EXPRESSION OF NOTCH-1 AND ITS LIGAND JAGGED-1 IN THE 2AAF/PHx OVAL CELL MEDIATED LIVER REGENERATION MODEL

By

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A THESIS PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE

UNIVERSITY OF FLORIDA

2005

This document is dedicated to my daughters Moira and Threnody Hatch.

ACKNOWLEDGMENTS

I would like to express my deepest gratitude to my mentor, Dr. Bryon Petersen, for his encouragement, support, and understanding throughout the course of my work for this thesis. I would also like to sincerely thank the members of my master's thesis committee: Dr. Ammon Peck, and Dr. Gregory Schultz. Their advice was crucial to my success in this research. I would also like to extend my thanks to Drs. Seh-hoon Oh, Rafel Witek, Jie Deng, Takahisa Fijikawa, and Thomas Shupe. I would like to thank Donghang Zhen, Youngmi Jung, Jennifer LaPlante, Marda Jorgensen, and Doug Smith for all of the experimental advice, friendship, and support they have shown me throughout my work at the University of Florida. Finally, I would like to give a special thanks to my husband, Adam Staple; my parents, Brad and Kim Hatch; and my two daughters, Moira and Threnody Hatch, for all of the support they have offered to me over the years.

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August 2005

Chair: Bryon E. Petersen
Major Department: Molecular Genetics and Microbiology

The Notch/Jagged signaling pathway has been shown to be directly involved in cellular differentiation and proliferation. Dysfunction of this pathway is associated with human pathologies in several tissues including the liver. In normal rat liver Notch-1 is expressed in hepatocytes, bile ductular and endothelial cells, whereas Jagged-1 is expressed in hepatocytes and bile ductular cells. The current study set out to examine the role of these transmembrane proteins in an oval cell mediated liver regeneration model. Utilizing the 2-acetylamino-fluorene/partial hepatectomy (2AAF/PHx) model for oval cell activation and reverse transcriptase polymerase chain reaction (RT-PCR), we show an upregulation of gene expression for both Notch-1 and its ligand Jagged-1 as compared to normal rat liver. These results are further confirmed through the use of quantitative real time PCR, which reveals a 6.7 fold increase for Notch on Day 11 and a 22.8 fold increase of Jagged on Day 13 during oval cell proliferation. Western blot analysis also demonstrates the expression levels of both proteins increasing as oval cell numbers rapidly expand. In addition, immunohistochemistry reveals the translocation of intra-

cytoplasmic Notch from the cytoplasm to the nucleus has occurred. Lastly, using both RT-PCR as well as quantitative real time PCR analyses revealed an upregulation of the HES-1 gene, a Notch downstream target. These data further illustrate the Notch/Jagged pathway had been activated.

To determine the role that Notch/Jagged plays during oval cell activation and liver regeneration, siRNAs were utilized to block expression of these proteins. Hematoxylin and eosin staining shows the presence of activated oval cells in all time points including Day 22 post PHx where oval cells usually are not seen. RT-PCR demonstrates a down regulation of Notch and Jagged in siRNA treated tissues. Down regulation of alpha-feto protein (AFP) is also demonstrated and confirmed in Northern blot analysis. Real-time PCR analysis shows a dysregulation of Notch/Jagged AFP mRNA expression. Immunohisotochemical staining for oval cell markers (i.e., OV6, Thy-1) indicates that the cells present in the siRNA treated tissues are oval cells. These data illustrate that the Notch/Jagged pathway not only is active within hepatic oval cell mediated liver regeneration, but that it may affect multiple signaling pathways which contribute to the differentiation of these progenitor cells toward a mature phenotype.

CHAPTER 1
LITERATURE REVIEW

Introduction

The Notch/Jagged Pathway

Several animal models of chemical hepatoxicity have been developed and utilized in the study of the mechanisms regulating the proliferative response to liver injury. However, in most models, the oval cell response is negligible and the role, which is played by these cells and their differentiation, cannot be studied. Using the 2AAF/PHx, the role of the Notch/Jagged pathway was explored in reference to the differentiation and replacement of acutely injured liver tissue.

A highly conserved set of homeobox genes encodes the group of heterodimeric transmembrane proteins that make up the four Notch receptors. These receptors, along with their ligands, Jagged-1, Jagged-2, and Delta, which are also highly conserved, are involved in various cell fate decisions, such as proliferation, differentiation, and apoptosis. Notch signaling regulates differentiation of many cell types, including hematopoietic progenitors, lymphoid, myeloid, and erythroid precursors; as well as B and T cells, monocytes, and neutrophils. Drawing from what is known about the cell populations in which the Notch pathway is active, it can be inferred that Notch signaling may also be involved in lineage decisions and differentiation at several stages throughout hematopoietic development.

The binding of these ligands triggers activation of the Notch pathway. Binding of the receptor induces cleavage of the transmembrane subunit which then in turn releases

the intracellular domain. The intracellular domain then translocates to the nucleus where it plays a role in transcriptional regulation of its target genes which include basic helix-loop-helix transcription factors. This is done largely through the transcriptional regulator C promoter binding factor-1/recombination signal sequence binding protein-JK (CBF1/RBP-J_K).

Notch is an important signaling receptor that contributes to proper development, influencing cell fate, proliferation and survival. The expression of Notch receptors on hematopoietic stem cells is well documented. Several reports have demonstrated that hepatic oval cells and hematopoietic stem cells share many of the same surface markers (Thy-1, CD34, c-kit) (1,2,3). Thus, it may be possible to show that these cells are capable of differentiating into hepatic cells *in vitro* through the interactions of the Notch/Jagged pathway and the microenvironment present within the injured liver.

Discovery of Notch

Drosophila malanogaster has played an integral role in the identification and study of the Notch gene and the pathway with which it is now associated. Notch made its first noted appearance during genetic experiments, in which several differing phenotypic variations were noted, one of which was expressed as a “notch” in the wing of flies that had a haploinsufficiency for this particular gene. The gene was later identified and cloned by Artavanis-Tsakonas *et al.* (4) in the late 1970s to mid-1980s.

Following its discovery, the expression of the Notch gene was studied in *Drosophila*. During these studies, the Notch gene 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.

Notch in Lower Organisms

Notch has been shown to be essential for the embryonic development in *Drosophila*. In *Drosophila*, normal neurogenesis, myogensis, wing formation, oogenesis, eye development, and heart formation are all dependant upon the proper expression of the Notch gene (4). Lateral inhibition was shown to function through research done during neurogenesis. The major target gene for Notch, achatete-scute complex (AS-C) was shown to be suppressed in proneural cells, which inhibits further differentiation into neural cells (5). AS-C also proved to be an activator of the Notch ligand, Delta (6). Delta was then shown to be suppressed in response to Notch activation (4) completing a feedback inhibition loop effectively controlling Notch signaling and amplifying the effect of "lateral inhibition." In this case, lateral inhibition refers to a mechanism in which two identical adjacent cells can be induced to differentiate down different lineages during development. This mechanism is thought to play a prominent role in the development of "boundaries" during embryogenesis and development.

Many cells can choose a "default" pathway during development. It is believed in general that Notch inhibits this selection and instead promotes an alternative fate. The signaling pathways involved are extensive and their interactions and their potential for influencing multiple outcomes is not as yet well understood. As with neurogenesis, Notch has been shown to suppress muscle cell differentiation (7). In wing formation, Notch triggers the wing cell proliferation, which is required for formation of the wing through the expression of a vestigial gene (8,9). The expression of the wingless gene, the homolog of the mammalian Wnt gene, has also been shown to be regulated through

Notch. Interaction between the Wnt and Notch signal pathways have recently been shown to take place, though it is believed to have distinctly different effects than those seen from Delta mediated Notch signaling (10,11).

Notch in Humans and Mammals: Structure

The human Notch receptor family is comprised of four members (Notch 1-4). Each of the members shares a high degree of structural homology with one another. This homology extends across species as diverse as *Drosophila*, *Mus,* and *Rattus* as well.

In general, the extracellular domain of Notch 1 and Notch 2 is comprised of 36 epidermal growth factor (EGF)- like repeats and 3 membrane proximal Lin-12/Notch/Glp-1 (LNG) repeats. Notch 3 and Notch 4 have 34 and 29 EGF repeats respectively (12-16). The intracellular domain of Notch receptors consists of a RAM domain, 6 ankyrin (ANK) repeats, 2 nuclear localization sequences (NLS), one immediately preceding and one following, a transcriptional activator domain (TAD), and a proline-glutamate-serine-threonine-rich (PEST) domain, except for Notch 4 . Notch 4 has a shorter intracellular domain because of the lack of the NLS (13,14).

The RAM domain is the primary binding site for the C promoter Binding Factor-1 (CBF1)/ Recombination signal Binding protein-J kappa (RBPJκ) (17), and a homolog of *Drosophila* Su(H). The ANK repeat domain can also interact with the CBF1 (18). ANK repeats are also binding sites for several other proteins such as Deltex and Mastermind, which modulate Notch signaling. Notch can also be bound by Numb; another membrane bound protein, which acts to inhibit Notch (19,20).

Notch 4 was first identified in murine cells as a common integration site (int3) for a retrovirus, Mouse Mammary Tumor Virus (MMTV), and is associated with tumors. Int3

encodes the intracellular domain of Notch 4 (13,14,21). This gene is directly involved in malignancy.

Notch in Humans and Mammals: Signal Transduction

Notch signaling requires three proteolytic events to occur. A glycosyltransferase, which is known to be a product of the Fringe gene, acts in the Golgi to modify the EGF modules in the extracellular domain of Notch. This cleavage is referred to as the S1 cleavage site and precedes embedding of the Notch receptor within the plasma membrane (22). The resulting receptor unit within the membrane is a non-covalent heteroduplex (23). This heteroduplex consists of a 180kDa fragment that contains the majority of the extracellular domain and a 120kDa fragment consisting of the membrane tethered notch intracellular domain (NICD) containing a short EC sequence (24). This action alters the ability of Notch to bind to a specific ligand and appears to regulate ligand specificity. In mammals, this heterodimeric form comprises the majority of the Notch 1 receptor at the cell surface and is responsible for signaling through the Su(H)/CBF1-dependant pathway (23,24).

The second proteolytic (S2) event to occur during Notch signaling is considered the ligand-dependant cleavage of the Notch receptor. This cleavage occurs following ligand binding to the extra-cellular domain (ECD) and is dependant upon a member of the enzyme family of disintegrins and metalloproteases (ADAMs). It is believed that ADAM17 is the active metalloprotease in mammals and possibly TACE TNF alpha converting enzyme though some questions still remain (25). It is possible that the S2 cleavage may involve the activities of more than one enzyme and inspection of the protein sequences suggests that the S2 cleavage mechanism may not be strictly conserved between members of the Notch family (26). This cleavage produces the membrane-

tethered form of the NICD, which is the constituitive substrate for the final proteolytic cleavage, S3 (27).

The final cleavage, S3, takes place prior to the signal-generating step in the Notch signal transduction pathway. This step takes place within the transmembrane domain and releases the soluble NICD (28). Presnilin proteins are required for this process to be completed (28,29). In mammals, presnilin 1 and 2 are associated with the gamma-secretase activity that cleaves the amyloid precursor protein (APP), also within its transmembrane domain (28, 30-33), which allows soluble NICD to be release from the membrane and transported to the nucleus.

The translocation of soluble NICD to the nucleus which then activates the Su(H)/CBF1-dependant signal. The NICD binds via the RAM domain and ankyrin repeats to the Su(H)/CBF1 transcription factor enabling transcription to occur. In the absence of the NICD, CBF1 can repress transcription through the recruitment of histone deacetylases (HDAC) (34). Alternative mechanisms for Notch signaling have been proposed and recently reviewed (35). Among these alternative mechanisms, Deltex, a cytoplasmic Notch binding protein, is associated with CBF1-independent functioning of the Notch signaling pathway (36). Deltex has also been shown to be a positive regulator of the CBF1-dependant Notch activity upstream pf the S3 cleavage in some developmental contexts (37).

Downregulation of Notch signaling also involves proteolytic cleavages that are mediated by another enzyme, ubiquitine ligase. Sel-10, a *C. elegans* substrate-targeting component of a Skip1-cullin-F-box (SCF) class E3 ubiquitin ligase, was originally identified as a negative regulator of Notch signaling (38). Mammalian homologues of

Sel-10 have been found to stimulate phosphorylation dependant ubiquitinization of nuclear NICD and trigger proteosome-dependant degredation (39,40). Phosphorylation and turnover of NICD can also be stimulated by the transcriptional activator Mastermind that is recruited by NICD into the CBF1 complex providing a feedback inhibition of the Notch signaling pathway (41).

