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The Role of H19, a Long Non-coding RNA, in Liver Development

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University of Connecticut, 2017

Liver development at the postnatal age has been understudied despite being a time when the liver is rapidly growing and changing its primary function as an organ involved in hematopoiesis to an organ responsible for metabolism. Long non-coding RNAs (LncRNAs) are important in many biological processes, including organogenesis, normal liver functions, and liver diseases. Expression levels of lncRNAs change in identifiable patterns in liver during development, and H19, due to its expression pattern, may be important for liver development.

H19 RNA is highly expressed at early postnatal ages and precipitously decreases at a specific time corresponding with increases in expression of genes important for mature liver function, such as drug metabolizing enzymes. H19's role in the regulation of liver maturation is currently unknown. Using an H19 knockout mouse model to determine the role of H19 in liver development, we quantified gene expression for insulin growth factor signaling, Wnt signaling, key cytochrome P450 (P450) enzymes known to change as the liver develops, and fetal and adult plasma protein produced in liver. In mice lacking H19 expression, liver weights were significantly increased immediately after birth and significant increases were found in the number of actively proliferating cells. Increases in cell proliferation may be due to increases in β-catenin protein affecting Wnt signaling, increases in insulin-like growth factor 2 (IGF2) expression, and/or increases in insulin-like growth factor 1 receptor (IGF1R) expression at the protein level. Loss of targeted inhibition of IGF1R by microRNA 675 (miR-675) may be the cause of IGF1R increases, as miR-675 expression is also abrogated with loss of H19 expression

in our model. P450 expression patterns were significantly altered for certain P450 genes at particular time points in development, but were largely unchanged. No change in the production of plasma proteins was found, indicating H19 may not be important for liver maturation despite its role in controlling cell proliferation during liver growth. H19 may be important for normal liver development, and understanding how the liver matures will assist in predicting drug efficacy and toxicity in pediatric populations.

The Role of H19, a Long Non-coding RNA, in Liver Development

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The Role of H19, a Long Non-coding RNA, in Liver Development

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DEDICATION

To Mom, Dad, and my sister, Alyssa

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ABCA1	ATP-binding cassette transporter
ACSL1	Acyl-CoA synthetase long-chain family member 1
APTR	Alu-mediated CDKN1A/P21 transcriptional regulator
ATB	Activated by TGF-ß
BCL2	B-cell lymphoma 2
C/EBPß	CCAAT/enhancer-binding protein ß
CTCF	CCCTC-binding factor
CDKN1C	Cyclin-dependent kinase inhibitor 1C
CNOT1	CCR4-NOT transcription complex subunit 1
CTNNB1	Catenin Beta 1
CUDR	Cancer upregulated drug resistant
CYFRA	Cytokeratin fragments
DANCR	Differentiation antagonizing non-protein coding RNA
DCN	Decorin
DILC	Downregulated in liver cancer stem cells
DLK1	Delta-like non-canonical notch ligand 1
DM	Diabetes mellitus
DMR	Differentially-methylated region
Dreh	Down-regulated expression by HBx
EGR1	Early growth response protein 1
EMT	Epithelial-mesenchymal transition
EPCAM	Epithelial cell adhesion molecule
EPS	Erythroid prosurvival
EZH2	Enhancer of zeste homolog 2
FOXO1	Forkhead box protein O1
FXR	Farnesoid X receptor

GAS5	Growth arrest specific 5
GSTP1	Glutathione S-transferase pi 1
GNAS	GNAS complex locus
H3K9	Histone H3 at lysine 9
H3K27	Histone H3 at lysine 27
HBV	Hepatitis B virus
HBx	Hepatitis B virus X protein
HCV	Hepatitis C virus
HCC	Hepatocellular carcinoma
HDAC	Histone deacetylase
HEIH	High expression in HCC
HnRNP	Heterogeneous nuclear ribonucleoprotein
HOTAIR	HOX transcript antisense RNA
HOTS	H19 opposite tumor suppressor
HOTTIP	HOXA distal transcript antisense RNA
HOX	Homeobox
HSP90	Heat shock protein 90
HULC	Hepatocellular carcinoma up-regulated long non-coding RNA
ICR	Imprinting control region
IGF	Insulin-like growth factor
IGF1R	Insulin-like growth factor 1 receptor
IGF2BP1	Insulin-like growth factor 2 mRNA binding protein 1
IGN	Imprinted gene network
IL	Interleukin
IVF	*In vitro* fertilization
KMT	Lysine methyltransferase
KRT19	Keratin 19
LALR1	Long noncoding RNAs associated with liver regeneration 1

