PH alone. Moreover, treatment with GSI XX resulted in annihilation of cytochrome p450 and G6Pase-α expression in hepatocytes that lasted through day 22 post-PH.
CHAPTER 4
MATERIALS AND METHODS

4.1 Animal Studies

The 2AAF/PH model was utilized to accurately assess the activation and fundamental biology of the liver stem cell. This model provided the basis for understanding oval cell biology with respect to growth, proliferation and differentiation, as well as in response to extrinsic interventions. The assessment of oval cell biology \textit{in vitro} can be informational; however, \textit{in vivo} evaluation holds greater value in the analysis of the liver stem cell’s inherent functions. To date, no substitute has been found that adequately replaces an animal model in examining the fates of oval cells.

4.1.1 Animals and Animal Housing Facilities

All animals utilized in this study were under approved animal protocols submitted to the University of Florida IACUC committee. All animals utilized in this study were Fisher 344 male rats obtained from Charles River Laboratories, Inc. (Wilmington, MA). Animals were housed in a barrier facility under sterile conditions at the Animal Care Services Facility in the Medical Science/Communicore Building. The Animal Care Services is a state-of-the-art animal facility that provides a pathogen-free barrier environment. The animal care program is accredited by AAALAC. The facility is supervised by veterinarians, which are always present at the facility or on call. Animals are checked several times per day, and a veterinarian is always available for consultation, particularly if decisions need to be made regarding euthanizing an animal prior to the sacrifice date. The University of Florida meets National Institutes of Health standards as set forth in the DHHS publication #NIH 86-23 and accepts as mandatory the PHS “Policy on Humane Care and Use of Laboratory Animals by Awardee Institutions” and the National Institutes of Health “Principles for the Utilization and Care of Vertebrate Animals Used in
Testing, Research and Training.” The University of Florida has on file with the Office for Protection Form Research Risks an approved Assurance of Compliance.

4.1.2 Animal Sacrifice and Tissue Collection

All animals utilized for tissue collection were euthanized by administration of an overdose (150 mg/kg) of Nembutal Sodium Solution (Ovation Pharmaceuticals, Inc., Deerfield, IL). This is consistent with the recommendations from the panel on euthanasia of the American Veterinary Medical Association, as well as the Guide for the Use and Care of Laboratory Animals (U.S. Department of Health and Human Services/NIH Publication #86-23).

After euthanasia, tissue from brain, heart, lung, liver, pancreas, spleen, kidney, and intestine was collected for paraffin embedding, frozen sectioning, and RNA and protein collection. Samples for protein and RNA were snap frozen in liquid nitrogen and kept at -80°C until isolation was performed. Tissues for paraffin embedding were fixed O/N in 10% Neutral Buffered Formalin (Richard-Allan Scientific, Kalamazoo, MI). The formalin was then exchanged for PBS and the tissue submitted for embedding by the University of Florida Molecular Pathology Core Facility. Tissue collected for frozen sectioning was immediately placed in Tissue-Tek O.C.T. Compound (Sakura Finetek U.S.A., Inc., Torrance, CA), snap frozen in liquid nitrogen, and stored at -80°C until sectioning. All paraffin and frozen sections were cut to a 5μm thickness.

4.1.3 Rat Model of Oval Cell Induction

4.1.3.1 2AAF pellet implantation

Continuous administration of 2AAF was used to suppress proliferation of mature hepatocytes prior to partial hepatectomy. Utilization of a 2AAF time release pellet alleviated undue stress to the animals associated with multiple 2AAF oral gavage and reduced the amount of human exposure to the 2AAF.
Briefly, the animals were anesthetized with isoflurane. The abdomen was shaved and scrubbed three times in a circular pattern emanating from the center outward with ethanol and three times with Betadine (Purdue Pharma L.P., Stamford, CT). The animal was then draped with a Steri-Drape (3M, St. Paul, MN) with only the incision site exposed. Then a very small, approximately 1/4 inch, incision was made in the lower right quadrant of the animal’s abdomen. A midline incision would not suffice because the pellet must be placed distal to the liver in order to prevent adherence of the pellet to the body of the liver. The fibrosis associated with adherence would complicate the subsequent partial hepatectomy.

After opening the abdominal wall, a small incision was made within the abdominal muscle to facilitate entry to the peritoneal cavity. A 70 mg/28 day release (2.5 mg/day) 2AAF time release pellet (Innovative Research Inc., Sarasota, Fl) was carefully introduced through the incision into the peritoneal cavity. The muscle tissue was then closed using 1-2 sutures of 3-0 Vicryl (Ethicon, Inc., Cornelia, GA). The skin was closed with the Autoclip Wound Closing System (Braintree Scientific, Inc., Braintree, MA). Rats were then placed in a warmed cage and monitored for complete recovery. This procedure yielded a survival rate of 100%.

4.1.3.2 Two-thirds partial hepatectomy

The removal of 70% of the liver was originally described by Higgins and Anderson. The animals were anesthetized with isoflurane. The abdomen was shaved and scrubbed three times in a circular pattern emanating from the center outward with ethanol and three times with Betadine (Purdue Pharma L.P.). The animal was then draped with a Steri-Drape (3M) with only the incision site exposed. A 1.5 cm longitudinal incision was made in the skin just below the xyphoid process. The incision was continued through the midline of the abdominal muscle, exposing the liver. The tip of the xyphoid process was excised to facilitate removal of the liver and limit liver injury during extrusion. Next the left medial, right medial and the left lobe of the
liver were gently extruded through the incision. The lobes were then tied off with a silk suture. The exposed lobes were excised and the remaining stump examined for excessive bleeding prior to replacement within the peritoneal cavity. Bleeding from the stump indicated incorrect tying off of the excised lobes. If bleeding occurred and was unable to be controlled the animal was euthanized. The muscle tissue was then closed using 4-5 sutures of 3-0 Vicryl (Ethicon, Inc.).

The skin was closed with the Autoclip Wound Closing System (Braintree Scientific, Inc.). The stainless steel staples were removed after 10 days. Rats were then placed in a warmed cage and monitored for complete recovery. This procedure yielded a survival rate of greater than 95%. The 5% death rate was usually associated with the aforementioned bleeding from the incorrectly tied liver stump. The difficulty obtaining the correct tension on the ligating suture should be noted. A ligature tied too tightly causes the liver proximal to the knot to tear and this situation is practically impossible to resolve. Conversely, insufficient tension on the ligature results in the inability to staunch the blood flow to the stump and subsequent bleeding. In successful procedures, hypothermia and dehydration were not an issue during recovery. In the event that any blood was lost during surgery, animals were injected i.p. with 1-3mL sterile saline. The animals were checked every six hours until fully recovered. The stainless steel staples were removed after 10 days. Animals were sacrificed at days 3, 5, 7, 9, 11, 13, 15, 17, and 21 days post-PH.

4.2 Model for Inhibition of Notch Signaling in the Rat

In order to confirm that Notch signaling is required for oval cell-mediated liver regeneration, Notch receptor activation was halted using GSI XX, an inhibitor of the cleaving enzyme γ-secretase and potent downregulator of the Notch cascade. By transiently blocking cleavage of the NICD from the transmembrane receptor duplex, transduction of the Notch signal
was successfully impeded; as a result, the capacity of oval cells to both proliferate and properly differentiate was greatly diminished.