The Liver

The liver is the largest and one of the most important organs in the body. It functions as a storage unit for vitamins, sugars, fats and other nutrients that the body requires and plays a critical role in regulating the glucose levels in the blood. It produces many molecules such plasma proteins and hormones that are released directly into the blood stream. It detoxifies the blood, removing substance such as alcohol and xenobiotics ingested with food. In short, the liver acts as a biotransformation machine, using enzyme systems to metabolize proteins, lipids, and glycogen, and conjugating dangerous metabolites so that they may be safely excreted from the body.

The liver plays a vital and irreplaceable role in the body. Despite this, it is on the front line in the daily assault caused by ingested toxins. Massive death of hepatocytes caused by free radicals and other highly reactive electrophiles released by the breakdown of toxins places enormous pressure on the liver. This pressure has created an evolutionary drive for quick and efficient regeneration.

Anatomy and Physiology

The liver is the largest glandular organ in the body. The smallest structural unit of the liver is the hepatic lobule that is comprised of hepatic plates. Hepatic plates are hexagonally shaped, plate-like structures made of a single layer of hepatocytes radiating from the central vein approximately 0.3-0.5mm in diameter (42,43). The edges of each

lobule are interconnected with those of others which drain into the portal triad made of the afferent vessels of the liver: branches of the portal vein and hepatic artery, and bilary ductules. The blood from the portal vein bring high concentrations of nutrients from the entire gastrointestinal tract, while blood form the hepatic artery provides highly oxygenated blood. In a hepatic lobule, the hepatic plates radiate from the terminal branches of the hepatic triad. Canaliculi that form between individual hepatocytes connect with the terminal biliary ducts in the portal triad. Capillary sized sinusoids line either side of the plate and circulate blood from the portal triad toward the central vein. This arrangement allows blood and fluid to flow from the periphery of the lobule toward the central vein. These lobules, arrays of hepatic plates, form tri-dimensional structures comprising the liver.

The functional unit in the liver is the hepatic acinus. This unit, as defined, recognizes the real pattern of blood flow in the liver and hepatocytes as secretory cells, secreting bile. The acinus is comprised of 3 zones. The overall organization of the acinus is characterized by a mass of parenchymal tissue surrounding and extending from the fine terminal branches of one portal triad toward another. The hepatocytes in the innermost zone or zone 1, which surrounds the portal triad, receive blood first. This blood originates from the hepatic artery meaning that these hepatocytes receive the highest concentration of oxygen and are the most metabolically active. The hepatocytes in the intermediary zone, zone 2, receive less oxygen than those in zone 1 and the hepatocytes in the final zone, or zone 3, receive the least oxygen. This arrangement dictates that metabolic potential decreases as distance from the portal triad increases (44).

The majority of blood circulating through liver passes through the gastrointestinal tract first carrying with it digested nutrients which will be further processed in the liver. The remaining blood found in the liver comes from the heart through the hepatic artery and is oxygen rich. The hepatic vein carries blood away from the liver into the inferior vena cava toward the heart. The microvasculature comprised of sinusoids and the small branches of the efferent and afferent circulatory system retain approximately 25% of cardiac output, making the liver an important blood reservoir.

Hepatic Cell Types

There are two major categories of cells in the liver. Each cell type expresses specific, characteristic phenotypic markers (*table 1*). The principle cell type in the liver responsible for most of hepatic function is the parenchymal cell. Parenchymal cells, or epithelial cells, include hepatocytes and biliary ductular cells arranged in hepatic plates. Non-parenchymal cells, found in the sinusoids, are comprised of sinusoidal endothelial cells, Kupffer cells, hepatic stellate cells, and pit cells. These individual cell types create through cell-cell contacts and contact with the extra cellular matrices (ECM) form a unique architectural arrangement that facilitates the many functions of the liver.

Liver Regeneration

Liver regeneration is accomplished through compensatory hyperplasia following cell loss or injury. This hyperplasia replaces the cell mass and general architecture of the liver as shown by Higgins and Anderson (Figure 1) with the two-thirds partial hepatectomy model in rodents (45). Morphologically and functionally, the remaining hepatocytes maintain an active urea cycle, albumin synthesis, and drug metabolism as well as exhibiting normal polarity of membrane domains during this process (46).

However, this hyperplastic response does not yield new lobes to replace those lost.

Instead, massive cell replication and remodelling of the remaining liver is undertaken.

Table 1. Characteristic Phenotypic Markers of the Liver.

Markers	oval cells	Hepatocytes	bile duct cells	Ito cells	endothelial cells
CK7	-	-	+	-	-
CK8	+	+	+	-	-
CK18	+	+	+	-	-
CK19	+	-	+	-	-
ALB	+/-	+	-	-	-
AFP	+	-	-	-	-
GGT	+	-	+	-	-
OV-6	+	-	+	-	-
OV-1	+	-	+	-	-
BDS7	+	-	+	-	-
BD1	-	-	+	-	-
HES6	-	+	-	-	-
OC.2	+	-	+	-	-
OC.3	+	-	+	-	-
H.1	-	+	-	-	-
HBD.1	-	+	+	-	-
A6	+	-	+	-	-
Desmin	-	-	-	+	-
GFAP	-	-	-	+	-
SE1	-	-	-	-	+

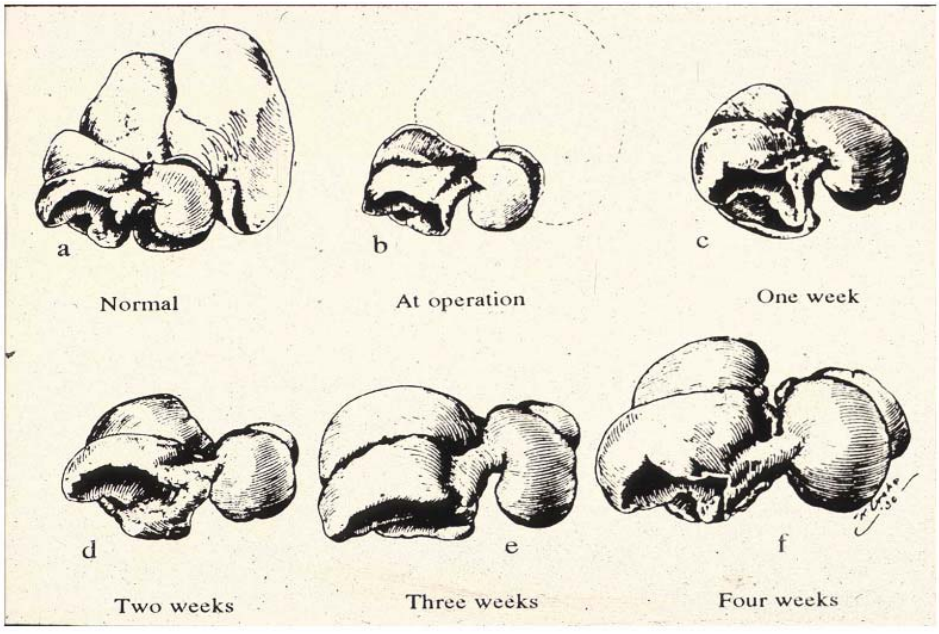


Figure 1. Higgins and Anderson, Cartoon of Liver Regeneration post Partial Hepatectomy. From Experimental Pathology of Liver Resection.

Liver regeneration following hepatic injury can take place using one of two mechanisms: replicative hepatocytes drive regeneration or progenitor dependant regeneration. Both regeneration mechanisms involve systematic replacement of lost tissue through a series of orchestrated events including: ECM rearrangement; resetting the cell cycle for mitosis; cellular division; shutting down the cell cycle and rearrangement of newly formed tissues. These processes are strictly regulated and end when the liver returns to its original mass.

Hepatocyte-Driven Regeneration

Under normal metabolic conditions, hepatocytes are quiescent in the G_0 phase of the cell cycle and exhibit minimal replicative activity. However, under circumstances where significant injury and/or cell death is incurred, synchronous entry into the cell cycle may be observed in the remaining hepatic cells. In the two-thirds partial hepatectomy (PHx) liver regeneration model, replication of the remaining hepatic cells can be seen emanating from the periportal region of the acinus in zone 1, followed by cells in the pericentral region in zone 2. The kinetics of cell proliferation and the growth factors produced by replicating hepatocytes suggest that hepatocytes provide the mitogenic stimuli leading to the proliferation of other hepatic cell types. There are two peaks of hepatocyte proliferation. The first within 24 hours of PHx, while the second follows at 48-72 hours post PHx and is much smaller in magnitude. Biliary ductal cells also show a peak in proliferation at the 48 hour time point followed closely by stellate and Kupffer cells. Sinusoidal endothelial cells have the slowest regeneration time at 3-8 days post PHx (47). Unlike the parenchymal cell types, DNA synthesis in non-parenchymal cell populations does not begin around the portal triads, but takes place

throughout the liver (48). The spatial distribution of proliferating hepatocytes in the regenerating liver has a predictable pattern during these processes (49).

Progenitor Cell Dependant Regeneration

When hepatic damage is too wide spread or cell proliferation is inhibited as a result of either hepatotrophic viral infection or hepatotoxic intermediates resulting from the metabolism of foreign compounds, progenitor cells such as hepatic oval cells (HOCs) are recruited to replenish the function of the liver (50). Morphologically, HOCs are characterized as being small in size ($\approx 10\mu m$), with a large nucleus to cytoplasm ration, and an oval shaped nucleus (51). Following activation, large numbers of HOCs can be seen to migrate from the bile ductules into the hepatic parenchyma (52).

Activated HOCs differentiate into basophilic small hepatocytes and then fully functioning mature adult hepatocytes (51,53). HOCs are also capable of differentiating into intestinal type epithelium *in vivo* (53), as well as bile ductular and pancreatic-like cells *in vitro* (54,55).

The origin of HOCs is controversial though it was widely accepted for some time that these cells originate in the transitional zone known as the canals of Herring located between periportal hepatocytes and the biliary cells lining the smallest terminal bile ducts (51). Petersen et al. demonstrated that selective damage of bile ductular epithelium in the periportal zone reduces HOC proliferation (56). HOCs in the rat have been found to express the bile ductular epithelium maker CK-19 as well as the hepatic marker, albumin. They also express alphafeto protein and are positive for monoclonal antibodies such as OV6, OC.2, and BD1. HOCs have also been found to express select hematopoietic stem cell markers such as CD-34, c-kit, Thy-1, AFP and Flt-3 (57-60). These findings raised the intriguing possibility that HOCs may originate within the bone marrow.

Evarts et al. demonstrated that the oval cell compartment is activated extensively in rats treated with 2-acetylamino-fluorene and two-thirds partial hepatectomy (2AAF/PHx) (61). This model of oval cell activation has been widely utilized in the study of the oval cell compartment over the past decade. This model is comprised of two surgeries. The first is the insertion of the 2AAF time-released pellet. This pellet is placed intraperitoneally where it releases a small and consistent dose of 2AAF over a period of 14-28 days. This dose inhibits hepatocyte proliferation through blockage of the cyclin D1 pathway and also acts to activate HOCs. The second surgery is a two-third PHx 5-7 day post 2AAF insertion. This severe mechanical injury induces massive proliferation, expansion and finally differentiation of the 2AAF induced HOCs within the parenchyma. 3 days post PHx, HOCs have been found to infiltrate the liver lobule and form elongated ductular structures. Approximately 7-11 days post PHx HOC differentiation peaks and sometime with the 10-11 day time frame differentiation begins forming small foci of hepatocytes and intestinal-type structures (62,63).

In HOC mediated liver regeneration injury induces changes in cytokines and growth factors definitively affect the fate of proliferating HOCs. Growth modulators such as stem cell factor (SCF), transforming growth factor alpha (TGF-a), epidermal growth factor (EGF), hepatocyte growth factor (HGF), urokinase-type plasminogen activator (uPA), leukemia inhibitory factor (LIF) and their receptors are involved in oval cell proliferation. Cellular localization of the different components indicates that an intricate web of paracrine and autocrine mechanisms are involved in the regulation of growth.

Specific Aims of this Study

Elaboration upon the Notch signaling pathway, which is involved in many cell processes, including differentiation, proliferation, and apoptosis, may prove integral in

learning to induce and/or control those processes involved in repair/regeneration and dysfunction of the liver. The goal of this research is to lay a basic foundation upon which other research in areas such as stem cell, cell therapy and gene therapy may build in order to correct abnormal function or restore function in the best interest of the medical patient.

Liver regeneration following injury is well organized. In cases in which hepatic cells are no longer able to replicate, HOCs must be activated and affect the repair of the liver. The Notch/Jagged pathway became of interest when it was shown to play a role in normal liver regeneration by Kohler *et al* (64). **For this study we asked specifically, what is the role of Notch and Jagged during regeneration in the 2-AAF/PHx oval cell mediated liver regeneration model?** Where are these proteins expressed in the 2AAF/PHx liver regeneration model and what are the consequences involved with interfering with their expression? To begin to answer these questions, experiments were performed as outlined in Kohler *et al.* This study incorporated methods from molecular genetics, molecular biology, toxicology, and pathology.