LincRNA	Long intergenic non-coding RNA
LncRNA	Long non-coding RNA
LSTR	Liver-specific triglyceride regulator
MALAT1	Metastasis associated lung adenocarcinoma transcript 1
MAPK	Mitogen-activated protein kinase
MBD1	Methyl-CpG-binding domain protein 1
MDM2	Mouse double minute 2 homolog
MDR1	Multidrug resistance protein 1
MeCP2	Methyl-CpG binding protein 2
MEG3	Maternally-expressed 3
MIRG	MicroRNA-containing gene
MLL	Mixed lineage leukemia
MVIH	Microvascular invasion in HCC
NAFLD	Nonalcoholic fatty liver disease
NASH	Nonalcoholic steatohepatitis
NOP2	Nucleolar protein 2
NTCP	Na^+-taurocholate cotransporting polypeptide
P450	Cytochrome P450
PEG1	Paternally-expressed gene 1
PCAF	P300/CBP-associated factor
PGK1	Phosphoglycerate kinase 1
PKM2	Pyruvate kinase isozyme M2
PLK1	Polo like kinase 1
PPAR	Peroxisome proliferator activated receptor
PRAL	P53 regulation associated lncRNA
PRC2	Polycomb repressive complex 2
RAC1	Ras-related C3 botulinum toxin substrate 1
RIAN	RNA imprinted and accumulated in nucleus

ROR	Regulator of reprogramming
SETDB1	SET domain bifurcated 1
SHP	Small heterodimer partner
SLC38A4	Solute carrier family 38 member 4
SPHK1	Sphingosine kinase 1
STAT3	Signal transducer and activator of transcription 3
SUV39H1	Suppressor of variegation 3–9 homolog 1
TERT	Telomerase reverse transcriptase
TGF	Transforming growth factor
VEGF	Vascular endothelial growth factor
WDR5	WD repeat domain 5
XIST	X-inactive specific transcript
ZEB	Zinc finger E-box-binding homeobox
ZHX2	Zinc fingers and homeoboxes 2

Chapter 1: Introduction/Literature Review

1.1 Liver Development

Liver development requires specific temporal molecular events leading to the generation of each specific cell type, and the formation of a precise three-dimensional architecture. In mammals, the liver forms one cell thick cords of polarized hepatocytes. Hepatocytes secrete hormones into the blood from their basolateral surface, and they secrete bile acids and bile salts across their apical surface into tight junctions that form canaliculus surrounding each hepatocyte (Si-Tayeb et al., 2010). The liver lobes are distinctively arranged containing one centrilobular vein at the lobe center and the portal triad consisting of the hepatic artery, portal vein, and one or two bile ducts (Mescher and Junqueira, 2013). The hepatic artery supplies blood from the heart and the portal vein supplies blood from the gastrointestinal tract, gallbladder, pancreas, and spleen. Blood leaves the liver to return to the heart via the centrilobular vein. The axis between the portal triad and the centrilobular vein separates liver cells into distinct zones. Zone 1 contains oxygen rich cells near the portal triad, zone 3 contains cells near the centrilobular vein, and zone 2 is comprised of cells in-between. Cell function and gene expression exhibit different patterns depending on zonal location. For example, the metabolism of xenobiotics mainly occurs in hepatocytes closer to the centrilobular vein in zone 2 and zone 3 while gluconeogenesis occurs more in the periportal region in zone 1. Canonical Wnt signaling plays a key role in maintaining hepatocyte zonation in mice (Monga and SpringerLink (Online service), 2011).

Liver ontogenesis is complex and tightly orchestrated. Hepatocytes and biliary epithelial cells are formed from the endoderm germ layer in the embryo. Stromal cells, stellate cells, Kuppfer cells, and blood vessels are derived from the mesoderm. In mice, the endoderm forms around embryonic days 6.5 to 7.5 and by embryonic day 15, hepatoblasts are formed which will

eventually differentiate into hepatocytes and biliary cells (Zorn, 2008). Prior to hepatoblast formation, the endoderm cells near the sinus venosus become columnar and begin to express genes indicating hepatic cell fate at embryonic day 8.5 (Bort et al., 2006). The initiation of liver ontogeny is when epithelial cells of the foregut endoderm commit to becoming the liver primordium at around embryonic day nine (Hata et al., 2007). And, after embryonic day 9.5, the matrix on the basal surface of the endoderm near the sinus venosus degrades, and hepatoblast cords migrate into the surrounding stroma (Bort et al., 2006). Between embryonic days 10 to 15 the liver becomes the major hematopoietic organ in the fetus (Zorn, 2008) and throughout ontogenesis until it is fully mature, gene expression profiles change in identifiable patterns (Li et al., 2009a).