Animals underwent 2AAF administration along with PH as previously described. 500ug of GSIXX was dissolved in a solution of 0.05% Methocel E4M and 0.01% Tween 80 as previously described. This solution was delivered via tail vein injection to each animal once daily beginning day 7 post-PH and continuing through day 14 post-PH. Twelve animals received the inhibitor solution while another six animals received a control solution (Methocel E4M and Tween 80 only). Animals were sacrificed at days 8, 11, 14, and 22 post-PH.

4.3 RNA Analysis

4.3.1 Isolation of RNA from Tissue

Tissue samples were homogenized in 1mL of RNABee Reagent (Tel-Test, Inc., Friendswood, TX) per 50-100mg of tissue using a sonic homogenizer. The homogenized samples were incubated for 5min at RT to allow for the complete dissociation of nucleoprotein complexes. 0.2mL of chloroform was then added per 1mL of RNABee Reagent and the tubes vortexed. The samples were then centrifuged at 12,000 x g for 15min at 4°C. The upper aqueous phase of the supernatant was transferred to a fresh tube and the RNA precipitated out by the addition of 600ul of isopropanol per 1mL of RNABee Reagent. The samples were incubated for 10min at RT and then centrifuged at 12,000 x g for 10min at 4°C. The RNA precipitate formed a gel-like pellet on the bottom of the tube. The supernatant was removed and the pellet washed with 1mL of 75% ethanol. The sample was then vortexed and centrifuged at 7500 x g for 5min at 4°C. The RNA pellet was then air-dried for approximately ten minutes (but not allowed to completely dry), then dissolved in RNase-free water. In order to accurately determine the concentration of RNA in each sample, 2ul of RNA were diluted in 98ul of DEPC-H2O, and
the absorbance analyzed at OD of 260 and 280nm in a spectrophotometer. Purity was determined based on an OD 260/280 of 1.6 < x < 1.9. RNA concentration was determined using the formula RNA (ng/ul) = OD260 x 50 ng/ul x 100 dilution factor. The RNA samples were then stored in fresh screw-cap tubes at -80°C until further use.

4.3.2 RT-PCR

5.0ug RNA from each animal sacrificed was utilized for cDNA production. First-strand cDNA was synthesized utilizing the SuperScript First-Strand Synthesis System (Invitrogen) as per manufacturer’s instructions. The following ingredients were brought together for the reaction: 1ul cDNA, 2ul 10X PCR Buffer, 0.25ul 10mM dNTP mix, 0.5ul 10uM Forward Primer, 0.5ul 10uM Reverse Primer, and 15.55ul Milli-Q H2O. 0.2ul Taq Polymerase was added to each sample just before placement into the thermocycler. The PCR reaction was run in the following manner with the annealing temperature specific to the primer set being utilized:

i. 94°C for 10min
ii. 31 cycles of 94°C for 30sec, annealing temp for 30sec, 72°C for 1min
iii. 72°C for 10min
iv. 4°C for ∞

Table 4-1 shows the reaction details for each primer set utilized for cDNA amplification. Following the PCR reaction, 2ul loading dye was added to each 20ul sample and 10ul of that loaded into a 2% agarose mini-gel containing 0.001% Ethidium Bromide, along with an appropriate molecular weight ladder (1kb, 100bp, etc). The gel was run at about 110 volts for one hour or until desired separation of bands was achieved, as visualized on a UV light box. Pictures of the final gels were taken using the GelDoc XR system (Bio-Rad, Hercules, CA).
4.3.3 Real Time RT-PCR (qPCR)

To accurately assess the variations in gene expression during 2AAF-PH, as well as to confirm successful delivery of GSIXX, levels of Notch1 and other Notch-pathway genes were analyzed quantitatively by real time RT-PCR. This was accomplished using ready-to-use gene plates specific for stem cell genes (SA Biosciences, Frederick, MD) and performed as per the manufacturer’s instructions.

4.4 Protein Analysis

4.4.1 Isolation of Protein from Tissue

A small amount of tissue was placed in the desired amount of RIPA buffer with Protease Inhibitor. The tissue was broken up then sheared with an 18 gauge needle and syringe, then pipetted up and down until thoroughly homogenized. The sample was vortexed and then centrifuged at 10,000 x g at 4°C to remove excess lipids and DNA. The supernatant was collected in a fresh screw-cap tube and placed at -80°C until further use.

4.4.2 Protein Quantification with DC Protein Assay

Blank and protein standards were made up in 1mL tubes as follows:

i. Blank: 25ul H2O
ii. Std #1: 1ul std + 24ul H2O
iii. Std #2: 2ul std + 23ul H2O
iv. Std #3: 4ul std + 21ul H2O
v. Std #4: 8ul std + 17ul H2O

Samples were made up with 1ul sample plus 24ul H2O. Separately, 125ul Reagent A per sample and 2.5ul Reagent S per sample were mixed; 125ul of combined solutions A and S were added to each sample. Just before measurement, 1mL of Reagent B was added and vortexed. After ten minute incubation the OD of the samples was measured in disposable cuvettes using a spectrophotometer set to 750nm.
4.4.3 Western Blot Analysis of Protein Levels

10% acrylamide precast gels were utilized for gel electrophoresis of protein samples. The amount of protein loaded per well was determined based on source of isolation (tissue) and also on the sensitivity of the primary antibody being used for detection. Samples were added to the appropriate amount of RIPA buffer to equal 12ul per lane and placed in a screw cap tube, along with 3ul Western loading buffer. Each sample was boiled for about 10min then incubated for 5min at RT. Individual wells were loaded with 15ul sample with dye with the exception of the first, which was loaded with 7ul of protein molecular weight standard. Any remaining protein solution was placed on ice and returned to storage at -80°C until the next use. Unused wells in the gel were loaded with 15ul of 1x Western loading buffer.

The gel was loaded in the running apparatus with the small plate facing inward. The inner chamber was filled to overflowing with 1X Running Buffer, and the outer chamber was filled approximately 2 inches. The gel was run at 60 volts until the loading dye was out of the stacking gel, then run at 100 volts for the remaining length of the gel.

Immuno-blot PVDF (Bio-Rad) membrane was prepared by dipping quickly in methanol, soaking in H2O for 5min, then 1X Transfer Buffer for 20min. Sponges and Whatman paper were also saturated in 1X Transfer Buffer. Upon completion of the electrophoresis the gel plates were removed and the stacking gel/wells removed from the gel, which was then transferred to 1X Transfer Buffer. A sandwich consisting of black assembly tray, sponge, Whatman paper, gel, PVDF membrane, Whatman paper, sponge and red assembly tray along with an ice block and a stir bar were placed in the transfer apparatus. The apparatus was then filled with 1X Transfer Buffer and the proteins transferred at 200milliamps for 90min.
Following the transfer, the membrane was blocked O/N at 4°C with a solution consisting of 5% skim milk and 2% glycine in PBS-T. The membrane was then probed with the appropriate concentration of primary antibody O/N at 4°C. After three 5min washes in PBS-T, the appropriate HRP-conjugated secondary antibody was applied in PBS-T for 30mins to 1hr with shaking at RT, followed by another three 5min washes in PBS-T.