CHAPTER 2
MATERIALS AND METHODS

Experimental Animals

Female Fischer 344 rats (120-150g) were used for all experiments. All animals were housed in pairs in air-conditioned rooms at temperatures between 22-25°C. Animals received ad libidum food and water and were kept on 10:14 hour dark/light cycles. Prior to sacrifice, animals were anesthetized using an intraperitoneal injection of sodium pentobarbital. All animal protocols followed recommendations of the Association for Assessment and Accreditation of Laboratory Animal Care and were approved by the University of Florida Institution Care and Use Committee.

Oval Cell Activation by 2-AAF/PHx

2-acetylamino-fluorene is metabolized through the cytochrome P450 pathway in hepatocytes.The three main metabolites of 2-AAF include N-hydroxy-AF, a sulfate ester, and an O-glucuronide. All of these compounds are considered to be mutagenic and carcinogenic.2-AAF implantation in conjunction with hepatic injury can activate the hepatic oval cell compartment (60). The inhibition of hepatocyte proliferation is believed to be required for efficient oval cell activation in the liver. A two-third partial hepatectomy is performed 5-7 days post 2-AAF implantation in order to activate the oval cell compartment. The procedure to remove two-thirds of the liver consists of removal of the median and left lateral lobes of the liver leaving intact the right lateral and caudate lobes.

Sample Collection

Animals were sacrificed at days 7, 9, 11, and 22 post PHx and their livers harvested. Each liver was cut into 1-2 cm slices. These livers were made into cryo-blocks using Thermo Shandon Optimal Cutting Temperature (OCT) compound, snap frozen, or soake in 10% buffered formalin for a period of 12 hours followed by 1X phoaphate buffered saline buffer and refrigeration until processing and embedding.

Tissue Sectioning

Five micrometer frozen sections were cut from OCT embedded tissues and placed on positively charged slides. These slides were allowed to air dry and tissue was fixed according to immunohistochemical or Immunofluorescence protocol to be observed.

Five micrometer paraffin sections were cut and stored for later use. When put to use, tissue sections were deparaffinized by two incubations in xylene for ten minutes followed by rehydration through bathing in a series of graded alcohols and then water.Endogenous peroxidase activity was quenched by incubation in 3% H_2O_2 for 10 minutes at room temperature.

Microscopy

Tissue sections mounted on glass slides were photographed using either bright field microscopy or fluorescence microscopy with excitation/emission wavelengths for Texas Red and FITC fluorochromes. Photographs were taken using constant exposure using a peltier-cooled Olympus digital camera.

RNA Isolation, PCR, and Real-time-PCR Analysis

Tissue (50mg) snap frozen in liquid nitrogen added to 1 ml RNAzol Bee (Friendly, TX) was used to isolate total RNA. DNAse I digestion and purification (Qiagen, Rneasy Minipreps) followed by reversed transcription reactions (Superscript II RNase H- reverse

transcriptase, Invitrogen, CA) were performed per manufacturer's protocol. The following primers (64) and reaction conditions were used for semi quantitative real-time polymerase chain reaction (PCR) using Applied Biosystems Core reagents: Notch mRNA was detected using primers 5'CACCCATGACCACTACCCAGTT3', which amplified a 186-bp fragment; Jagged-1 mRNA was amplified with 5'AACTGGTACCGGTGCGAA3' and 5'TGATGCAAGATCTCCCTGAAAC3' primers that generated a190-bp fragment. A 174-bp fragment of Hes-1 was amplified using the primers: 5'CGACACCHHACAAACCAAA3' and 5'GAATGTCTGCCTTCTCCAGCTT3' . The QuantumRNA Classic18s Internal standard (Ambion, TX) was used as a standard for real-time PCR according to manufacturer's protocol. The standard conditions used for real-time PCR were as follows: 50°C for 10 minutes and 95°C for two minutes followed by 50 cycles of 15 seconds denaturation at 95°C, 45 s annealing/elongation at 58°C or 60°C. SYBR® Green signal was measured in each step. Baseline was set between cycles three and 15. Each 96-well plate carried the same standard curve based on the 18s internal standard. Normal Rat Liver was used as further control and expression was put equal to 1.0. Further statistical calculations were carried out with the mean fold gene expression being calculated through the use of Applied Biosystems software utilizing the standard curve as well as the 2 (Delta Delta CCT) Method.

BrdU Incorporation In Vivo

Hepatocyte and HOC proliferation were measured in treated animals at each time point. Two hours prior to sacrifice of the animal, a 50mg/kg dose of BrdU was administered intraperitoneally. At the time of sacrifice, tissue was prepared and fixed in 10% normal buffered formalin, transferred to PBS and then processed and embedded in

paraffin. 5μm section were cut from these blocks and stained for BrdU incorporation according to the protocol provided with the Dako Animal Research Kit in conjunction with the anti-BrdU antibody from Dako. All sections were counterstained using light green so as to maintain the clarity of the nuclear staining.

Characterization of Freshly Isolated, D11 Hepatic Oval Cells

The expression of several putative HOC markers as well as the presence of both Notch-1 and Jagged-1 proteins were tested on freshly isolated HOCs prepared as cytospins. Each slide was prepared by fixation in acetone for 10 minutes at -20°C after which they were blocked using Dako protein block and the Vector Labs Avidin/Biotin blocking kit according to recommendations. Primary antibody incubation was carried out using the antibodies and concentrations listed in *table2* for 1 hour at room temperature at which time the slides were then incubated with secondary antibodies at their respective concentrations for 30 minutes at room temperature.

Table 2. List of Antibodies.

Antibody	Source	Catalog Number	Concentration
OV-6	00 One Inch Spacer	gift	1:50
Thy-1/CD.90 FITC conjugated	BD Pharmingen	555595	1:250
Thy-1/CD.90 purified	BD Pharmingen	550402	1:250
Notch-1	Sanat Cruz	Sc-6014	1:100
Jagged-1	AbCam	Ab20580-33	1:100
Notch-1	Upstate	05-557	1:100
Anti-goat Texas Red	Vector Labs	TI-5000	20μg/mL
Anti-Hamster FITC	Vector Red	FI-9100	20μg/mL

Localization of Notch and Jagged Proteins in Rat Liver

The cellular distribution of both the Notch receptor and the Jagged ligand in the rat liver was investigated through use of formalin-fixed and paraffin embedded liver tissue taken from 2AAF/ PHx treated animals. Goat anti-Notch-1 (sc-6014) antibody at a 1:100 concentration was used in conjunction with the Vectastain ABC Peroxidase kit (Goat

IgG) following the protocol provided. Jagged-1 was detected/ localized using a goat polyclonal primary antibody from AbCam (ab10580-33, lot 45327) at a concentration of 0.2µg/ml. This was followed with the Vectastain ABC Peroxidase kit (Goat IgG) following the protocol provided.

Fluorescent confocal microscopy was utilized to further show the Localization of both Notch and Jagged and demonstrates the translocation of the NICD. 20µm cryosections were cut and dried at room temperature for 30 minutes, followed by fixation in 2% paraformaldehyde for 30 minutes followed by treatment with 3% H2O2 in MeOH for another 60 minutes both at 4□C. The sections were then blocked in 10% BSA at room temperature for 60 minutes and washed in PBS for an hour at room temperature. A dual primary antibody incubation was completed overnight at 4□C using the AbCam goat polyclonal antibody for Jagged-1 and Upstate hamster monoclonal anti-Notch antibody (05-557, lot 24918). The primary incubation was followed by a four-hour wash with two changes of PBS pH 7.4 at 4□C. A dual secondary incubation was done with Vector anti-Goat TEXAS Red-conjugated antibody and a Vector anti-Hamster fluorescein-Conjugated antibody at recommended dilutions overnight at 4□C followed by a 2-hour wash in PBS and cover slipping with Vectashield with DAPI.

Protein Extraction and Western Blotting

Analysis of protein upregulation and compartmentalization was carried out through the use of whole rat liver tissue, which was collected at specified time points and snap frozen in liquid nitrogen. Each time point contains equal portions of liver tissue from three separate animals. This pooled tissue was then separated into individual cellular compartments through the use of the CNM Compartment Protein Extraction kit from BioChain (K3012010, lot A612008) according to the protocol provided. Each of the

resulting liver lysates, cytoplasmic, membrane, and nuclear, were then assayed for total protein content using the Bio-Rad DC Protein Assay (500-0114). 100µg of total protein was denatured and loaded into a 7 % mini-gel and run at 100V until the dye front reached the bottom of the gel, transferred to 0.22µm PVDF membranes at 100V for one hour and blocked in a 5% milk solution overnight at 4°C. Each membrane was washed 3 times 15 minutes and incubated with the primary antibody for 1 hour at room temperature. Following primary antibody incubation the blots were washed three times for 10 minutes each in PBS pH 7.4, followed by a 30 minute incubation at room temperature with the secondary antibody and three more 10 minute washes in PBS. The AbCam and Upstate antibodies were used at the recommended dilutions for Western Blotting applications with the respective Santa Cruz secondary antibody being used at a concentration of 1:2000.

Silencing Jagged-1 and Notch-1 Using a siRNA Vector and *In Vivo* Transfection

For silencing experiments *in vivo*, the psiRNA-hH1neo plasmids containing specific Jagged-1 and Notch-1 sequences prepared by Kohler et al. was obtained, grown up, purified, and utilized. A scrambled sequence was used as a negative control. Each of the synthesized oligonucleotides was designed to contain a short sequence- TCCAAGAG- to transcribe a dsRNA with a hairpin structure. Competent GT116 *E. coli* were transformed and plasmid expressing E. coli were selected and grown up in LB broth containing 50µg/mL kanamycin. Plasmids were isolated using a Maxipreps (Qiagen). Animals on the 2-AAF/ PHx protocol were transfected with 50µg of plasmid using the *In vivo* jetPEI system (Q-Biogene, Irvine, Ca.) via the tail vein at days 7, 9, 11, and 13 post partial hepatectomy. Silencing of *Notch-1* and *Jagged-1* was controlled by

immunohistochemical detection of Notch -1 and Jagged-1 protein in normal rat liver sections.

CHAPTER 3
RESULTS: HEPATIC OVAL CELL EXPRESSION PROFILE OF NOTCH AND JAGGED

Introduction

Liver regeneration involves waves of proliferation, migration, and differentiation which is influenced by a complex mixture of cytokines and chemokines to restore liver mass and function (47). When hepatocyte proliferation is inhibited by hepatotoxic agents, a distinct liver progenitor cell, known as the hepatic oval cells, is recruited to aid in liver regeneration (53,61). This phenomenon is demonstrated well through the Solt-Farber liver injury model in which 2-AAF administration in concert with PHx initiates oval cell activation and proliferation in the periportal region of the liver lobule. 2-AAF induced oval cells have been shown to originate from within the biliary ductal epithelia and are characterized by expression of phenotypic markers such as AFP, CK-19, and Thy-1 (54,59).

Preliminary studies were undertaken to determine whether Notch and Jagged were expressed in the 2-AAF/PHx oval cell mediated liver regeneration model.

Notch and Jagged Localization in the 2-AAF/PHx Model

In order to determine whether further study of this model of liver regeneration was worthy of further study in reference to the Notch/Jagged signal transduction pathway, immunohistochemical staining was completed.

5μm sections of tissue from animals place on the 2-AAF/PHx oval cell activation protocol were tested for the presence of the Notch receptor and its ligand Jagged. The

results demonstrate the presence of both proteins (Figure 2). Panels A and B demonstrate that Notch is expressed by both hepatocytes and cells that appear to be hepatic oval cells. Hepatocytes that are positive are in close relation to the smaller HOC-like cells. Panels C and D demonstrate that Jagged is also expressed in the 2-AAF/PHx model though it appears to expressed on smaller hepatocytes and ductular formations.

To further justify the study of the 2-AAF/PHx model in reference to the Notch/Jagged pathway translocation of the Notch intracellular domain (NICD) to the nucleus needed to demonstrated. Figure 3d shows Immunofluorescence staining for Notch and the translocation of the NICD to the nucleus. This indicated that the Notch/Jagged pathway is active in the 2-AAF/PHx oval cell activation model and called for further investigation to be conducted.

Characterization of Day 11 Activated Hepatic Oval Cells

A number of cell types comprised of parenchymal hepatocytes as well as non-parenchymal bile duct, stellate, Kupffer, and endothelial cells may be involved in generating the oval cell response. We are interested in whether the hepatic oval cell express proteins that would indicate that they are influenced by the Notch/Jagged signal transduction pathway.