Wnt signaling plays important roles in proper fetal liver organogenesis. Acting to repress *Hhex* in the posterior endoderm during early somite stages, Wnt signaling prevents ectopic hepatic development. Repression of Wnt signaling allows *Hhex* expression in the anterior endoderm, allowing liver formation. After hepatic specification, Wnt signaling acts in the opposite manner to promote hepatogenesis (McLin et al., 2007). In rats, Wnt2 increases proliferation of sinusoidal endothelial cells by targeting VEGF receptor-2 (Klein et al., 2008), and activation of the VEGF receptor in mice results in sinusoidal endothelial cell secretion of mitogenic factors increasing hepatocyte proliferation and liver mass (LeCouter et al., 2003). Wnt signaling also promotes stem cell specification to cholangiocytes in embryonic mouse liver cultured *ex vivo* (Hussain et al., 2004). Before birth, this pathway clearly acts to instruct liver development, and after birth, as the liver continues to grow and mature, Wnt signaling remains a key factor.

Wnt signaling is important for postnatal liver growth and maturation. ß-Catenin has been shown to be critical for early postnatal liver growth, as knockout results in a reduction in cell proliferation (Apte et al., 2007). Aside from growth, Wnt signaling impacts normal liver functions including affecting expression of drug metabolizing enzymes and influencing bile acid homeostasis. For example, ß-catenin activates P450s (Loeppen et al., 2005), and knockout of ß-catenin results in lower expression of P450s (Sekine et al., 2006). ß-Catenin knockout mice exhibit a decrease in bile flow and mild intrahepatic cholestasis with an inability to respond to increases in bile acids (Yeh et al., 2010). Acting before and after birth, Wnt signaling directs proper liver growth and maturation.

Insulin-like growth factor (IGF) signaling is important for proper development. IGF signaling is due to two ligands, IGF1 and IGF2, capable of activating receptors, IGF1R and IGF2R. IGF1R responds to both ligands, while IGF2R only responds to IGF2. Growth hormone from the pituitary stimulates the liver to release IGF, and binding to the tyrosine kinase receptor, IGF1R, results in receptor autophoshorylation activating phosphorylation of various intracellular substrates. Positive signaling requires IGF binding to IGF1R, but IGF2 binding to IGF2R serves to limit IGF2 to dampen positive signaling (Baker et al., 1993). Most tissue in human and mouse express IGF1R and respond to each isoform of IGF, which are dominantly expressed at different stages of liver development. Human and mouse livers express IGF2 early in life, and levels decline as the liver matures. IGF1 exhibits the opposite expression, and is not highly expressed until the liver is fully mature. Therefore, IGF2 has been described as a fetal growth factor while IGF1 is considered an adult growth factor. Mouse knockout studies have elucidated the influence of IGF1, IGF2, and IGF1R on total body weight indicating IGF signaling is important for normal growth. Disruption of either IGF1 or IGF2 in mice results in a reduction in total body

weight to 60% of normal weight at embryonic day 18.5. Disruption of IGF1R expression has an even more dramatic impact on total body weight. Mice embryonic day 18.5 are only 40% normal wild type weight (Baker et al., 1993).

Hematopoiesis occurs in site specific locations depending on the stage of development. Prior to liver formation, hematopoiesis occurs in the aorta-gonad-mesonephros region in the embryonic mesoderm. Here, oncostatin M acts to stimulate development of both hematopoietic cells and endothelial cells. After the liver begins to form, it accepts hematopoietic stem cells and begins to function to support the production (erythropoiesis) and decomposition of red blood cells. Fetal liver is the major hematopoietic organ in the body before birth. Hematopoietic cells of the liver produce oncostatin M, and this promotes liver maturation through differentiation (Miyajima et al., 2000). After the liver gains maturity, the differentiated cells are no longer able to support hematopoiesis, and the formation of blood stem cells shifts from being produced in liver to being produced in bone marrow. However, after this switch, the liver continues to be important for blood functions producing plasma proteins including albumin, the most abundant protein in blood serum, and coagulation factors. Liver also filters the blood and metabolizes various compounds included unneeded hormones and harmful chemicals.