For development, excess liquid was removed from the membrane and it placed inside a plastic bag. 25ul Solution A along with 1mL of Solution B ECL Plus Reagents (GE Healthcare, Piscataway, NJ) was incubated on the membrane for 5min. Excess reagent was removed, and film (Kodak) exposed to the membrane for 30s to 10min depending on the intensity of the banding pattern.

If further probing was necessary, the membrane was then stripped. 714ul of 2-mercaptoethanol was added to 100mL 1X Stripping Buffer and the solution along with the membrane sealed inside a plastic bag. The bag was incubated at 55°C for 30min with intermittent shaking. The membrane was then washed 5-7 times of ten minutes each with 1X PBS-T until all residual 2-mercaptoethanol was removed. Membranes were then re-blocked with milk and reprobed as per the above protocol. Table 4-2 demonstrates the primary antibodies and conditions utilized for western blot analysis.

4.5 Histology and Immunohistochemistry

4.5.1 Hematoxylin and Eosin of Paraffin-Embedded Tissue

Tissue sections 5um in size were cut and placed in a 42°C water bath, then lifted from the bath on a Superfrost Plus (Thermo Fisher Scientific Inc, Waltham, MA) positively-charged slide, which were then allowed to dry O/N at RT. The paraffin was removed and the tissue rehydrated by incubating the slides in Xylene 2 x 5min, 100% Ethanol 2 x 2min, 95% Ethanol 2 x 2min and
finally distilled H2O for 1 min. Nucleic acids and other positively charged molecules were then stained with Hematoxylin 7211 (Richard Allan Scientific) for 2.25 min, then rinsed with distilled H2O for 2 x 1 min. The blue of the hematoxylin was intensified by incubating the slides in Clarifier 1 (Richard-Allan Scientific) for 1 min, distilled H2O for 1 min, Bluing Reagent (Richard-Allan Scientific), distilled H2O for 1 min, and 80% Ethanol for 1 min. Proteins were then stained a pink color with Eosin-Y (Richard-Allan Scientific) for 1.5 min. The tissue was then dehydrated for coverslipping with 95% Ethanol 2 x 1 min, 100% Ethanol 2 x 1 min, and Xylene 3 x 1 min. Coverslips were then applied with Cytoseal XYL (Richard-Allan Scientific).

4.5.2 Hematoxylin and Eosin of Frozen Tissue

Tissue sections 6 μm in size were cut and placed on Superfrost Plus (Thermo Fisher Scientific Inc., Waltham, MA). The slides were air dried for 30 min at RT, then fixed in acetone for 30 sec, then washed in distilled H2O for 1 min. Nucleic acids and other positively charged molecules were stained with Hematoxylin 7211 (Richard-Allan Scientific) for 45 sec then rinsed with distilled H2O for 1 min. The blue of the hematoxylin was intensified by incubating the slides in Clarifier 1 (Richard-Allan Scientific) for 25 sec, distilled H2O for 30 sec, Bluing Reagent (Richard-Allan Scientific) for 30 sec, distilled H2O for 30 sec, and 80% Ethanol for 30 sec. Proteins were stained a pink color with Eosin-Y (Richard-Allan Scientific) for 30 sec. The tissue was then dehydrated for coverslipping with 95% Ethanol 2 x 1 min, 100% Ethanol 2 x 1 min, and Xylene 3 x 1 min. Coverslips were then applied with Cytoseal XYL (Richard-Allan Scientific).

4.5.3 Immunohistochemistry: Chromagen Staining

All staining of paraffin and frozen sections was performed with Vector ABC Kits using DAB (Vector Laboratories, Burlingame, CA) and carried out as per manufacturer’s instructions. Tissue was deparaffinized and rehydrated as in H & E staining. Frozen sections were fixed in acetone unless otherwise noted. Slides were incubated with primary antibody O/N at 4°C and 1
hour for secondary antibodies. Any special retrieval method utilized is indicated, along with the
details for each antibody used, in Table 4-3.

4.6 Index of Solutions

10X Agarose Gel Loading Buffer
1. 15.0mg bromophenol blue
2. 15.0mg xylene cyanol
3. 8.0g sucrose
4. Milli-Q H2O qs to 10mL

10X PBS
1. 80.0g NaCl
2. 2.0g KCl
3. 11.5g Na2HPO4 × 7H2O
4. 2.0g KH2PO4
5. Milli-Q H2O qs to 1L

RIPA Buffer and Protease Inhibitor Solution
RIPA Buffer
1. 1.5mL 1M NaCl
2. 0.5mL 1M Tris-Cl pH 8.0
3. 1.0mL 10% NP-40
4. 1.0mL 10% NaDeoxycholate
5. 5.5mL Milli-Q H2O
Protease Inhibitor Solution (added to RIPA just prior to use)
1. 100μl 10mg/mL PMSI in isopropanol
2. 300μl Aprotinin
3. 100μl 100mM NaOrthovanadate
Total = 10.0mL

5X TBE
1. 54.0g Tris base.
2. 22.5g Boric acid
3. 4.7g EDTA
4. Milli-Q H2O qs to 1L

10X TBS
1. 80.0g NaCl
2. 2.0g KCl
3. 30.0g Tris base
4. 800mL H2O
5. Milli-Q H2O qs to 1L
Adjust pH to 7.4 using 1M HCl

5X Western Loading Buffer
1. 1.5mL 0.5M Tris-HCl
2. 1.0g 10% SDS
3. 2.5mL β-mercaptoethanol
4. 1.5mg Bromophenol Blue
5. Milli-Q H$_2$O $qs$ to 10mL

**10X Western Running Buffer**
1. 144.0g Glycine
2. 30.0g Tris-Base
3. 10.0g SDS
4. Milli-Q H$_2$O $qs$ to 1L

**5X Western Stripping Solution**
1. 37.83g Tris-Base
2. 1g SDS
3. pH to 6.8
4. Milli-Q H$_2$O $qs$ to 1L

**10X Western Transfer Buffer**
1. 115.0g Glycine
2. 24.0g Tris-Base
3. Milli-Q H$_2$O $qs$ to 1L

When diluted to 1X, 80mL of 10X Transfer Buffer was added to 720mL Milli-Q H$_2$O and 200mL Methanol.
Figure 4-1. Model for oval cell induction. Diagrammatic representation of the 2AAF-PH protocol in the rat including pellet implantation, 2/3 partial hepatectomy and dates of sacrifice.