Utilizing the Solt-Farber liver injury model followed by a 2-step liver perfusion at day 1 post PHx, activated oval cells were isolated from the liver. To separate parenchymal cells from non-parenchymal cells, a centrifugation method was used. Once the single-cell perfusate was passed through a 70 micrometer nylon mesh, it was centrifuged at 500 rpm. This speed causes the heavier parenchymal cells to pellet at the bottom of the centrifuge vessel. The cloudy fluid above the pranechymal cells contains the smaller non-parenchymal cells. This fluid can then be incubated with FITC

conjugated Thy-1 antibody (59) and once complete passed through a column containing magnetic beads conjugated with an anti-FITC antibody. Cells isolated in this manner may be further purified by subsequent passages through clean columns.

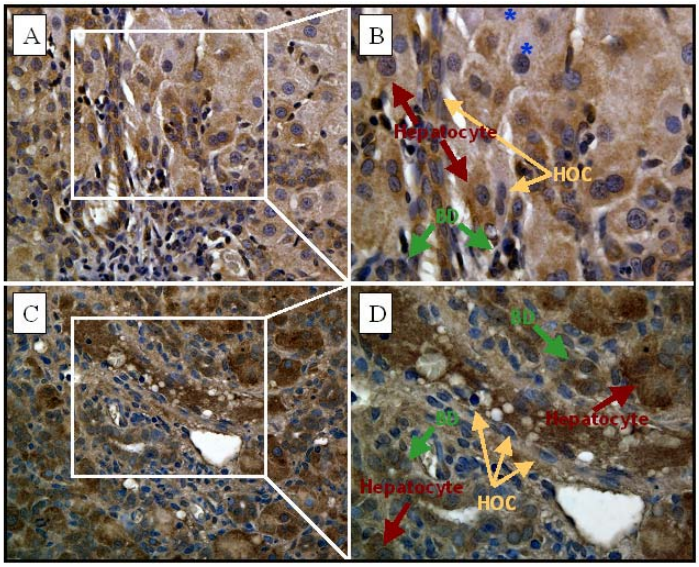


Figure 2. Immunohistochemistry for Notch (A and B) and Jagged (C and D) liver sections from 2AAF/ PHx treated rats. Notch expression appears to be strongly expressed on hepatocytes in close proximity to the proliferating oval cells (black arrows). The staining of Jagged appears to be located on oval cells and ductular formations created by the proliferating oval cells. At higher magnification, oval cells (green arrows) are clearly present. Asterisks in B indicate hepatocytes negative for Notch away from oval cell proliferation

Purified oval cells were washed and concentrated by centrifugation at 1000 rpm. Re-suspension and dilution so that there were approximately 100,00 cells per 80μl of Iscove's media were accomplished so that cytospins could be prepared. Preparation of cytospins was made through the use of a Thermo-Shandon Cytospin 4 and positively charged slides were used to avoid cell loss during staining. Once cytospins were made, they were air dried under bright light to allow cells to settle tightly onto the slide and to effect quenching of the FITC in the conjugated antibody. Immunohistochemical analysis for CD-45, OV6, Thy-1, Notch, and Jagged. Immunofluorescence was also completed for Notch and Jagged as described in Materials and Methods.

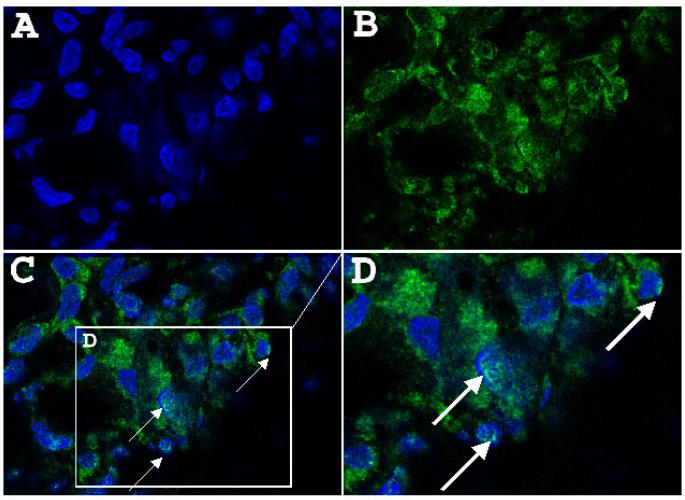


Figure 3. Immunohistochemistry for Notch translocation A) DAPI alone showing nucleus B) Notch Cytoplasmic localization. Merged images showing green flourescence now in the nucleus (C), indicating Notch intracytoplasmic domain has translocated to the nucleus. D) Higher magnification of boxed area in (C). Arrows show nuclei with positive signal.

An isotype control was set as a negative control to show that no unexpected staining was to be seen (Figure 4a). CD-45 was used to show that the population isolated for this experiment contained no inflammatory cell contamination (Figure 4b), which was indeed the case, and OV6 and Thy-1 were used to show that the isolated cells were hepatic oval cells as defined by prior research (Figure 4e and f). Isolated day 11 oval cells were very distinctly positive for the expression of the Notch receptor protein. These cells had a dark staining in the cytoplasm, in some regions of the nucleus, as well as on the plasma membrane (Figure 4d). However, there was very little Jagged staining and what appeared to be present was located in or very closely associated with the nucleus, indicating that this protein was not yet active in the role of ligand since it was not located on the cell surface (Figure 4c). These results demonstrated that HOCs could be affected by the Notch/Jagged signal transduction pathway.

Immunofluorescence also definitively showed the presence of the Notch receptor in the day 11 isolated oval cell population (Figure. 5b). The micrographs also demonstrate the presence of the NICD in the nucleus of this population, indicating that Notch is not only present, but is also actively taking part in the signal pathway (Figure 5d). Probing for the ligand, Jagged, was unsuccessful in this instance. No expression of Jagged was seen using these methods (Figure 5c).

RT-PCR for Expression of Notch and Jagged in the 2-AAF/PHx Model

Reverse transcriptase polymerase chain reaction (RT-PCR) was utilized using primers for Notch, Jagged, and Hes-1 published by Kohler et al. to test for transcription of these gene in snap frozen tissue from the 2-AAf/PHx model. Transcription of Notch, Jagged and Hes-1 were seen at all time points (Figure 6). Hes-1, found in many tissues (65), was found to be well expressed in normal and PHx treated liver tissue by Kohler et al. and was used as an indicator of Notch activation. In this study, it was found that Hes-1 activation followed the trend seen in Notch activation in 2AAF/PHx treated liver. This expected upregulation demonstrated that the 2AAF/PHx treatment does not interfere with the Notch/Jagged pathway allowing further investigation in this model to commence

Western Blot Analysis of the 2-AAF/PHx Model

Western blot analysis of the 2AAF/PHx tissue time points was undertaken using the same tissues that were utilized for RT-PCR. Western blot analysis of whole cell lysates was performed as outlined in the Material Methods and the results are shown in Figure 7.

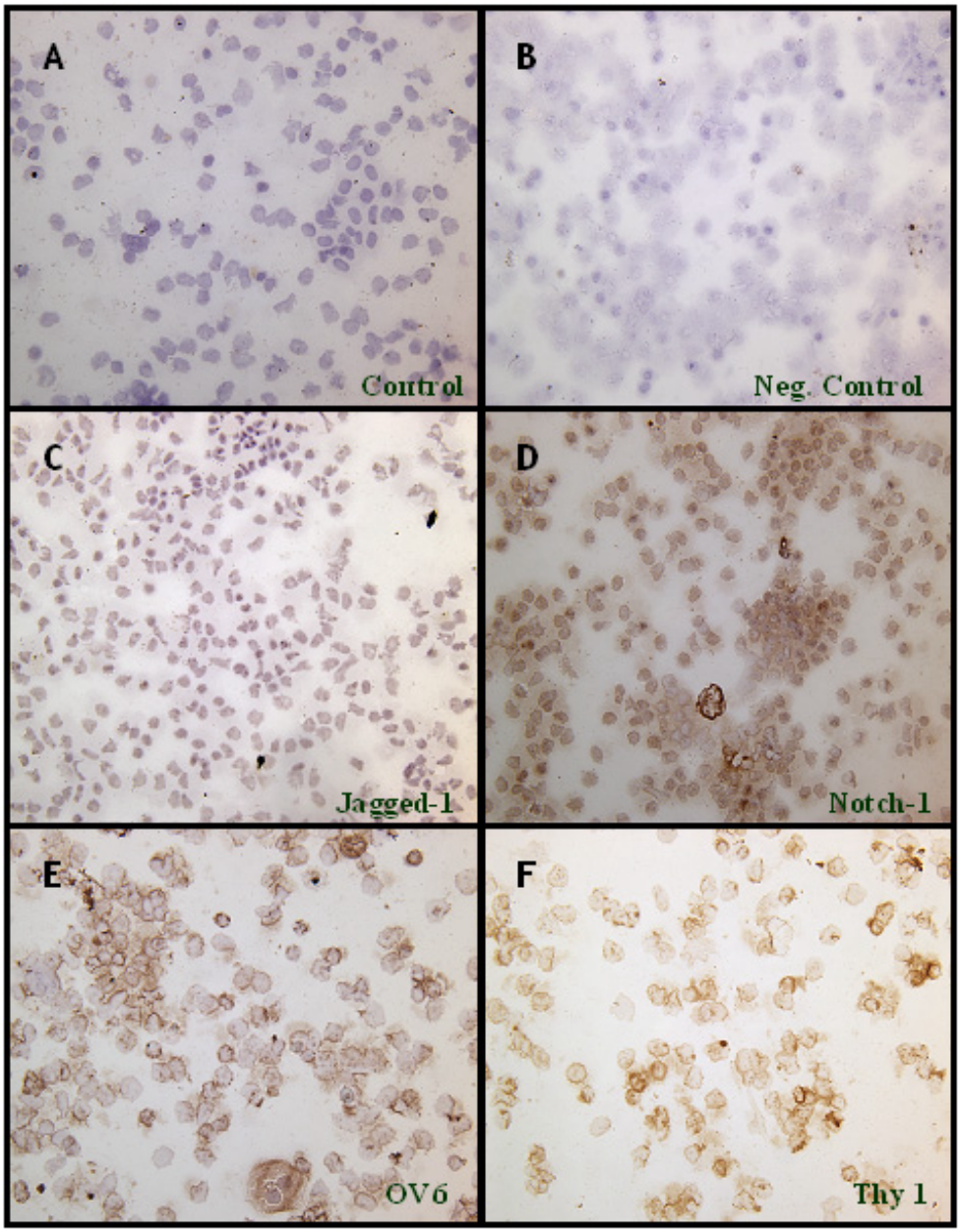


Figure 4. Characterization of D11 Sorted Hepatic Oval Cells. Oval Cells were harvested from a 2AAF/ PHx treated animal at day 11. Panels A and B represent controls where A has IgG in place of primary antibody and B is stained for CD-45 which serves to exclude inflammatory cells. Panel C and D are stained for Jagged and Notch respectively, demonstrating that both proteins are present on Oval Cells though Notch-1 is, at this time point, preferentially expressed. Panel E and F are stainings which are representative and consistent with proteins accepted as being expressed by stem-like cells in the liver.

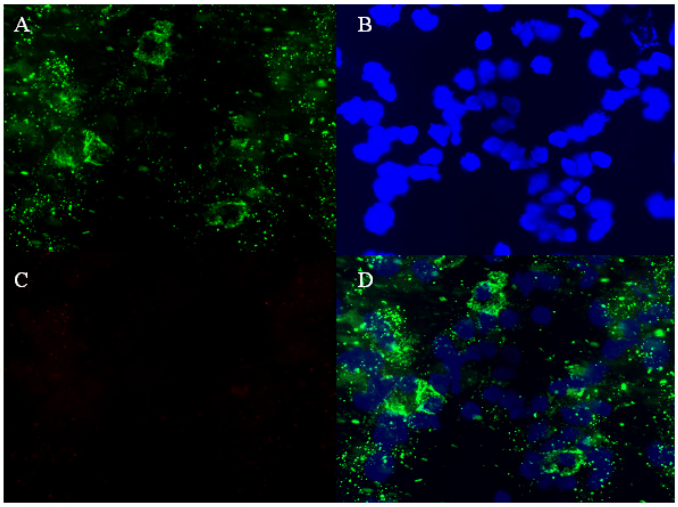


Figure 5. Thy-1 Positive Sorted Oval Cells (a) Thy-1 sorted positive cells stained with FITC conjugated secondary against Thy-1 antibody, (b) DAPI for nuclear staining, (c) staining with Texas Red secondary to Jagged antibody, (d) merge. No obvious colocalization of Notch and Jagged on 2AAF/PHx D11 Thy-1 sorted cells.