A major function of the liver is to metabolize endogenous and exogenous compounds to be cleared by the body so they do not accumulate and reach potentially toxic levels. Exogenous compounds such as xenobiotics are metabolized by P450s, and expression of specific P450s depends on the maturity of the liver. Certain isoforms of different P450s are expressed before life and early life while other forms are present only in fully mature adult liver. For example, in mice, CYP3A11 is not highly expressed before or immediately following birth. CYP3A11 gradually increases in expression as the liver matures. Opposite to this, CYP3A16 is only highly

expressed in fetal and early postnatal livers of mice (Peng et al., 2012). Similarly in human, the major CYP3A isoform is CYP3A4 in the adult liver, but during early liver development, CYP3A7 is the dominate CYP3A isoform (Lacroix et al., 1997). The major CYP3A isoform expressed depends on the stage of development of the liver. CYP2C29 and CYP2B10 are important xenobiotic metabolizing P450s that also exhibit a distinct pattern of expression during liver development. CYP2C29 is not highly expressed until the adult ages, while CYP2B10 expression is at its highest during the postnatal adolescent stage (Peng et al., 2012). Comparing the normal pattern of expression of well-characterized P450s to experimental conditions can allow for the determination of proper liver development.

Another function of liver is production of serum proteins which bind cations, fatty acids, and bilirubin. Two serum proteins, albumin and α-fetoprotein are produced at different levels during liver maturation, and comparing their patterns of expression in experimental conditions may allow examination of changes in proper liver development. In human, α-fetoprotein serum levels are high only in the embryo and fetus and drop to low levels after birth. Human albumin serum levels rise from low expression in the fetus to high expression in adult, but are always expressed at a higher level than α-fetoprotein even in early development (Nayak and Mital, 1977). Albumin functions as the main protein in blood to regulate oncotic pressure, however, the function of α-fetoprotein is less clear, despite being heavily expressed in fetal liver.

1.2 Long Non-Coding RNA in Liver Development

Long non-coding RNA (LncRNA) are transcripts greater than 200 nucleotides that do not code for protein. Despite not being translated, approximately 35,000 lncRNAs have been discovered as of 2005. LncRNAs exhibit characteristics of mRNA such as being 5' capped, spliced, and poly-adenylated (Carninci et al., 2005). LncRNAs are also generated similarly to

proteins having similar histone-modification profiles, splicing signals, and exon and intron lengths (Derrien et al., 2012). LncRNAs are highly abundant comprising 80% of all transcription (Kapranov et al., 2007), and their expression levels are highly tissue specific (Derrien et al., 2012) indicating their potential importance in regulating cell differentiation.

Many lncRNAs have been discovered as being regulators of gene expression. For example, HOTAIR binds to histone modification complexes including Polycomb Repressive Complex 2 (PRC2) capable of repressing transcription of the HOXD locus through epigenetic modification (Tsai et al., 2010). And XIST, one of the first characterized lncRNAs capable of manipulating gene expression, inactivates an X-chromosome in female placental mammals controlling gene dosage between XY males and XX females by first accumulating on the chromosome followed by recruitment of PRC1 and PRC2 leading to epigenetic gene silencing (Wutz and Gribnau, 2007). LncRNAs have also been implicated in more broad biologically relevant functions including controlling cell lineages by maintenance of stem cell quiescence (Venkatraman et al., 2013), pluripotency (Fatica and Bozzoni, 2014), and differentiation (Fatica and Bozzoni, 2014). This section highlights liver lncRNAs and their importance in normal development, normal liver functions, and in disease states as well as describes the various molecular mechanisms liver lncRNAs use to achieve their biological function.

LncRNAs are important regulators of normal liver development. LncRNAs are differentially expressed throughout different stages of development, and our laboratory has previously characterized, using whole transcriptome analysis, the expression of liver lncRNAs by RNA-sequencing in mice before birth to adult (Peng et al., 2014). We have discovered that there are three major oncogenic patterns of differential expression that occur at the neonatal, adolescent, and adult stages indicating differential lncRNA expression highlighting their

important role in growth and development. Another lab has done similar work to characterize mouse liver transcriptomes during development. They examined mouse livers at embryonic days 14.5 and 18 as well as adult livers, and have found similar results indicating that lncRNA expression is temporal and exists in patterns throughout development (Lv et al., 2014). For example, as the liver ages, changes in transcription of lncRNAs occurs. In mice, it was discovered lncRNAs MEG3, RIAN, and MIRG all increased in mice aged 28 months compared to young adults, four months old (White et al., 2015). Differences in lncRNA expression is not limited to different developmental stages. Many liver lncRNAs are differentially expressed due to growth hormone regulation. This results in sex-biased expression of lncRNAs (Melia et al., 2015). Liver lncRNAs may be important in regulating different developmental programs in liver due to having precise timing in their expression at particular stages during development.