Figure 4-2. Model of oval cell induction with Notch inhibition. Diagrammatic representation of the 2AAF-PH protocol combined with γ-secretase inhibitor in the rat including pellet implantation, 2/3 partial hepatectomy, dates of inhibitor injection and dates of sacrifice.
Table 4-1. PCR Primer Sequences. Individual primer sets utilized for RT-PCR, together with the reaction and product details for each primer set.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence (5’ → 3’)</th>
<th>Annealing Temp (°C)</th>
<th>Product Amplified (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Notch1</td>
<td>Fw:ATCCATGGCTCCATCGTCTA</td>
<td>55</td>
<td>422</td>
</tr>
<tr>
<td></td>
<td>Rev:TTCTGATTGTCGTCATCAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Notch2</td>
<td>Fw:TTTGCTGTCGAAGACGACC</td>
<td>62</td>
<td>403</td>
</tr>
<tr>
<td></td>
<td>Rev:GCCCATGGTTCCTGGCGT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Jagged1</td>
<td>Fw:TCCAGCCTCCAGCAGTGAA</td>
<td>61</td>
<td>201</td>
</tr>
<tr>
<td></td>
<td>Rev:GGAAGGCTCACAGGCTATGT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hes1</td>
<td>Fw:CAACACGACACCGGACAAACC</td>
<td>55</td>
<td>349</td>
</tr>
<tr>
<td></td>
<td>Rev:AGTGCACCTCGGTGTTAAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>Fw:TGAGGGAGATGCTCAGTGTT</td>
<td>58</td>
<td>490</td>
</tr>
<tr>
<td></td>
<td>Rev:ATCAGTGCATCTCAGAAGAC</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### Table 4-2. Antibodies utilized for western blot analysis.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Host</th>
<th>Concentration</th>
<th>MW (kDa)</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Notch1</td>
<td>Rabbit</td>
<td>1:1000</td>
<td>120</td>
<td>Cell Signaling</td>
</tr>
<tr>
<td>p21</td>
<td>Mouse</td>
<td>1:200</td>
<td>21</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>G6Pase-α</td>
<td>Goat</td>
<td>1:200</td>
<td>36</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>β-actin</td>
<td>Mouse</td>
<td>1:2000</td>
<td>42</td>
<td>AbCam</td>
</tr>
</tbody>
</table>

### Table 4-3. Antibodies utilized for immunohistochemical analysis.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Host</th>
<th>Concentration</th>
<th>Section</th>
<th>Retrieval</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Notch1</td>
<td>Rabbit</td>
<td>1:100</td>
<td>Paraffin</td>
<td>Citrate</td>
<td>Millipore</td>
</tr>
<tr>
<td>Jagged1</td>
<td>Goat</td>
<td>1:100</td>
<td>Paraffin</td>
<td>Citrate</td>
<td>AbCam</td>
</tr>
<tr>
<td>Ki67</td>
<td>Mouse</td>
<td>1:100</td>
<td>Paraffin</td>
<td>Citrate</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>AFP</td>
<td>Mouse</td>
<td>1:500</td>
<td>Paraffin</td>
<td>Trilogy</td>
<td>Dako</td>
</tr>
<tr>
<td>OV-6</td>
<td>Rabbit</td>
<td>1:150</td>
<td>Frozen</td>
<td>None</td>
<td>Gift from S. Sell</td>
</tr>
<tr>
<td>CYP3A2</td>
<td>Rabbit</td>
<td>1:100</td>
<td>Paraffin</td>
<td>Citrate</td>
<td>Fitzgerald</td>
</tr>
</tbody>
</table>
5.1 Analysis of Notch Family Expression During Oval Cell-Mediated Liver Regeneration

As there are multiple isoforms of both the receptor (four) and ligand (two), we set out to determine which isoforms were upregulated during oval cell-mediated liver regeneration. RT-PCR analysis across the 2AAF-PH timecourse shows an upregulation of Notch1 and Jagged1 during the peak of oval cell induction (Figure 5-1), approximately 9-11 days post-partial hepatectomy. Expression of Notch2, Notch3, Notch4 and Jagged2 remained unchanged during the timecourse. Examination of Notch effector Hes1 also revealed an increase in expression during oval cell-induction, demonstrating transduction of the Notch signal downstream and true activation of the pathway. Based on these data, we ascertained that Notch1 signaling via Jagged1 activated the downstream effector Hes1 during the peak of oval cell induction, indicating that Notch signaling may play a critical role in oval cell-mediated liver regeneration.

To further confirm Notch1 signaling during oval cell induction, we performed an in situ PCR on liver sections taken from animals on day 11 of the 2AAF-PH protocol. This was accomplished using a DIG-labeled UDP for the otherwise normal PCR reaction, followed by incubation with a DIG-labeled secondary antibody and subsequent development. Human lung adenocarcinoma was used as a positive control for Notch activation. Notch1 DNA was found to mainly be localized in the cytoplasm and nuclei of hepatocytes, while membranous Jagged1 expression was seen in primarily in biliary cells as well as some hepatocytes at day 11 (Figure 5-2). This is indicative of Notch1 receptor activation by Jagged1 present on adjacent cells, as well as of subsequent translocation of the NICD to the nucleus of the hepatocytes (key indicator of pathway activation).
Next, western blot analysis was used to investigate activation of the pathway at the protein level (Figure 5-3). Protein taken from animals at standard timepoints in the oval cell induction protocol confirmed upregulation of Notch1 from day 9 to day 13 of the timecourse, with a return to basal levels by day 22 post-PH. To examine receptor-ligand protein localization, immunohistochemical analysis was performed on liver sections taken from animals on day 11 of the 2AAF-PH protocol (Figure 5-4). The Notch1 antibody used is specific to the NICD portion of the receptor, while the Jagged1 antibody is specific to the protein’s carboxy terminus. Here again we find activation of Notch1 receptor in the hepatocytes, as well as expression of Jagged1 on hepatocytes and cholangiocytes present. This is indicative of Notch1-Jagged1 receptor-ligand interaction between adjacent cells, and further demonstrates translocation of the NICD to the nucleus in the Notch1 activated hepatocytes. Immunofluorescence, also performed with antibody specific to the NICD, further demonstrates activation of the receptor and translocation of the NICD to the nucleus in activated hepatocytes (Figure 5-5).

Investigation of all the isoforms of Notch and Jagged during 2AAF-PH reveals upregulation of Notch1 and Jagged1 at the peak of oval cell induction. As the peak mRNA and protein expression of both receptor and ligand occurs with the influx of oval cells at days 9-11 post-PH, it can be concluded that activation of Notch1 by its ligand Jagged1 is necessary for oval cell-mediated liver regeneration. Persistent upregulation of Notch1 protein through day 13 of the timecourse indicates a role for Notch signaling in the differentiation of the activated oval cells.
5.2 *in vivo* Inhibition of Notch Signaling During Oval Cell Induction

\(\gamma\)-secretase is the enzyme responsible for the cleavage and subsequent activation of the Notch receptor. Administration of gamma secretase inhibitor (GSI) XX prevents this cleavage and thereby terminates the Notch signal before it is able to take effect in the nucleus. 2AAF-PH treated rats were injected with GSIXX and the oval cell response was examined at day 11 and day 22 following PH. Hematoxylin and Eosin staining shows that there remains a massive activation of the oval cell compartment at day 11 post-PH upon treatment with GSIXX; however the architecture is more disorganized and there is a larger immune infiltrate, likely due to the solution used to administer the inhibitor, as compared to 2AAF-PH alone (Figure 5-6). Also significant is the number of oval cells remaining in the periportal regions of the liver at day 22 post-PH after GSIXX treatment as compared to 2AAF-PH alone. The presence of oval cells at this late stage of regeneration indicates their failure to differentiate into a mature phenotype, either hepatocyte or cholangiocyte.