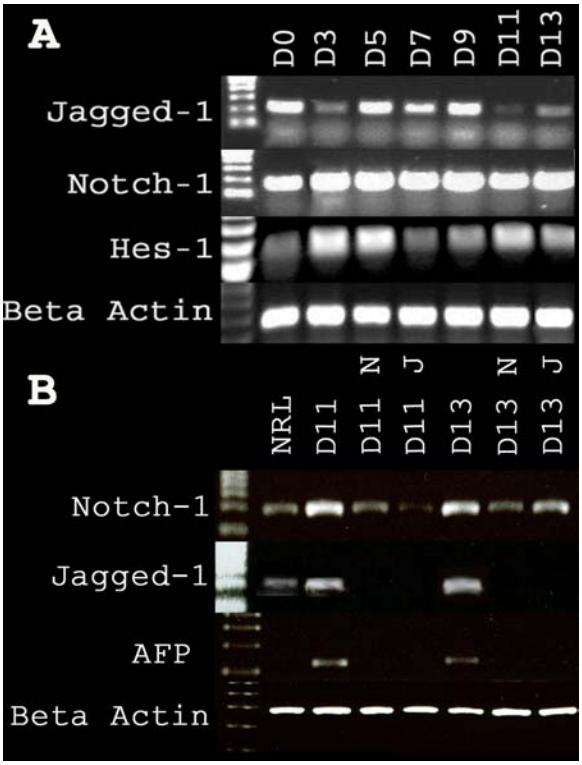


Figure 6. Reverse Transcriptase PCR (A) gene expression for Notch, Jagged and it's down-steam target gene HES-1 in 2AAF/PHx treated total RNA. (B) RT-PCR for gene expression of Notch, Jagged, and AFP in 2AAF/PHx siRNA treated tissue where N and J represent the siRNA injected.

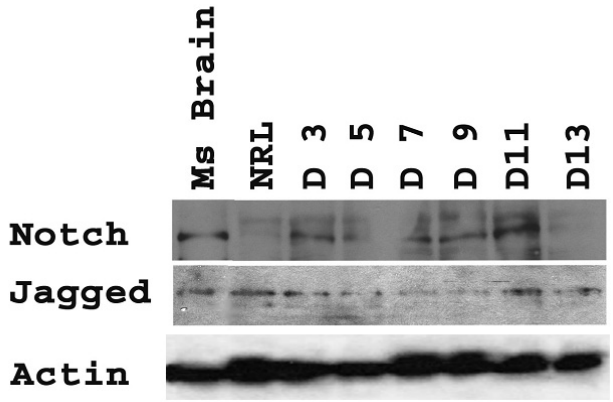


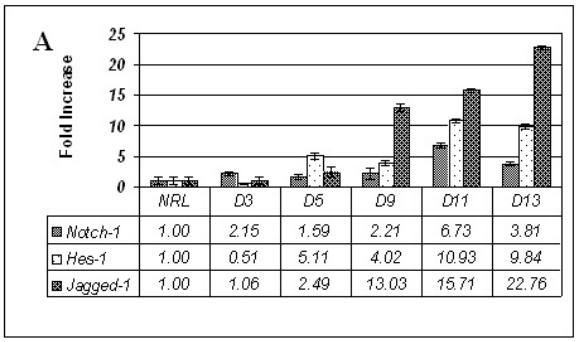
Figure 7. Western Blot Analysis. Whole cell fractions from 2AAF/ PHx liver tissue were used. Both Notch and Jagged are shown to be upregulated. Notch peaks at day 11, while Jagged follows at day 13, corresponding with hepatic oval cell activation

Mouse brain was used a positive control for the presence of both Notch and Jagged as recommended. Normal rat liver was also used as a control to shown normal expression of Notch and Jagged. In experimental samples, Notch and Jagged protein was shown to increase and peak around the time that oval cell activation is also at its peak. The corresponding peaks of Notch, Jagged and oval cell activation indicate that there may a possible link between the Notch/Jagged pathway and oval cell activation or differentiation. However, this data does not conclusively indicate whether hepatocyte associated Notch is activated through Jagged expression by other hepatocytes or through expression by other cell types such as oval cells.

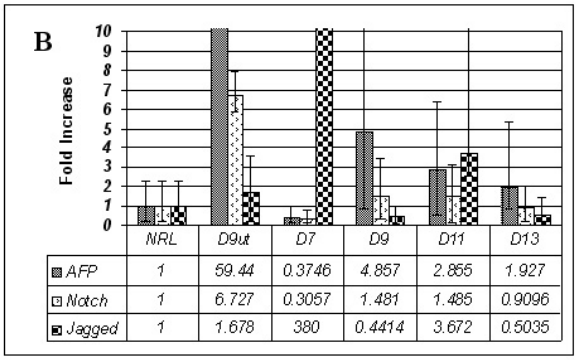
Quantitative Real-Time PCR for Notch and Jagged in the 2-AAAF/PHx Model

Semi-quantitative real-time PCR was used to examine the expression of Notch, Jagged, and Hes-1 mRNA in 2AAF/PHx treated animals (Figure 8a). Real time PCR further demonstrated that Notch, Jagged, and Hes-1 seemed to be intimately involved in the oval cell mediated liver regeneration model. A rise in the expression of all three signaling factors was seen, which corresponds with the rise of oval cell numbers within

this model. This rise demonstrated a 2 to 7 fold increase in Notch spanning from day 3 to day 11. Hes-1, a downstream gene of Notch and regulated by Notch, also demonstrated a half fold increase at day 3 to an 11 fold increase by day 11 (Figure 8a). Jagged gene expression was also seen to increase, however, with a slightly different time frame of upregulation spanning from day 5 through day 13 at which time the upregulation continues reaching a 23 fold increase. The decrease in the expression of both Notch and Hes-1 at this time may indicate that the translocation of the NICD and activation of the Notch/Jagged pathway has tapered toward basal levels once again while other repairs are being affected in the liver.



	NRL	D3	D5	D9	D11	D13
Notch-1	1.00	2.15	1.59	2.21	6.73	3.81
Hes-1	1.00	0.51	5.11	4.02	10.93	9.84
Jagged-1	1.00	1.06	2.49	13.03	15.71	22.76



	NRL	D9ut	D7	D9	D11	D13
AFP	1	59.44	0.3746	4.857	2.855	1.927
Notch	1	6.727	0.3057	1.481	1.485	0.9096
Jagged	1	1.678	380	0.4414	3.672	0.5035

Figure 8. Semi-Quantitative Real-Time PCR (a) 2-AAF/ PHx model. Notch expression peaks on day 11, while Jagged peaks two days later. The Notch target gene HES-1 appears to peak on or shortly after day 11, but is still extremely elevated at day 13. The upregulation of these genes coincides with oval cell activation. (b) 2-AAF/PHx siRNA treated model. Notch and Jagged both appear to be down regulated post injection of siRNAs. AFP, however, looks to be completely dysregulated.

CHAPTER 4
RESULTS: INTERFERRANCE IN THE NOTCH/JAGGED SIGNAL TRANSDUCTION PATHWAY DURING OVAL CELL ACTIVATION

Introduction

RNA interference (RNAi) is an intracellular mechanism for post-transcriptional gene silencing that functions in the regulation of gene expression (66). RNAi is triggered by double-stranded RNA (dsRNA) which is cleaved by Dicer, an enzyme with RNAse activity. Dicer cleaves the dsRNA into fragments of ~21nt termed short interfereing RNA (siRNA). These siRNAs associate with with several proteins to form and RNAi solencing complex (RISC). The minus strand of the siRNA targets a particular mRNA based on sequence homology. This sequence directed removal of specific mRNA transcripts yields a knockdown of expression of the affected gene. Extensive research is ongoing in the field of RNA interference in order to gain a more detailed understanding of this useful technology.

Application of RNAi technology has been used for the study of gene function and large scale analyses (67-69). A great deal of excitement has been generated by RNAi's possible use in both therapeutic and genomic research. This is in large part is due to the potentials that exist for the treatment of a wide spectrum of genetic and transcriptional disorders, such as HIV (70,71), spinocerebellar ataxia type 1 and Huntington's disease (72), certain cancers (73-75) and hypercholesterolemia (76,77), as well as its demonstrated use in functional genomics via controlled gene knockdown (78-80).

In this study, the knockdown of the Notch and Jagged genes was sought using constructs provided by Kohler et al. We hoped to be able to compare typical liver regeneration with progenitor cell mediated liver regeneration in order to shed light on mechanisms that may yet be unknown. We also sought to determine what extent the Notch/Jagged pathway plays a role in the proliferation, differentiation and perhaps apoptosis mechanisms that are active during oval cell mediated liver regeneration.

Effects of Silencing RNA for Notch and Jagged on Oval Cell Activation During Liver Regeneration

Each experiment which was completed for the 2AAF/PHx oval cell mediate liver regeneration model were again run, but with tissues from animals that had been treated with small interfering RNAs. Each animal was first placed on the 2AAF/PHX protocol. Following PHx, each animal was given a cumulative dose of 200μg of one of two experimental siRNAs. These siRNAs were given in dosages of 50μg at days 7, 9, 11, and 13 and three animals per time point were sacrificed at days 9, 11, 13, 15, and 22.

Gross Histology of siRNA Treated Rat Liver

In order to assess the gross affects of the siRNA treatment, hematoxylin and eosin (H&E) staining was prepared on experimental tissues from each time point. We noted that the histology of siRNA treated 2-AAF/PHx rat liver appeared to have the same characteristics seen in the 2-AAF/PHx model through day 15 (Figure 9c and d). However, at day 22, when 2-AAF/PHx treated tissues have returned to a normal histological profile, the siRNA treated tissues demonstrated a back up of apparently undifferentiated oval cells (Figure 9e and f).

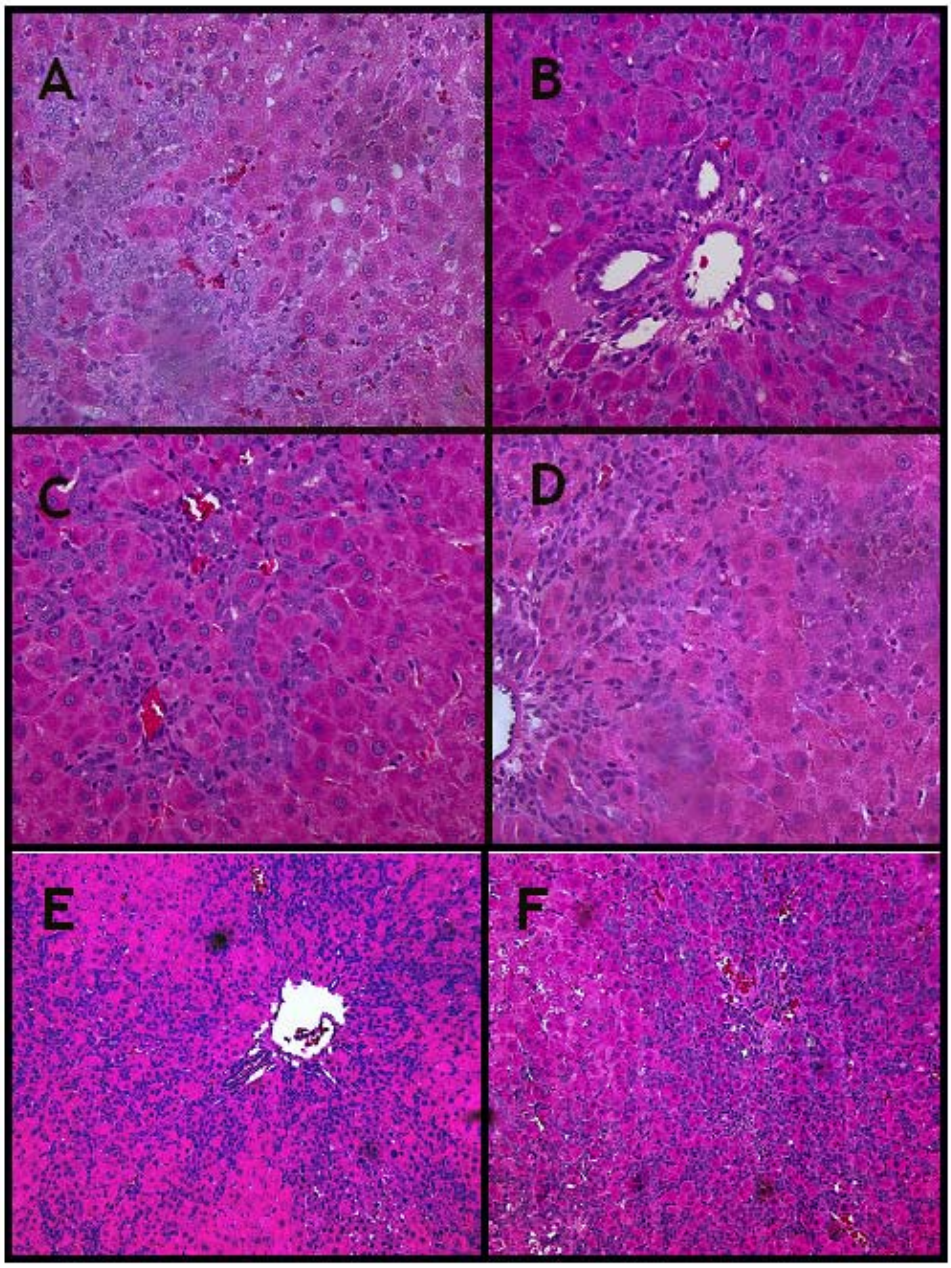


Figure 9. Hematoxylin and Eosin Staining of siRNA Treated 2AAF/PHx Tissues (a) Notch siRNa treated Day 9 tissue 40X (b) Jagged siRNA treated Day 9 tissue 40X (c) Notch siRNA treated Day 11 tissue 40X(d) Jagged siRNA treated tissue 40X (e) Notch sirNA treated Day 22 tissue 20X (f) Jagged siRNA treated Day 22 tissue 20X. Arrows indicate oval cells which have been recruited to the periportal region. In panels e and f, a back up of small cells can be seen where under normal circumstances these sections would appear to be normal liver.