1.3 Long Non-Coding RNA in Liver Functions

1.3.1 LncRNAs in Hematopoiesis

LncRNAs have been shown to play important roles in hematopoiesis. Erythropoiesis is regulated by the survival of red blood cell lineage-committed precursors and lincRNA-EPS has been shown to inhibit the apoptosis of red blood cell precursors in fetal liver (Paralkar and Weiss, 2011). There is a balance between the inhibition and promotion of apoptosis in this lineage. Erythropoietin, the major erythropoietic cytokine, binds the Epo receptor initiating signaling pathways that inhibit apoptosis of erythroid precursors (Paralkar and Weiss, 2011), while signal transduction through Fas and tumor necrosis factor receptors promotes apoptosis. LincRNA-EPS was shown to promote erythropoiesis through the prevention of apoptosis of red blood cell precursors by potentially binding *Pycard* to inhibit its transcription by recruiting

transcriptional repressor complexes (Paralkar and Weiss, 2011). PYCARD normally activates caspases to induce apoptosis, but is inactive leading to lineage progression.

MALAT1 is another lncRNA that controls lineage progression in hematopoiesis. MALAT1 expression is high in early-stage progenitor cells, and low in late-stage progenitor cells. Knockdown of MALAT1 inhibits erythroid myeloid lymphoid cell (a mouse multipotent hematopoietic cell line) proliferation after the tumor suppressor, p53, binds to the *Malat1* promoter to repress its transcription (Ma et al., 2015). MALAT1 is also a good example of a lncRNA having multiple functions. MALAT1 plays roles in liver regeneration (Li et al., 2017), glucose homeostasis, liver fibrosis (Yu et al., 2015a), HCC, and can be used as a predictive biomarker for disease in humans (Konishi et al., 2015). LncRNAs controlling differentiation have been widely reported in literature for various cell types and organs, including the liver.

1.3.2 LncRNAs in Lipid Metabolism and Homeostasis

Metabolism of endogenous compounds is also an important function of liver, and there have been many studies examining the roles of lncRNAs in lipid metabolism and lipid homeostasis with a focus on disease states regarding lipid metabolism disorder. Fatty acids, including the most common non-toxic form, triacylglycerols, are the most commonly stored and circulating forms of energy. The liver is the hub of fatty acids synthesis, lipogenesis, and lipid circulation (Nguyen et al., 2008). Improper lipid homeostasis can result in diseases, including metabolic syndromes such as obesity and type 2 diabetes.

LncLSTR has been shown to be a regulator of triglycerides by interacting with TDP-43, a transcriptional suppressor of CYP8B1 expression. This interaction leads to increased CYP8B1 activity, resulting in changes in bile acid ratios which induce apolipoprotein C2 expression

through FXR. Increased apolipoprotein C2 leads to lipoprotein lipase activation and increased plasma triglyceride clearance (Li et al., 2015b).

Lnc-HC regulates cholesterol metabolism by forming a complex with hnRNPA2B1 (Lan et al., 2015). This protein-RNA complex then binds to CYP7A1 and ABCA1 mRNA inhibiting their expression. CYP7A1 and ABCA1 are implicated in cellular cholesterol excretion, so when the level of lnc-HC is increased, a risk for cholesterol disorder is also increased. Furthermore, high cholesterol upregulates lnc-HC expression through the activator C/EBPß (Lan et al., 2015).

1.3.3 LncRNAs in Liver Regeneration

Aside from normal hepatic growth during development, the liver also has the ability to grow after injury after either physical (surgical removal) or chemical (hepatotoxicity) insult (Michalopoulos and DeFrances, 1997). This is a remarkable ability owing to being the only visceral organ capable of regeneration and needing only as little as 33% normal tissue for re-growth to its original mass with minimal disturbance in liver functions such as glucose regulation, blood protein synthesis, bile synthesis, and drug metabolism (Michalopoulos and DeFrances, 1997).

Activation of the cell cycle is needed for liver regeneration. To replace injured tissue, the organ needs to participate in massive cell-division. Two lncRNAs have been proven to be important in this process both using the same mechanism to activate the cell cycle. LncRNA-LALR1 (Xu et al., 2013) and MALAT1 (Li et al., 2017) enhance cell proliferation during liver regeneration by activation of Wnt/ß-catenin signaling facilitating the expression of cyclin D1 through suppression of Axin1. Mechanistically, lncRNA-LALR1 suppresses expression of *Axin1* by recruiting CTCF to its promoter (Xu et al., 2013).