To further demonstrate downregulation of Notch signaling via GSI XX, we performed a real time PCR analysis of mRNA isolated from livers of animals on the 2AAF-PH protocol, both with and without inhibitor treatment. Figure 5-7 shows graphical representations of the fold change in expression of Notch pathway genes during 2AAF-PH alone. Notch1 expression levels increase significantly at day 11 post-PH as compared to control and day 22 post-PH (** P < .001). As expected, no change was seen in Notch2 expression. Analysis also revealed a drastic upregulation of downstream effector Hes1, demonstrating significant Notch signal transduction at the peak of oval cell infiltration.

Figure 5-8 demonstrates fold change in expression for Notch pathway genes in animals on the 2AAF-PH protocol with GSIXX. As the inhibitor targets Notch at the protein level, we do
not expect to see a decrease in Notch1 gene expression at day 11 in the GSI XX-treatment group, but should see a change in expression levels of downstream effectors. In the GSIXX-treatment group there was no increase in Hes1 expression at day 11 post-PH, demonstrating interruption of the Notch signal. While we do see a small increase in Notch2 expression levels, it is insignificant as compared to Notch1 and likely due to functional redundancy that is subsequently eliminated by inhibitor targeting. To verify downregulation of Notch signaling we performed a western analysis with an antibody specific to the cleaved form of Notch1 (Val1774). Figure 5-9 demonstrates a significant decrease in the levels of the activated receptor (** P < .001) at days 11 and 22 post-PH with GSIXX.

In order to fully characterize the oval cells activated by 2AAF-PH when Notch signaling is simultaneously inhibited, we examined the expression levels of standard oval cell surface markers. Immunohistochemical analysis of OV-6 expression (Figure 5-10) demonstrates the activation of oval cells in the periportal regions in response to injury at day 11 post-PH alone, which have generally differentiated into mature phenotypes by day 22 post-PH alone. However in the absence of Notch signaling, while there is a similar infiltration of oval cells at day 11 we see a large number of OV-6 positive cells persisting out to day 22 post-PH.

Further evidence of this abnormal oval cell differentiation is exhibited in the staining for AFP (Figure 5-11). Note the typical elevated level of expression at day 11 post-PH both with and without treatment, but also that there is minimal AFP expression at day 22 when Notch is inhibited. This lack of AFP expression is echoed at the gene level, where mRNA expression of AFP at day 22 in the GSIXX-treated group is almost equivalent to control (Figure 5-8). The fact that the oval cells present at day 22 post-PH are OV-6 positive and AFP negative is indicative of
a short-circuiting within the differentiation programming of these progenitors when Notch signaling is inactivated.

As it appeared that termination of the Notch signal had an inhibitory effect on the differentiation capacity of oval cells, we decided to investigate other markers of differentiation. Western analysis of protein isolated from livers of GSIXX-treated animals demonstrated a dramatic decrease in levels of the biliary marker CK19 upon treatment with GSIXX at day 22 as compared to 2AAF-PH alone (Figure 5-12). We therefore also decided to examine levels of HNF-1β, a transcription factor regulated by Notch signaling and which is involved specifically in biliary differentiation. Western blot analysis reveals a marked downregulation in the expression of this factor during oval cell-mediated liver regeneration when Notch signaling was concomitantly downregulated (Figure 5-13). Morphological evidence as well as the decreased expression of markers of differentiation by the cells present at day 22 in the GSIXX-treated group is evidence that dysregulation of the Notch pathway results in a delay in the differentiation of activated oval cells.

There have been conflicting reports in the literature describing the effects of Notch downregulation on proliferation, particularly in the liver. We therefore examined cell cycle progression and growth during regeneration by analysis of hepatocellular mitoinhibition index, a ratio between expression of p21\textsuperscript{Waf1} (cell cycle inhibitor) and Ki67 (a marker of cellular proliferation). Figure 5-14 shows a mass of proliferating cells by Ki67 staining at day 11 post-PH during 2AAF-PH alone, with a similar response seen during 2AAF-PH combined with GSIXX treatments. While the cells remaining at day 22 post-PH alone have ceased proliferating, the hepatocytes seen at this timepoint appear to remain mitotically active when Notch signaling is
terminated. Semi-quantitative analysis of these data can be seen in Figure 5-15, demonstrating the enhanced effects on proliferation seen during Notch inhibition.

We also investigated expression of p21, a well known cell cycle inhibitor and downstream target of Notch signaling. Figure 5-16 shows a western analysis of protein isolated from livers of GSIXX-treated animals, demonstrating a dramatic decrease in expression levels of p21 upon treatment with GSI XX at day 22 as compared to 2AAF-PH alone. Expression of phospho-AKT, another marker of cell cycle inhibition, also revealed an interesting result; treatment with GSIXX significantly reduced levels of phosphorylated AKT (Figure 5-17). This drastic downregulation of cell cycle inhibitors signifies dysregulated cellular growth and cell cycle progression.

To fully determine the extent of growth during 2AAF-PH with and without GSI XX, we examined the hepatocellular mitoinhibition index. This ratio of replicative arrest/proliferation was determined by dividing the number of p21-positive nuclei by the number of Ki67-positive nuclei. This ratio, as represented graphically in Figure 5-18, was found to be significantly different between the two groups at both day 11 and day 22 post-PH (** P < .001). These results demonstrate that diminished Notch signaling leads to an aberrant regulation of factors that works to promotes cell cycle progression and proliferation during regeneration of the liver following 2AAF-PH.

Mature hepatocytes are responsible for carrying out the functions of the liver that are required for homeostasis. Typically during 2AAF-PH, the hepatocytes produced in response to the injury are mature and functional by day 22 post-PH; however when Notch signaling was inhibited, the activated oval cells did not properly differentiate into a mature phenotype. We therefore examined the functional characteristics of the hepatocytes produced in the two
treatment groups. Immunohistochemical analysis of CYP3A2 (Figure 5-19), an isoform of cytochrome p450 expressed only by mature hepatocytes, shows a typical decrease in expression at day 11 post-PH with an upsurge back to basal levels by day 22 post-PH (Figure 7A and B). However in the GSI-treated group, there is a drastic decrease in CYP3A2 expression at day 11 that remains in effect through day 22 post-PH.