BrdU Incorporation In Vivo

Hepatocyte and HOC proliferation were measured using BrdU incorporation at each time point to determine whether the back up of cells seen in the H&Es of the treated tissues were proliferating. The micrographs indicate that there is limited replication taking place at all time points. At day 22, the replication that is seen predominately in isolated hepatic cells (Figure 10e and f). There is no relation between the location of these replicative cells and those seen in the H&E micrographs.

OV6 and Thy-1 Verification of Oval Cell Presence

Immunohisotchemistry for both OV6 and Thy-1 were utilized to verify the presence of HOCs within the siRNA treated tissues. Since a build up of HOC-like cells was shown in the H&Es and no replication was seen using BrDU incorporation, it was important to ascertain whether those cells were indeed HOCs.

Immunohisotchemistry for OV6 was completed on day 22 frozen tissues in order to identify the HOC-like cells. The results indicate that the cells present in those tissues are expressing the HOC markers in both treatments (Figure 11c and d), though the presence of OV6 expressing cells in day 22 siRNA treated tissues is not as evident as in the 2-AAF/PHx day 11 control tissue (Figure 11b).

In order to further show that the cells seen in the OV6 positive slides are indeed HOCs, Thy-1 staining was also initiated. The staining that resulted indicates that the cells positive for OV6 are indeed HOCs due to the similarity in area and cell type stained for Thy-1 (Figure 12e and f). Both Notch and Jagged siRNA treated tissues demonstrate staining for the presence of Thy-1, where in normal liver tissue and 2-AAF/PHx day 22 tissue there would be no staining (59).

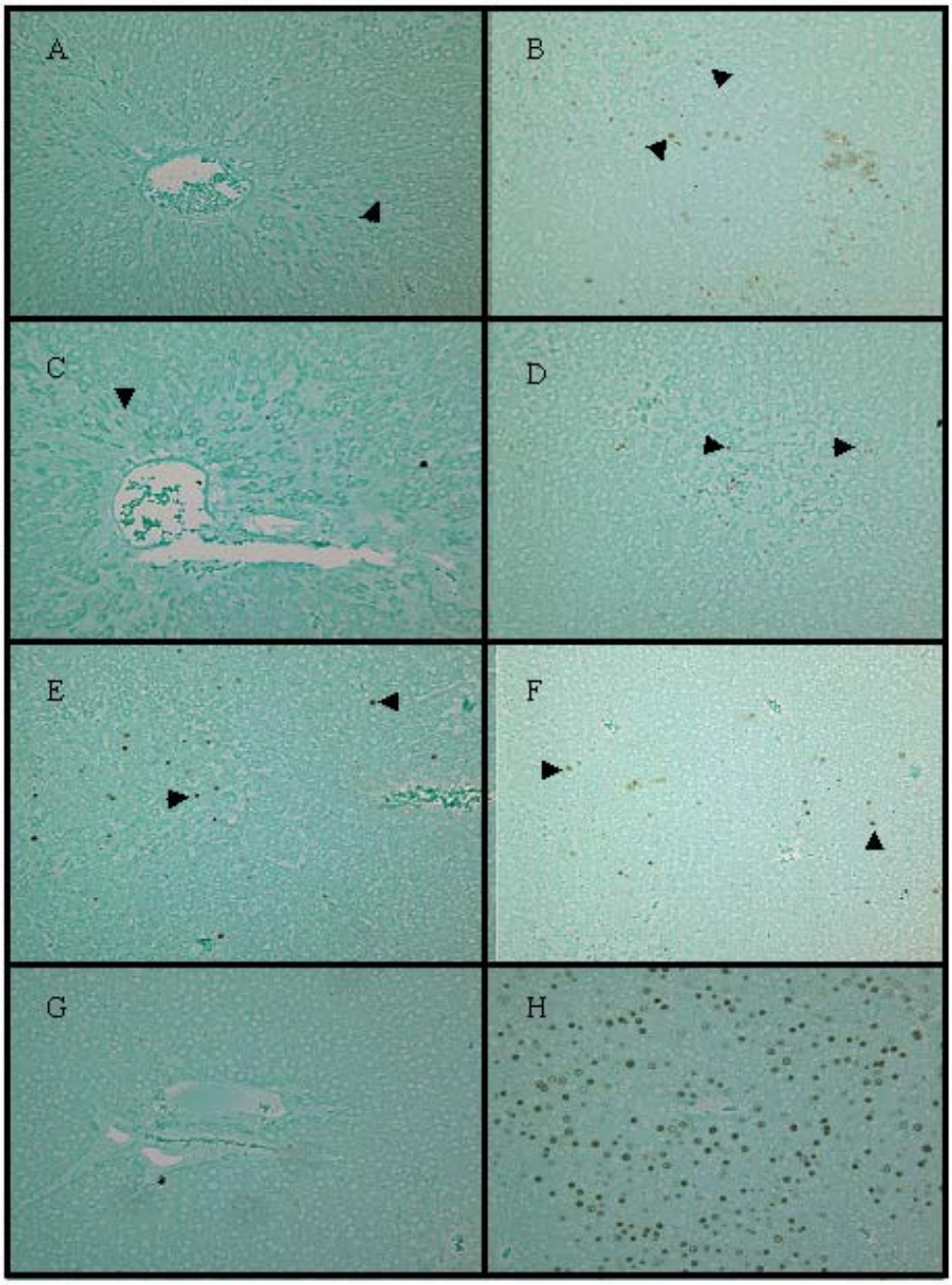


Figure 10. *BrDU* Immunohistochemistry on 2AAF/PHx, siRNA Treated Tissue (a) Notch treated Day9 tissue (b) Jagged treated Day9 tissue (c) Notch treated Day 11 tissue (d) Jagged treated Day11 tissue (e) Notch treated Day11 tissue (f) Jagged treated Day 11 tissue (g) Normal Rat Liver (h) Day 11 2-AAF/PHx tissue. Arrows indicate replicative cells. All micrographs are taken at 20X magnification

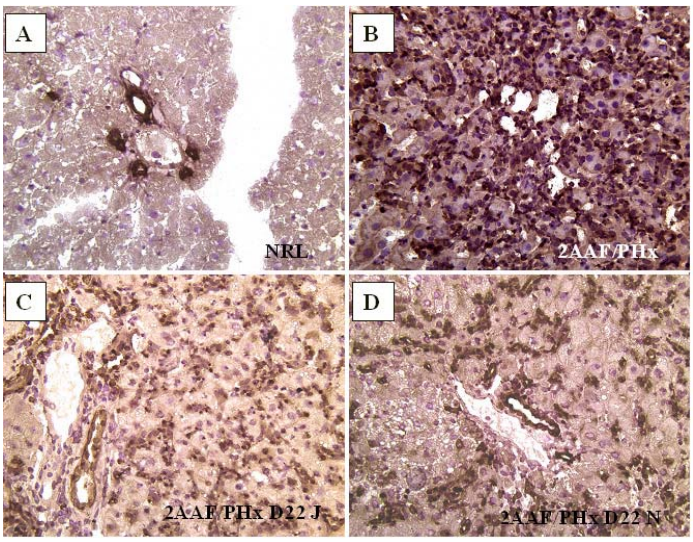


Figure 11. OV6 Staining in Day 22 siRNA Treated Tissue (a) Normal rat liver (b) 2-AAF/PHx day 11 tissue (c) 2-AAF/PHx day 22 Jagged siRNA treated tissue (d) 2-AAF/PHx day 22 Notch siRNA treated tissue.

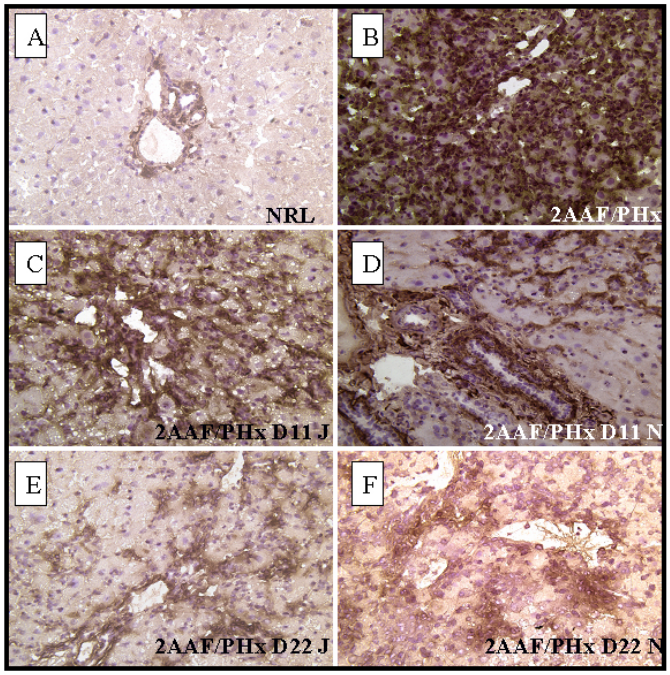


Figure 12. Thy-1 Staining of srRNA Treated Tissue. (a) Normal rat liver (b) 2-AAF/PHx day 11 tissue (c) 2-AAF/ PHx day 11 Jagged siRNA treated tissue (d) 2-AAF/ PHx day 11 Notch siRNA treated tissue (e) 2-AAF/ PHx day 22 Jagged siRNA treated tissue (f) 2-AAF/ PHx day 22 Notch siRNA treated tissue.

Notch and Jagged Localization in the siRNA Treated 2-AAF/PHx Model

The cellular distribution of Notch and Jagged in siRNA treated 2AAF/PHx rat liver tissue was investigated through immunohistochemical staining.

Notch Protein Staining

Notch protein levels did not appear to increase above what was seen in normal liver tissue regardless of which siRNA was employed. Day 11 post PHx tissue which had been treated with 100 micrograms of Notch siRNAs did demonstrate a knockdown of the Notch protein (Fidure 13c). Day 22 post PHx with the full cumulative dosage of 200 micrograms of Notch siRNA having been delivered 8 days prior to tissue harvest still shows a knockdown of the protein. The protein that is present is largely localized in the bile ductular epithelium and hepatocyte nucleic (Figure 13e). However, this was not the case with Notch expression in Jagged siRNA treated tissues.

Treatment with Jagged siRNAs did not block the expression of normal levels of Notch protein at day 11 post PHx (Figure 13d). Notch levels in the Jagged siRNA treated day 22 time point do seem to be affected. The localization seen is similar to that seen in Notch siRNA treated tissue with one notable difference: notch is still being expressed in the nuclei of hepatocytes and the biliary ductal epithelium. It also appears to be expressed in the small intermediary cells that are found around the biliary ductules and between mature hepatocytes (Figure 13f).

Jagged Protein Staining

Jagged protein levels did not increase above what was seen in normal liver tissue regardless of which siRNA was injected. Day 11 post PHx tissue which had been treated with 100 micrograms of Notch siRNAs demonstrate what appears to be complete knockdown of the Jagged protein (Figure 14c). Day 22 post PHx with the full cumulative dosage of 200 micrograms of Jagged siRNA having been delivered 8 days prior to tissue harvest continues to show a knockdown of the protein though levels have begun to rise. The protein that is present is largely localized in the cytoplasm of hepatocytes near bile

ducts and activated HOCs (Figure 14e). However, this was not the case with the expression of the Jagged protein.