1.4 Long Non-Coding RNA in Liver Diseases

1.4.1 LncRNAs in Glucose Homeostasis

Glucose homeostasis is an important function of liver and is influenced by lncRNA regulation. Many lncRNAs have been implicated in controlling glucose homeostasis (Sun and Wong, 2016). In particular, the previously discussed MALAT1 also appears to influence the pathogenesis of diabetes. MALAT1 is upregulated in diabetic rat and mice models and participates in crosstalk with the p38 MAPK signaling pathway regulating endothelial cell function (Liu et al., 2014). Another multifunctional lncRNA, MEG3, is most often studied for its role in cancer (Anwar et al., 2012; Zhuo et al., 2016), but has been shown to enhance insulin resistance in type 2 diabetes (Zhu et al., 2016). MEG3 increases FOXO1 expression, and FOXO1 is associated with characteristics of type 2 diabetes including hyperglycemia and hypertriglyceridemia. MEG3 is a well-studied lncRNA also having been studied in multiple liver diseases and disorders including fibrosis (He et al., 2014).

1.4.2 LncRNAs in Liver Fibrosis, Fatty Liver Diseases

Many lncRNAs work in tandem to regulate liver fibrosis. For example, lincRNA-p21 inhibits the activation, proliferation, and cell cycle progression of stellate cells to reduce fibrosis and is decreased in patients with fibrosis and cirrhosis (Zheng et al., 2015), and MALAT1 is upregulated in liver fibrosis acting as a competing endogenous RNA for miR-101b which normally represses RAC1 leading to an increase in stellate cell proliferation, activation, and progression of the cell cycle (Yu et al., 2015a).

H19 was recently implicated in being important in bile acid homeostasis. Bile is produced in liver and functions to aid digestion of lipids by emulsification in the small intestine.

Cholestatic liver fibrosis can occur after small heterodimer partner (SHP) degradation by BCL2, an antiapoptotic regulator protein. SHP normally acts to transcriptionally repress H19, and H19 is not found in normal healthy adult liver. If H19 not repressed, liver injury, fibrosis, and inflammation occurs alongside an increase in serum bile acids and bilirubin (Zhang et al., 2016).

Another lncRNA, HULC, is upregulated in HCC and promotes lipogenesis to increase triglyceride and cholesterol levels in cancer cells. HULC activates PPARA by inhibiting expression of miR-9, an inhibitory microRNA against PPARA mRNA. PPAR activates the *ACSL1* promoter when not inhibited due to HULC coordinating methylation of the *miR-9* promoter. ACSL1 generates cholesterol which further promotes HULC's ability through positive feedback (Cui et al., 2015).

Specific lncRNAs implicated in liver fibrosis have been shown to affect the profibrogenic factor TGF-β1 (Yang et al., 2015; Yu et al., 2015b). APTR is increased in the stellate cells of fibrotic liver, and APTR promotes upregulation of α-smooth muscle actin in stellate cells through TGF-β1 signaling (Yu et al., 2015b). TGF-β1 signaling also downregulates MEG3 to promote liver fibrosis. A downregulation of MEG3 is achieved by promoter methylation (Yang et al., 2015).

LncRNA GAS5 inhibits the activation of stellate cells and is another regulator of liver fibrogenesis. miR-222 targets GAS5 and inhibits its expression, and reciprocally, GAS5 binds miR-222 to reduce its expression. Both reduction of miR-222 and GAS5 occur at the RNA level. Therefore, due to competition, higher levels of GAS5 results in greater inhibition of miR-22 expression. As a result of the reduction of miR-222 by GAS5, p27 increases, because miR-222 also binds competitively to inhibit this protein. An increase in p27 results in inhibition of the activation and proliferation of stellate cells (Yu et al., 2015c).

Berberine, a compound isolated from Chinese herbal medicines, reduces hepatic steatosis through lncRNAs to ameliorate Non-Alcoholic Fatty Liver Disease (NAFLD). Steatotic liver has reduced levels of both lncRNA MRAK052686 and Nrf2, but these levels recover with berberine treatment. Interestingly, berberine was found to change the expression profiles of 538 lncRNAs in steatotic livers of mice (Yuan et al., 2015).

1.4.3 LncRNAs in Liver Injury

Hematopoietic stem cell transplantation after radiation and chemotherapy can cause damage to hepatocytes and sinusoidal endothelial cells leading to hepatic veno-occlusive disease, and lncRNAs have been shown to be affected. LncRNAs were found to be dysregulated in hepatocytes (2,918 upregulated and 1,911 downregulated) after hematopoietic stem cell transplantation, and pathway analysis revealed increased T-cell receptor signaling and a decrease in VEGF signaling (Qiao et al., 2016).