We also decided to examine expression of another functional enzyme, glucose-6-phosphatase (G6Pase)-α. Protein analysis via Western Blotting revealed a dramatic decrease in the amount of enzyme being expressed after GSI XX treatment as compared to 2AAF-PH alone (Figure 5-20). These results are indicative of altered glucose metabolism and call into question the functionality of the hepatocytes present at this timepoint. Although the decrease in G6Pase upon Notch inhibition is a novel finding, the downregulation of CYP has been previously documented in other tissue systems when Notch signaling was inactivated {Rodriguez, 2008}. Altogether this data suggests that inhibition of Notch signaling may lead to functionally impaired cellular progeny.
Figure 5-1. RT-PCR analysis of normal rat liver as well as liver taken at standard timepoints following 2AAF-PH for mRNA expression of various Notch pathway components.
Figure 5-2. Expression of Notch and Jagged via *in situ* PCR. A. Notch DNA expression on human lung adenocarcinoma (positive control). B. Expression of Notch DNA on paraffin sections of liver at day 11 post-2AAF-PH. C. Expression of Jagged DNA on paraffin sections of liver at day 11 post-2AAF-PH.
Figure 5-3. Analysis of Notch1 protein expression during 2AAF-PH. A. Western blot analysis of Notch1 using an antibody specific to the cleaved receptor shows a significant increase in activated Notch1 expression during the peak of oval cell induction. B. Semi-quantitative analysis of A. All data was normalized to β-actin and then compared to control.
Figure 5-4. Immunohistochemistry for Notch1 and Jagged1. A. Antibody IgG negative control. B. Localization of Notch1 protein expression using an antibody specific to the Notch Intracellular Cytoplasmic Domain. C. Localization of Jagged1 protein expression using an antibody specific to the protein’s carboxy terminus. Notch1 appears to stain hepatocytes while Jagged1 positivity is seen primarily on oval cells and bile duct cells, but also in some hepatocytes.
Figure 5-5. Immunofluorescence for activated Notch1 at day 11 post-2AAF-PH.  A. Nuclear localization with DAPI.  B. Localization of activated Notch1 receptor using an antibody specific to Notch Intracellular Cytoplasmic Domain.  C. Merge Notch1 and DAPI.  D. Higher magnification of C.
Figure 5-6. H&E staining of liver from animals on the 2AAF-PH protocol or 2AAF_PH combined with GSIXX. A. 2AAF-PH control; B. Day 11 post-PH; C. Day 22 post-PH; D. 2AAF-PH-GSIXX control; E. Day 11 post-PH with GSIXX; F. Day 22 post-PH with GSIXX. Day 11 shows a massive infiltration of oval cells in both treatment groups. During 2AAF-PH alone, the oval cells have differentiated into mature phenotypes by day 22; however in the GSIXX-treated group, we see oval cells persisting through day 22 post-PH. Magnification A&D 200x; B-C&E-F 400x
Figure 5-7. Real time PCR analysis for expression of Notch pathway members during 2AAF-PH. Significant differences in expression occur during the peak of oval cell induction as compared to control for both the Notch1 receptor as well as the downstream effector Hes1.
Figure 5-8. Real time PCR analysis for expression of Notch pathway members during 2AAF-PH with GSIXX. The inhibitor targets Notch at the protein level, and therefore we do not see downregulation of message of the receptor; however there is a significant decrease in Hes1 expression, indicative of interruption of Notch signaling.
Figure 5-9. Analysis of Notch1 protein expression during 2AAF-PH with GSIXX. A. Western blot analysis of Notch1 using an antibody specific to the cleaved receptor shows a significant decrease in activated Notch1 expression during the peak of oval cell induction that remained in effect through day 22, indicating that the treatment effectively inhibited Notch signaling. B. Semi-quantitative analysis of A. All data was normalized to β-actin and then compared to control.
Figure 5-10. OV-6 staining during the 2AAF-PH protocol or 2AAF-PH with GSIXX. A. 2AAF-PH control; B. Day 11 post-PH; C. Day 22 post-PH; D. 2AAF-PH-GSIXX control; E. Day 11 post-PH with GSIXX; F. Day 22 post-PH with GSIXX. OV-6 expression significantly increases at day 11 in both treatment groups, but only in the GSIXX-treated group do we still see positivity at day 22 post-PH. Magnification 200x.
Figure 5-11. AFP staining during the 2AAF-PH protocol or 2AAF-PH with GSIXX. A. 2AAF-PH control; B. Day 11 post-PH; C. Day 22 post-PH; D. 2AAF-PH-GSIXX control; E. Day 11 post-PH with GSIXX; F. Day 22 post-PH with GSIXX. AFP expression significantly increases at day 11 in both treatment groups; however while OV-6 expression remains in effect at day 22 in the GSIXX-treated group we do not see AFP expression at day 22 post-PH. Magnification 200x.
Figure 5-12. Analysis of CK19 protein expression during 2AAF-PH or 2AAF-PH with GSIXX. 
A. Western blot analysis of CK19 shows an increase in expression at day 11 that remains in effect through day 22. However in the GSIXX-treated group, there is a significant decrease in expression levels at day 22 post-PH. B. Semi-quantitative analysis of A. All data was normalized to β-actin and then compared to control.
Figure 5-13. Analysis of HNF1β protein expression during 2AAF-PH or 2AAF-PH with GSIXX. A. Western blot analysis of HNF1β shows an increase in expression at day 11 that remains in effect through day 22. However in the GSIXX-treated group, there is a significant decrease in expression levels at day 11 that remains in effect through day 22 post-PH. B. Semi-quantitative analysis of A. All data was normalized to β-actin and then compared to control.
Figure 5-14. Ki67 staining during the 2AAF-PH protocol or 2AAF-PH with GSIXX.  A. 2AAF-PH control; B. Day 11 post-PH; C. Day 22 post-PH; D. 2AAF-PH-GSIXX control; E. Day 11 post-PH with GSIXX; F. Day 22 post-PH with GSIXX.  Ki67 expression significantly increases at day 11 in both treatment groups; however while cells have stopped proliferating at day 22 after 2AAF-PH alone, mitotically active cells persist through day 22 in the GSIXX-treated group.  Magnification 200x.
Figure 5-15. Semi-quantitative analysis of Ki67 staining during the 2AAF-PH protocol or 2AAF-PH with GSIXX.
Figure 5-16. Analysis of p21 protein expression during 2AAF-PH or 2AAF-PH with GSIXX.
A. Western blot analysis of p21 shows a decrease in expression at day 11 due to liver resection with a return to basal expression by day 22. However in the GSIXX-treated group, there is a significant increase in expression levels at day 22 post-PH. B. Semi-quantitative analysis of A. All data was normalized to β-actin and then compared to control.
Figure 5-17. Analysis of phospho-AKT protein expression during 2AAF-PH or 2AAF-PH with GSIXX. A. Western blot analysis of p-AKT shows a decrease in expression at day 11 due to liver resection with a return to basal expression by day 22. However in the GSIXX-treated group, expression of p-AKT does not return to anywhere near basal levels by day 22 post-PH. B. Semi-quantitative analysis of A. All data was normalized to β-actin and then compared to control.
Figure 5-18. Hepatocellular mitoinhibition index for 2AAF-PH alone as well as 2AAF-PH with GSIXX. This is calculated by dividing the number of p21 positive nuclei by the number of Ki67 positive nuclei.
Figure 5-19. CYP3A2 staining during the 2AAF-PH protocol or 2AAF-PH with GSIXX.  
A. 2AAF-PH control; B. Day 11 post-PH; C. Day 22 post-PH; D. 2AAF-PH-GSIXX control; E. Day 11 post-PH with GSIXX; F. Day 22 post-PH with GSIXX.  CYP3A2 expression significantly decreases at day 11 in both treatment groups due to liver resection; however upon restoration of the liver mass by day 22 expression of CYP3A2 returns after 2AAF-PH alone. No expression of CYP3A2 is seen at day 22 in the GSIXX-treated group. Magnification 200x.
Figure 5-20. Analysis of Glucose-6-Phosphatase-α protein expression during 2AAF-PH or 2AAF-PH with GSIXX. A. Western blot analysis of G6Pase shows a decrease in expression at day 11 due to liver resection with a return to basal expression by day 22. However in the GSIXX-treated group, there is no return of G6Pase protein to basal levels by day 22. B. Semi-quantitative analysis of A. All data was normalized to β-actin and then compared to control.
Figure 5-21. Diagram depicting involvement of Notch signaling in the differentiation of stem cells/progenitors. A. In the case of a general bipotential cell, the up- or downregulation of Notch signaling decides the fate of that cell. In the absence of the Notch signal, the cell will assume a primary fate; however when the signal is activated the cell will develop into its’ secondary phenotype. Applying this concept to the oval cell system during liver regeneration, we find that Notch signaling is necessary for the proper ratio of differentiation to occur. B. When Notch signaling is active we see that oval cells assume both hepatocyte and cholangiocyte linages. C. In the absence of the Notch the cells do not appear to be able to differentiate properly into a mature lineage.
CHAPTER 6
DISCUSSION AND FUTURE STUDIES