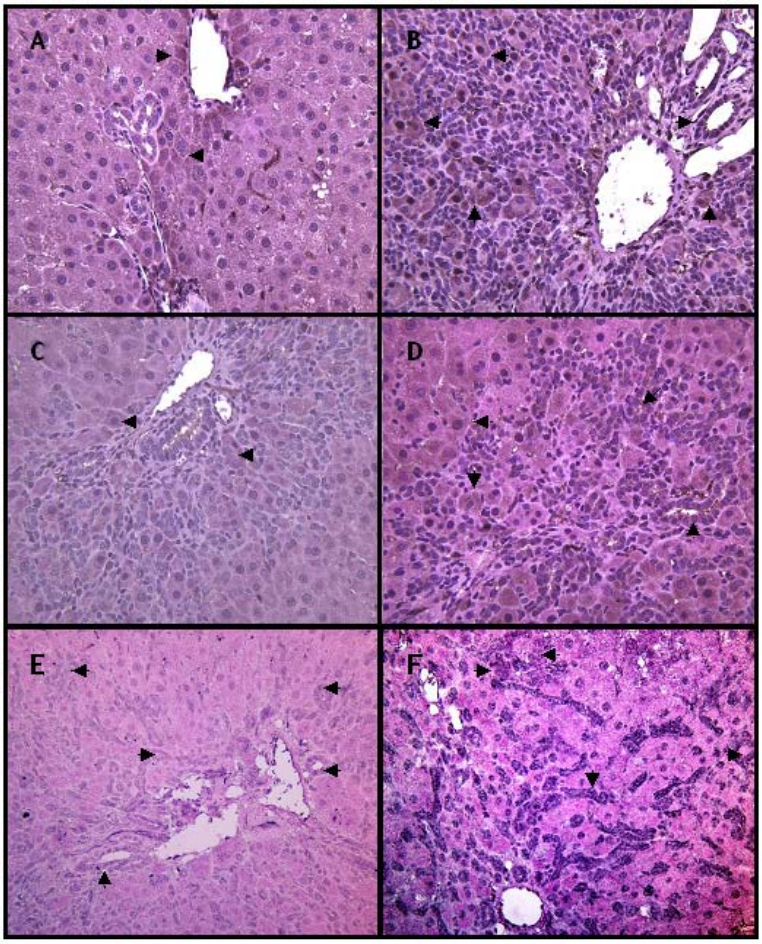


Figure 13. Notch Protein Levels (a) Fetal Liver as positive control Jagged staining as recommended (b) 2AAF/Phx D9 liver without siRNA treatment, many oval cells are in evidence and hepatocytes show positivity for Jagged (c) 2AAF/PHx D11 liver treated with total of 100ug of Notch siRNA. Oval cell proliferation has been minimally affected with little Notch expression (d) treatment of 2AAF/PHx liver with 100ug Jagged siRNA fails to curtail oval cell recruitment to the same extent as Notch siRNA and Notch expression is also little affectd (e) D22 post PHx with a total of 200ug Notch siRNA injected, the 2AAF/PHx liver appears disorganized, with a backlog of oval cells remaining and very little Notch being expressed in comparison to D11 2AAF/PHx tissue (f) D22 post PHx with a total of 200ug Jagged siRNA injected, the 2AAF/PHX liver appears intact, also with a backlog of oval cells remaining and Notch staining appearing in and around transitional oval cells.

Treatment with Jagged siRNAs completely blocked the expression of normal levels of Jagged protein at day 11 post PHx (Figure 14d). Jagged levels in the Jagged siRNA treated day 22 time are also minimal. The protein that is present is localized in the cytoplasm of hepatocytes near infiltrating HOCs (Figure 14f).

RT-PCR for Expression of Notch and Jagged in the 2-AAF/PHx Model

RT-PCR was utilized using the same primers for Notch and Jagged as were used in the 2-AAF/PHx model to test for transcription of these genes in snap frozen tissue from both the Notch siRNA and Jagged siRNA treated 2-AAF/PHx model. AFP primers were also used since it appeared that oval cell differentiation was being inhibited as seen in Figure 8 by the massive back up of oval cells. Transcription of Notch and Jagged were seen in normal rat liver (Figure 6). Transcription of the Notch protein was demonstrated to be down regulated by both Notch and Jagged siRNAs as compared with the 2-AAF/PHx treated alone at identical time points. Jagged gene transcription appears to have been completely down-regulated by both the Notch and Jagged siRNAs in comparison to matched time points in the 2-AAF/PHx model. AFP transcription was also down regulated in the siRNA treated tissues.

Western Blot Analysis of the 2-AAF/PHx Model

As with the 2-AAF/PHx model, Western blot analysis was undertaken using snap frozen tissues harvested from siRNA injected 2-AAF/PHx treated animals. Unlike the 2-AAF/PHx model, no signal was recovered from these tissues for either Notch or Jagged (data not shown).

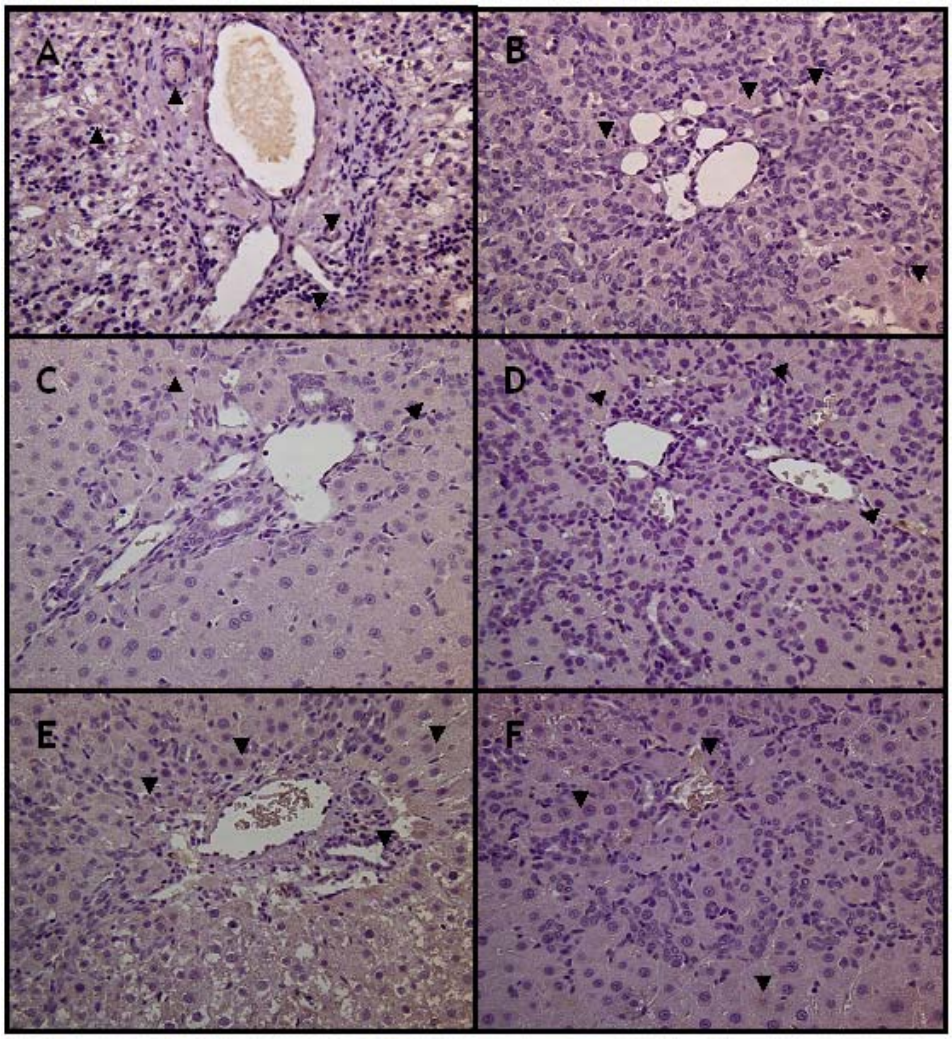


Figure 14. Jagged Protein Levels (a) Fetal Liver as positive control Jagged staining as recommended (b) 2AAF/Phx D9 liver without siRNA treatment, many oval cells are in evidence and hepatocytes show positivity for Jagged (c) 2AAF/PHx D11 liver treated with total of 100ug of Notch siRNA. Oval cell proliferation has been curtailed and very little positivity is seen for Jagged expression (d) treatment of 2AAF/PHx liver with 100ug Jagged siRNA fails to curtail oval cell recruitment to the same extent as Notch siRNAs did, but Jagged expression remains nominal (e) D22 post PHx with a total of 200ug Notch siRNA injected, the 2AAF/PHx liver appears disorganized, with a backlog of oval cells remaining (f) D22 post PHx with a total of 200ug Jagged siRNA injected, the 2AAF/PHX liver appears intact but with a backlog of oval cells remaining.

Quantitative Real-Time PCR for Notch and Jagged in siRNA Treated 2-AAF/PHx Liver Tissue

Semi-quantitative real-time PCR was used to examine the expression of Notch, Jagged, and AFP mRNA in siRNA treated 2AAF/PHx animals (Figure 8b). Real time PCR indicates that Notch, Jagged, and AFP seemed to be intimately involved in oval cell differentiation though there is no indication as to how that may be. The results demonstrate a dysregulation of both Notch and Jagged with counter-intuitively low transcription of AFP. Notch gene transcription levels were demonstrated to be increased by 1.5 in contrast to the 6.7 fold increase seen in the day 9 of the 2-AAF/PHx model used as a comparison. Jagged transcription levels were not as expected. There was a 3.7 fold increase in day 11 Jagged siRNA treated tissue as opposed to a 1.7 fold increase in untreated 2-AAF/PHx tissue. Taken together, these results are conflicting and point to either poor siRNA design or to AFP itself being regulated or in some other way affected by the Notch/Jagged signal transduction pathway.

Alphafeto Protein Expression in siRNA, 2AAF/PHx treated liver

In exploring the regeneration of liver post siRNA and 2AAF/PHx treatment, expression of alphafeto protein (AFP) was investigated. AFP is an abundant serum glycoprotein in developing mammals (81). During embryonic development, AFP is first detected in the yolk sac and later in the fetal liver (82). The full-length AFP RNA and protein are highly expressed in the primitive hepatoblasts and postnatal hepatocytes (65-68). Hence, AFP expression can be used as an indicator for an early hepatic lineage, and has also served as an important marker for the activation of the hepatic stem cell compartment. The expression profile for AFP in the 2AAF/PHx model demonstrates a drastic upregulation from approximately day 9 through day 11 indicating the presences of

oval cells which are undifferentiated cells that are believed to take up residence within the injured liver, differentiating to effect repairs and restoration of tissue loss/damage. In the siRNA, 2AAF/PHx treated liver, AFP levels were shown to be lower at days 9 and 11 by quantitative real-time PCR (Figure 6b). Northern Blot analysis also demonstrated a similar down regulation of AFP when compared to 2AAF/PHx days 11 and 13 (Figure 13). With this in mind, it may be concluded that the siRNAs which blocked the Notch/Jagged signaling pathway by binding to the Notch protein prior to expression, also affected oval cell recruitment to the sight of injury decreasing the AFP found within the siRNA, 2AAF/PHx treated liver during regeneration.

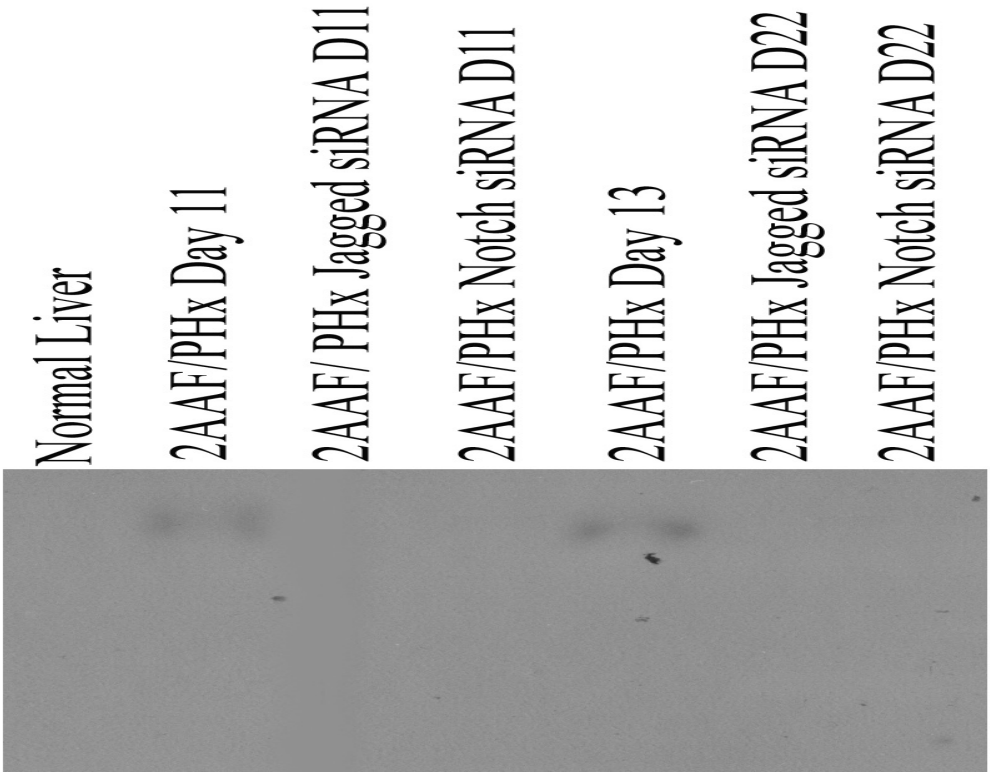


Figure 15. Alphafeto Protein Northern 20ug of RNA from each sample was loaded in each well. Normal was negative for AFP as expected with Days 11 and 13 from the 2-AAF/PHx model being positive as expected. Neither time point, 11 or 22 days post PHx in the siRNA treated models gave positive results.