Injury to liver tissue after liver is harvested and placed in cold storage prior to transplantation is an important concern. TUG1 has been found to be decreased during cold storage, and overexpression of TUG1 in mouse livers has been found to decrease injury after cold storage by preventing mitochondrial apoptosis and inhibiting endoplasmic reticulum stress pathways in hepatocytes and sinusoidal endothelial cells leading to increased graft survival (Su et al., 2016a).

1.4.4 LncRNAs in HBV and HCV Infection

LncRNAs also have consequences in liver diseases caused by viral infection. Both hepatitis B virus (HBV) and hepatitis C virus (HCV) have the potential to change cellular regulatory mechanisms that lead to hepatocellular carcinoma (HCC). HBV and HCV can impact

chromosomal instability and alter gene expression. HCV, an RNA virus, affects liver and acute and chronic symptoms resemble HBV symptoms. In contrast to HBV, HCV infection tends to lead to chronic infection. There is no approved vaccine for HCV, but recently drug treatment has been shown to cure the disease with a greater than 95% effective rate (Hoofnagle and Sherker, 2014). In HCV related HCC, lncRNA expression profiles are altered including upregulation of LINC01419 and AK021443, while lncRNA AF070632 is downregulated (Zhang et al., 2015b). In HBV, hepatitis B virus X protein (HBx) is a well cited transactivating viral protein capable of dysregulating many liver cell functions including cell cycle progression and apoptosis leading to HCC. Infection with HBV, a DNA virus, can result in a few weeks of acute symptoms including vomiting, yellowish skin, tiredness, dark urine, and abdominal pain. Chronic HBV may result in cirrhosis and liver cancer with death in 15 to 25% of patients. Since 1982, infection by HBV has been preventable by vaccination (Pungpapong et al., 2007). LncRNA-Dreh may be important in HBV related liver diseases as it is downregulated by HBx. LncRNA-Dreh was found to be a tumor suppressor inhibiting tumor growth and metastasis in HCC caused by HBV. Mechanistically, LncRNA-Dreh was able to repress vimentin by binding the protein which results in inhibition of tumor metastasis (Huang et al., 2013).

LncRNA HOTAIR and PLK1 kinase are also increased in HBV-induced liver carcinogenesis. HOTAIR binds both the repressive factors SUZ12 and ZNF198 to enhance PLK1 ubiquitination and subsequent protesomal degradation of SUZ12 and ZNF198. Their downregulation leads to epigenetic reprogramming in HBx-expressing cells, notably the modification of the *EPCAM* promoter, a gene important for cell adhesion and transformation (Zhang et al., 2015a).

1.4.5 LncRNAs in Progression of Hepatocellular Carcinoma

HCC is the most studied liver disease, and there have been many newly discovered roles that various lncRNAs play in its progression. HCC is the fifth most common cancer (Parkin et al., 2005) and the most common liver cancer accounting for 75% of all primary cases (Ahmed and Lobo, 2009). HCC has many known risk factors, including HBV and HCV (Tanaka et al., 2011). Treatment includes liver transplantation with a survival rate ranging from 67% to 91% from studies performed in the late 2000's (Vitale et al., 2007), pharmacological intervention with a tyrosine kinase inhibitor, sorafenib, that inhibits tumor-cell proliferation (Llovet et al., 2008), or surgical resection (Ang et al., 2015).

LncRNA research in liver diseases has focused on HCC and many lncRNAs have been discovered that are important for disease initiation and progression. Many studies utilize transcriptome and sequencing analysis to discover lncRNAs that are upregulated or downregulated at the transcriptome level in HCC compared to normal tissue (Esposti et al., 2016). Other studies examine variations in lncRNA copy number at the DNA level (Zhou et al., 2015). From there, the identified differentially expressed lncRNAs are studied further to determine their exact roles in disease often discovering one or more clear molecular mechanism for their actions. General characterization studies use tools that indicate regulation pathways where lncRNAs participate. For example, one particular study finds a total of 5,525 lncRNAs in 23 liver tissue samples comprised of controls, cirrhosis, and HCC and finds 57 differentially regulated lncRNAs that participate in cell cycle regulation, TGF-β signaling, and liver metabolism (Esposti et al., 2016). Alternatively, examination of lncRNAs expressed in fetal liver compared to adult can point to potential oncofetal genes. Often lncRNAs that are ectopically expressed in adult tissue indicates dysregulation and increased cell proliferation as seen in cancer or liver regeneration after injury. Many lncRNAs have been discovered to be

important in liver cancer by this method including lncRNA PVT1 (Wang et al., 2014) and H19 (Ariel et al., 1998).