6.1 Summary of Results

The role of the Notch signaling pathway in stem cell-mediated liver regeneration was examined using the 2AAF-PH oval cell induction protocol. Examination of the multiple isoforms of Notch receptor and Jagged ligand via RT-PCR, immunohistochemistry and western analysis revealed that at the peak of oval cell infiltration, approximately 9-11 days post-PH, there was a significant increase in expression of Notch1, Jagged1 and the downstream effector Hes1. These elevated expression levels abated by day 22 post-PH, the accepted endpoint of regeneration by this model, indicating a potential necessity for Notch signaling during oval cell-mediated liver regeneration.

We therefore decided to employ a model of Notch inhibition in vivo to examine what effects, if any, the absence of Notch signaling might have on the oval cells and their capacity for rapid regeneration of the liver after injury. Although a robust response was exhibited by the oval cells at day 11 post-PH, they were still present in the portal regions of the liver at day 22 post-PH upon treatment with GSIXX, indicating that Notch inhibition caused a delay in oval cell differentiation. Examination of oval cell markers OV-6 and AFP revealed interesting results: although the oval cells present at day 22 post-PH with GSIXX were OV-6 positive, they were not positive for AFP. Further analysis for markers of differentiation showed a decrease in expression of biliary marker CK19 via western blot at day 22 post-PH with GSIXX, a result that was abrogated by a significant decrease in the biliary transcription factor HNF-1β. These experiments demonstrate an asynchronous and skewed differentiation response by the oval cells when Notch is inhibited during 2AAF-PH.
Oval cell differentiation was not the only process to be affected by Notch inhibition. Analysis of proteins such as Ki67, p21 and AKT at day 22 post-PH with GSIXX revealed drastic dysregulation of these markers of growth and cell cycle progression, indicating that Notch signaling may be partly responsible for regulation of proliferation upon 2AAF-PH. The dysregulation of p21 upon Notch inhibition also supports this idea, as p21 is a well-known downstream effector of Notch signaling.

Lastly, as the absence of Notch signaling had a profound effect on the oval cell differentiation response, we decided to examine the functionality of the cellular progeny at day 22 post-PH with GSIXX. Investigation of functional enzyme markers CYP3A2 and G6Pase-α showed a significant decrease in their expression levels upon inhibitor treatment. This indicates not only a failure of the oval cells to differentiate into a mature phenotype but also that any differentiation that does occur will not be “normal”.

6.2 Interpretation of Results

Notch is an evolutionarily conserved local cell-signaling mechanism that participates in the regulation of cell fate specification, cellular growth and proliferation\(^1\). Here we have demonstrated activation of the Notch pathway during liver regeneration mediated by oval cells via the 2AAF-PH protocol, as well as showing that Notch inhibition during 2AAF-PH has significant consequences for liver regeneration. The data presented here provide novel evidence that Notch signaling is necessary in order for proper regulation of oval cell differentiation to occur, and further implies Notch signaling in a functional capacity with regard to the progeny produced by the activated oval cells.

Here we demonstrate disruption of the Notch signal upon treatment by both a decrease in activated Notch receptor as well as by a decrease in expression of the downstream effector Hes1.
Because GSIXX targets all forms of Notch receptor, functional redundancy of receptor/ligand is not called into question. It has been shown that when Notch signaling is upregulated during 2AAF-PH the bipotential oval cells do take on both hepatocyte and cholangiocyte fates; however, in the absence of the signal the number of oval cells committed to primary versus secondary fates is altered. Morphological analysis of GSIXX-treated samples shows incomplete differentiation of oval cells into mature lineages upon regeneration after 2AAF-PH, an idea that is further supported by persistent expression of oval cell markers OV-6 and AFP at day 22 post-PH.

The abnormal expression levels of CK19 and HNF-1β are also suggestive of an asynchronous differentiation response by these oval cells, potentially skewing the ratio of differentiating oval cells in favor of a hepatocyte versus a biliary fate. It is possible that the absence of the appropriate signal (i.e. Notch) forces the oval cells to choose a primary fate, but upon making that choice the cells find that they are incapable of fully differentiating into that functional phenotype. The cells present at day 22 post-PH after Notch inhibition were negative for functional markers of maturity such as CYP and G6Pase-α. This demonstrates a stunted attempt at differentiation and indicates that Notch is required for functional differentiation of oval cells during regeneration of the liver following 2AAF-PH. More specifically, it can be concluded that Notch signaling is necessary for the appropriate ratio of progenitors to assume primary versus secondary phenotypes (hepatocyte vs. biliary, respectively).

In 2004 Tanimizu et al. published work demonstrating that expression of NICD in progenitors inhibits the differentiation leading to hepatocytes and induces characteristics of cholangiocytes. Furthermore studies performed by Zong et al. give evidence that hepatocytes retain biliary competence in response to Notch signals, suggesting that hepatobiliary remodeling
after injury is regulated by Notch. The data presented in this paper corroborate these results and we have outlined our interpretation of Notch involvement in stem cell differentiation during 2AAF-PH in Figure 8, which diagrams the general effects of an increase or decrease in Notch signaling on the process of oval cell fate determination.

In the last decade conflicting reports have been seen in the literature describing the effects of Notch signaling on proliferation, particularly in the liver. For example, a study by Kohler et al. suggests that Notch1 inactivation has a direct role in inhibiting hepatocyte proliferation; in contrast, another study by Croquelois et al. suggests that Notch signaling plays a role in promoting rather than inhibiting regeneration and proliferation. Our experiments are further evidence of the necessity for Notch signaling in regulation of these processes. Inhibition of Notch during oval cell-mediated regeneration resulted in an aberrant hepatocellular mitoinhibition index (p21\textsuperscript{Waf1}:Ki67) after regeneration. It is likely that the absence of Notch1 during regeneration leads to altered cell cycle regulation, particularly because differentiation and transition through cell cycle checkpoints are closely linked. In fact, studies performed by Carlesso et al show that the relative proportion of cells in specific phases of the cell cycle were consistently altered in the presence of activated Notch1; conversely it is completely plausible that inhibition of Notch1 would also lead to a visible change in cell cycle kinetics during oval cell mediated liver regeneration, allowing for increased cell cycle progression. This idea is supported by decreased expression of p21 and increased expression of p-AKT (data not shown). It has further been suggested that results such as these along with the function of Notch1 to inhibit hepatocyte proliferation under non-pathological conditions could mean that Notch signaling is part of a tumor suppressor-like program in the liver. Although no data has been provided to show that Notch1 inactivation leads to the development of carcinomas in the liver,
the spontaneous proliferation of hepatocytes when Notch is inhibited may represent the first “hit” in a model of tumorigenic development, where further hits are required in order for hepatocyte growth to be completely disregulated. Determining if inactivation of Notch signaling promotes tumorigenesis could prove to be an exciting avenue to pursue.