CHAPTER 5
DISCUSSION

The notch/jagged signal transduction pathway is important for cellular differentiation and proliferation. The dysfunction of this pathway is associated with human pathologies in many tissues including the liver. The healing process within the liver requires replication and regeneration of injured cells and is influenced by a very complex variety of growth factors, cytokines, and cell-cell interactions (83). During simple mechanical injury, two-thirds PHx alone, the baseline expression of Notch and Jagged has been shown to be widespread with hepatocytes and the biliary epithelium showing slightly higher expression levels (64). The endothelial cells of the sinusoids and small vessels also express Notch. These findings in rat are comparable to what has been described in the human liver.

The role of the Notch/Jagged signaling pathway was investigated in the oval cell mediated regenerative process using the standard 2AAF/PHx model. Kohler *et al.* reported widespread expression of Notch and Jagged throughout the liver in different cell types. They also demonstrated that under circumstances where the liver was undergoing regeneration, higher expression of Notch was seen in the endothelial cells of the sinusoids and small vessels. These results were also seen in the oval cell mediated regeneration model, but with a slightly different time that may be attributed to the delay in the replication of cells caused by the introduction of the 2AAF to the remaining hepatocytes and the time required for the hepatic oval cells to mature and take over this function.

At the beginning of oval cell activation, seen at approximately day 3 post PHx in the 2AAF/PHx model, Notch is minimally expressed as detected by Western Blot analysis (Figure 7) and quantitative real-time PCR (Figure 8a). The activated form of Notch reaches peak levels within the liver by day 11 following PHx in the oval cell mediated regeneration model. This is accompanied by a peak in the expression of the Notch dependant gene, Hes-1, suggesting induction by the transport of the NICD to the nucleus. It cannot be determined whether this pathway is set in motion by the expression of the Jagged on hepatocytes or on other cell types. It must be recognized however that the increase in the NICD is probably not limited to the hepatocytes and is seen more generally throughout the liver in other cell types that also express Notch, such as the biliary epithelium and sinusoidal cells. The morphology of both the cells and nuclei in figure 2 demonstrate that at least some of the cells positive for NICD immunofluorescence are hepatocytes (Figure 3). The translocation of the NICD to the nucleus may be easier to demonstrate in hepatocytes due to the much larger size of the nuclei as compared with other hepatic cells types. This is especially important to note with the hepatic oval cell, which though it has a large nuclei to cytoplasmic ration is still very small.

An important finding of this study in relation to liver regeneration is that the increase of Notch and Jagged within the oval mediated regeneration model coincides with the influx of hepatic oval cells and that when the Notch protein is blocked through the use of siRNAs, these cells appear to become backed up or unable to differentiate to affect regeneration though liver mass has been physically restored. Multiple studies have indicated that regeneration proceeds from the direction of the periportal region toward

pericentral region in the liver. This progression is marked by a wave of mitosis (82), expression of metalloproteinases (83), and TGF-β1 (84). Our findings indicate that there is a dramatic increase in expression of both Notch and Jagged in the periportal regions at the time points which coincide with oval cell proliferation. This increase, as shown by Western blot analysis and Quantitative RT-PCR, begins as early as day 3 but peaks at day 11 for Notch and continues to increase through day 13 for Jagged protein levels. This change appears to affect the proliferation and/or differentiation of any of the cells expressing either of the two proteins. In hepatocytes, however, it is likely that Jagged and Notch reside on the same cells and that their co-localization on the plasma membrane, as shown in Supplemental figure 1, may be stimulating an autocrine pathway within that cell as well as juxtacrine pathway with the adjacent cells. It also seems likely that the expression of Notch and Jagged in hepatocytes mediates events in adjacent cell types.

Although hepatic oval cell proliferation peaks at days 9 through 11 in the 2AAF/PHx model there is a gradual increase in Notch and Jagged expression toward their respective peaks at days 11 and 13. It is known that in the PHx model, endothelial cells begin their proliferation at day 3 post PHx and that this proliferation continues until approximately day 6 (3). Endothelial cells express Notch and it is possible that the expression of Jagged by hepatocytes or other injured cells may affect proliferation or other events dependant upon Notch signaling in endothelial cells. Since it was observed that Notch expression was beginning to rise at approximately day 3 in the 2AAF/PHx model, it seems that this may be one of the first steps in the recruitment of hepatic oval cells to regions of the liver that must undergo restructuring of the micro-architecture(49,85,86) in order to support further infiltration and differentiation of stem

like cells. In the siRNA treated model, Notch signaling was still present though dysregulated and this may account for the general recruitment of hepatic oval cells. Along with the dysregulation of Notch signaling it may be that further restructuring was not possible due to lack of synchronicity in signaling that is required to complete this event and allow differentiation. It is clear that overall oval cell proliferation was not impeded.

It seems clear that hepatic oval cells express Notch preferentially over its ligand Jagged. It also appears that as time passes and the hepatic oval cell (HOC) begins to differentiate, there is a change in the overall character of the regeneration process in the 2AAF/PHx model. Instead of a large number of small oval cells expressing AFP and OV6 within the periportal regions of the damaged liver, larger cells that represent small, immature hepatocytes begin to appear (34). These cells express both AFP and albumin identifying them as intermediary cells; cells that are no longer stem-like, but are not fully differentiated to fulfill the hepatocyte role (59). This indicates that though the Notch/Jagged pathway is not required for activation of HOCs, it does affect the differentiation of oval cells, as seen when siRNAs were introduced and a build up of undifferentiated oval cells resulted. The rise in the expression of Jagged over time suggests that another signal or set of signals may be required as a catalyst to push these stem-like progenitors toward a more permanent shift, causing a new protein expression profile to emerge.

The expression of Notch in endothelial cells indicates that the presence of Jagged in other cell types may also affect the proliferative capacity of these cells. Recent evidence suggests that Notch decreases the proliferation of endothelial cells, but increases

angiogenesis, which in turn suggests a role in the re-vascularization and remodeling of the hepatic micro-architecture of the injured liver. The Notch receptor expressed on endothelial cells in the liver may be stimulated by its ligand Jagged which is expressed on proliferating hepatic oval cells. Given the findings from other studies, the presence of Jagged on oval cells may cause a decrease in endothelial cell proliferation, but increase the capacity of the injured liver to accept differentiating oval cells which then replace injured and non-replicative hepatocytes in order to repair the damaged liver.

Kohler *et al.* (64) reported that activation of the Notch signal transduction pathway is in itself important in the regenerating liver and enhances hepatocyte proliferation. Their study also demonstrated that Jagged is equally important in the liver regeneration process. Though the findings that were presented in the PHx model with silencing RNAs are specific, they don't seem to pertain to damaged liver in which the existing cell population has been disabled through the use of 2AAF. These cells are no longer capable of proliferation and therefore when further insult is delivered to the liver as a PHx, cannot fully participate in the regeneration process requiring that another source for cell proliferation be found, hence the oval cell mediation of liver regeneration. In this case, several key elements come into play: upregulation of Notch and Jagged, which is subsequently knocked down by siRNAs and a dysregulation of the pathway induced, recruitment of oval cells, and a build up of oval cells over the 21 day time period in which the 2AAF is active. These key elements can be seen to have the end effect of non-differentiation where under the same circumstances, minus the silencing RNAs, full recovery of liver mass and functionality are seen in the 2AAF/Phx treated liver by day 21.

Unfortunately, RNAi use is not without some complications (87). RNAi is shown to function in many different organisms. However, some organisms such as *Sacchromyces cerevisiae*, are considered to be RNAi negative based on the lack of experimental observations in which specific knockdown may have occurred. These organisms typically lack integral components such as DICER and RISC as well (88-90). Complications in the use of RNAi have been noted in mammals as well (91). It has also been recognized that the sequence of siRNA may not be perfect, affecting what was initially regarded to be highly specific means of gene repression, reducing its specificity (87-91).RNAi can still direct siRNA to target mRNa sequences that lack complete sequence identity (90). New methods for design of RNAi have been recommended through computational studies of the off target effects seen with their usage (92).

CHAPTER 6
SUMMARY AND CONCLUSIONS

The aim of this study was to gain better insight into the mechanisms that are involved in oval cell mediated liver regeneration. The Notch/Jagged pathway became of interest when it was shown to play a role in liver regeneration by Kohler et al. In this study a definitive correlation between the Notch/Jagged pathway and oval cell mediated liver regeneration was established through morphological, biochemical, and genetic characterization of Notch and Jagged *in vivo*.

1. The role of Notch and Jagged in oval cell activation during liver regeneration in the 2-AAF/PHx model.

The induction pattern of both Notch and Jagged were examined through both protein and RNA. The expression levels of both proteins increased post PHx and the activation of the pathway was linked to the peak seen in oval cell activation in the 2-AAF/PHx model supporting the hypothesis that the Notch/Jagged pathway plays a role in oval cell mediated regeneration as well as in hepatocyte drive regeneration.

Western blot analysis, RT-PCR, and real-time PCR demonstrated that Notch begins to rise shortly following PHX and this rise continues and peaks at approximately day 11 which corresponds to the peak of oval cell proliferation. Jagged was also shown to increase over this time period, with a continual rise indicating that perhaps a switch from oval cell to a more differentiated cell type ensues following day 13.

2. The Notch/Jagged pathway becomes dysregulated with the introduction of siRNAs and liver regeneration is hindered.

When siRNAs are used to down-regulate the expression of Notch or Jagged into the oval cell mediated regeneration model, a dysregulation of the Notch/Jagged pathway occurs causing a backlog of oval cells to build up by day 22 post PHx. These findings indicate that the Notch /Jagged pathway may be an integral part of the progenitor cell response when other methods of regeneration are not an option due to toxicity or massive injury.

3.AFP expression is affected through application and *in vivo* transfection of Notch and Jagged siRNAs.

RT-PCR and semi-quantitative PCR demonstrated the lack of AFP expression in siRNA treated tissues during oval cell activation. This observation appears to be a new insight into the importance of the Notch/Jagged/AFP pathways during progenitor cell mediated regeneration. This observation may be due in part to off-target effects caused by poor specificity in the siRNAs used. This poor specificity may in turn be expected because the genes, which encode the Notch and Jagged receptors, are highly conserved and show a great degree of homology between receptors as well as between species. Computational analysis has shown that there is a possibility of designing specific and working siRNAs with better understanding in the future (92). Alternatively, the lack of expression of AFP may be due to an interaction of the Notch/Jagged pathway with that of the AFP pathway. This line of thought begs the question of how well the developmental pathways have been defined.

In summary, our studies present evidence that the Notch/Jagged signal transduction pathway is activated and plays an important role in oval cell mediated liver regeneration, most specifically in the capacity of differentiation of these stem-like precursor cells into the more mature cells that lend to the functionality and reconstruction of the damaged

liver. The precise sequence of events, the cellular pathways and types of pathways affected need to be better understood. Developmental biology has suggested that these pathways are important in many aspects throughout the lifespan of an organism. Evidence from many other systems of tissue development support that these changes are also likely to be important. Further studies and more specific investigation into the impact of differing Notch receptors and their ligands is needed to more fully understand the roles that this pathway may play. It is also important that the interaction between other pathways and the Notch/Jagged pathway be investigated as to the possibilities in their commingled roles in tumorigenicity and carcinogenicity within the liver when aberrant expression occurs since proliferation seems to be one of the most notable consequences of this particular pathway.

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BIOGRAPHICAL SKETCH

Heather Hatch was born in Mesa, Arizona, in January 1973. She graduated from Gulf Coast Community College with her Associate of Science degree in the spring of 1997 and went on to be accepted and graduate from the University of Florida with her Bachelor of Science degree in wildlife ecology and conservation in the summer of 1999. Following graduation, she began working with Dr. Bryon Petersen as his laboratory manager. After learning much in the adult stem cell field and sharpening her research skills, she entered the College of Medicine's graduate program in the fall of 2003 to achieve her Master of Science degree in medical science while maintaining her position with Dr. Petersen and continuing her research in the mechanisms of adult stem cell activation.