As Notch is known to be involved in the mediation of binary cell fate decisions in other systems $^{100-103}$, it is logical to conclude that interruption of the signal would lead to impaired integration of cellular fate by the activated oval cells. However much remains to be understood about the mechanisms by which Notch signaling regulates oval cell development and growth. Our studies provide evidence that Notch signaling is activated and plays an important role in the differentiation and proliferation of oval cells after 2AAF-PH. Moreover the data presented here demonstrates that when Notch signaling is inhibited, other downstream signals are also influenced thereby leaving the newly formed hepatocytes functionally deficient. Further studies will be required to fully delineate the effects of Notch signaling on these processes.

6.3 Future Studies

6.3.1 Exposure of Oval Cells to Notch1

Isolation of oval cells and exposure of them *in vitro* to Notch1 may induce cholangiocyte differentiation in culture faster than current differentiation methods, and might also aid in protocols calling for oval cell transplantation and engraftment. Recombinant rat Notch1 protein is now available from R&D Systems and could easily be employed in an *in vitro* model. Furthermore, portal injections of Notch1 during 2AAF-PH could potentially enhance the oval cell response *in vivo*.

6.3.2 Conditional Knockout of Notch1 *in vivo*

Changes in Notch levels during embryogenesis results in severe and drastic malformations of numerous tissues and/or failure of the embryo to fully develop, therefore development of a
conditional Notch1 knockout animal could further define the role of Notch1 in oval cell-mediated liver regeneration. Creation of inducible-Notch-signaling systems has already been described for analysis of the necessity of Notch signaling for such diseases as Ewing Sarcoma and Kaposi’s Sarcoma. Controlling Notch1 expression with a tetracyclin (Tet) on/off system using the albumin promoter would result in a conditional knockout that would only be active in the liver when desired, i.e. at specific timepoints during the 2AAF-PH protocol. This knockout would confirm the results seen in this study and provide alternative methods for looking at the role of Notch1 in the liver.

Furthermore, use of the Tet on/off system would allow for a model of Notch recovery during 2AAF-PH, where Notch is initially turned off but then activated at the appropriate timepoint after partial hepatectomy. If the oval cells proceed to differentiate properly and are functional after Notch signaling is recovered, that would be irrefutable proof that Notch1 is required for functional differentiation of oval cells during liver regeneration.

6.3.3 Notch and Wnt: Oval Cell Fates Hang in the “Balance”

Crosstalk between the Notch and the Wnt signaling pathways has been demonstrated in a multitude of models in the literature. Inhibitory cross-communication between the two pathways was previously thought to occur at the level of the Notch intracellular cytoplasmic domain binding to Wnt and at the level of the Notch intracellular cytoplasmic domain binding to disheveled. However it has recently been shown that interpathway communication can also occur at the level of processing molecules such as presenelin, or even at the transcription factor level.
6.3.4 Notch and Liver Tumorigenesis

There has been no data published thus far implicating Notch in liver tumorigenesis, giving this area of research quite the potential. As Notch can exert tumor-suppressive and differentiation-promoting activities, if its signaling does have a role in tumorigenesis it is as yet unclear. It is possible that tumor progression might select for cells that are refractive to Notch signaling in tissues in which absence of this pathway promotes growth, enhances survival and/or inhibits differentiation. Also as previously stated, Notch alone is a poor oncogene and needs a “partner in crime” so to speak, one that would allow the cells to override the G1/S checkpoint. There are several likely candidates for this other oncogene, including Myc and Ras.\textsuperscript{110,111}

Furthermore, the inability of Notch1 to allow for override of the G1/S checkpoint indicates that it has some other tumorigenic capability, such as transferring resistance to anoikis or p53-mediated apoptosis.\textsuperscript{112,113} The latter seems to be the most likely scenario, one that could easily be investigated by analysis of expression levels of p53 upon Notch inhibition as well as a TUNEL assay for cell death or immunohistochemistry for such markers of apoptosis as the caspases (3, 8). However, resistance to anoikis could be investigated in isolated primary oval cells treated with GSIXX or other Notch inhibitor \textit{in vitro}.

6.3.5 Summary of Proposed Experiments

Each of the experiments proposed above would help to confirm the results seen in this study, as well as enhancing the knowledge of the Notch pathway’s role in oval cell-mediated liver regeneration. Complete evaluation of the contribution of Notch signaling to the regeneration process, as well as its communication with other signaling modulators, is essential for full understanding of oval cell-mediated liver regeneration. With this study and future experiments we hope to demonstrate the necessity for Notch signaling in oval cell-mediated liver regeneration, as well as how disregulation of Notch plays a role in disease and malignancy.


88. Lefort K, Dotto GP. Notch signaling in the integrated control of keratinocyte growth/differentiation and tumor suppression.


BIOGRAPHICAL SKETCH

Houda A. Darwiche was born in Merrillville, Indiana in 1982. She moved to Florida with her parents in 1985, where she has remained ever since. Houda attended Winter Haven High School in Winter Haven Florida, where she was very active in the Drama department as well as a member of the National Junior Honor Society and Future Business Leaders of America. Houda graduated in the top ten of her class in 2000, and was awarded a full scholarship to Florida Southern College (FSC) in Lakeland, Florida.

While attending FSC she was an active member of several groups and honoraries including Omicron Delta Kappa, Phi Eta Sigma, Beta Beta Beta and Mu Alpha Theta. She also served as both president of the FSC Student Chapter of the American Chemical Society, as well as president of the chemistry honorary Gamma Sigma Epsilon for two years. Under her leadership the student chapter of ACS was given awards at the SouthEastern Regional Meeting of the American Chemical Society in 2003. In 2004, Houda graduated magna cum laude with a Bachelor of Science in both biology and chemistry with a minor in mathematics.

Houda was accepted to the Interdisciplinary Program in Biomedical Sciences at the College of Medicine in the University of Florida in 2004. During her graduate school work, Houda was active in student organizations on campus, most notably the College of Medicine’s Graduate Student Organization (GSO). Houda also served as GSO treasurer for two years and was involved in organizing the monthly Biomedical Research Seminar hosted by the GSO. For her graduate work, Houda studied under Dr. Bryon E. Petersen in the department of Pathology, Immunology, and Laboratory Medicine. Her project concerned ascertaining the role of the Notch signaling pathway in stem cell based-liver regeneration. A portion of this research was presented in Snowmass, Colorado at the 2008 Federation of American Societies for Experimental Biology (FASEB) conference in liver biology.
Upon completion of her dissertation requirements, Houda received her Ph.D from the University of Florida in the summer of 2009. She went on to accept a two-year post-doctoral position in the laboratory of Dr. Chen Liu under the direction of Dr. David Nelson in the department of Medicine (Gastroenterology) at the University of Florida.