An example of an HCC liver lncRNA discovered using genome sequencing is lncRNA-PRAL. Variations in the copy number of this gene were discovered. Deletion of *lncRNA-PRAL*, a lncRNA that induces apoptosis through p53 regulation, has been associated with a decrease in HCC survival. In mice where tumors have been induced, delivery of lncRNA-PRAL reduces tumor volume (Zhou et al., 2015). To highlight the various mechanisms lncRNAs can achieve, we focus here on the molecular mechanisms involved in HCC with emphasis on the similarities and differences of their molecular functions.

1.4.6 Molecular Mechanisms of LncRNA in Hepatocellular Carcinoma

Recent studies have suggested that initiation of HCC beings with progenitor cells of the liver rather than the parenchyma. The lncRNA CUDR accelerates liver cancer stem cell growth by binding cyclin D1 and as a complex, binding both the promoters of *H19* and *c-myc*. Increased expression of H19 promotes excessive TERT enhancing telomerase activity and c-myc increases liver cancer stem cell proliferation (Pu et al., 2015). CUDR also interacts with another lncRNA, HULC, in tumorigenesis (Gui et al., 2015). CUDR enhances embryonic stem cell differentiation using epigenetic modifying mechanisms. It was shown to inhibit histone H3K27 trimethylation and to decrease promoter methylation of *HULC*. CUDR is also capable of changing chromatin looping promoting recruitment of RNA polymerase II and P300 via CTCF. HULC is also implicated in promoting angiogenesis by upregulating SPHK1 through sequestering miR-107 which normally targets and inhibits the transcription factor E2F1 (Lu et al., 2016b). LncRNA DILC represses self-renewal and expansion of liver cancer stem cells and has been found to be decreased in HCC. DILC binds the *IL6* promoter to inhibit its transcription leading to decreased

STAT3 activation (Wang et al., 2016c). LINC00152 activates the mTOR pathway and binds the *EPCAM* promoter to potentially promote cell proliferation (Ji et al., 2015). H19 has been widely implicated as being important in HCC. H19 knockdown in cancer cell lines, such as Hep3B, has been shown to decrease tumor weight and tumor volume (Matouk et al., 2007). A microRNA that is transcribed within the first exon of H19 has been shown to upregulate H19 expression in HCC. miR675 inhibits HP1α causing histone modifications in the EGR1 promoter (reduced H3K9 trimethylation, reduced H3K27 trimethylation, and increased H3K27 acetylation) enhancing its transcription. EGR1 upregulates H19 which activates PKM2 promoting tumorigenesis (Li et al., 2015a).

Previously mentioned lncRNA PVT1 is increased in HCC and indicates poor prognosis. PVT1 binding to NOP2 is needed for this lncRNA to increase cell proliferation, cell cycling genes, and generate a more stem-cell like property of cells. Stabilization and upregulation of NOP2 is also stabilized by this binding (Wang et al., 2014).

Epigenetic modifying enzymes impart histone modifications or chemically alter DNA itself, such as simply adding methylation, influence the accessibility of the transcriptional machinery towards particular genes therefore influencing gene expression. Guide lncRNAs act by bringing epigenetic modifying enzymes to impact transcription. A clear example of a guide lncRNA is lncRNA-HEIH. LncRNA-HEIH binds the enhancer EZH2, a component of PRC2, the histone remodeling complex capable of repressing target genes through methylation of H3K27 (Yang et al., 2011). LncRNA-HEIH essentially guides the repressive complex to genes targeted by EZH2. Another example of a guide lncRNA is HOTTIP. However, HOTTIP interacts instead with a transcriptional activating complex, WDR5/MLL, that promotes HOXA

genes through H3K trimethylation (Quagliata et al., 2014) showing that liver lncRNAs can act as both positive and negative regulators of gene expression.

Many liver lncRNAs function as decoys limiting expression of proteins by binding and sequestering them. For example, lncRNA-Dreh is capable of altering the filament and cytoskeleton structure of cells by binding vimentin, an intermediate filament protein, to inhibit its expression (Huang et al., 2013). Similarly, lncRNA-MVIH associates with PGK1, a glycolytic enzyme, to disallow its function in angiogenesis (Yuan et al., 2012). In converse, the binding of proteins to lncRNAs can also serve to enhance expression of that protein such as the scaffold/guide lncRNA-PRAL. LncRNA-PRAL binds HSP90 and p53 promoting HSP90-p53 interaction which stabilizes p53 by disallowing MDM2 binding and ubiquitination of p53 (Zhou et al., 2015). These mechanisms are also examples of lncRNAs that do not directly bind DNA to regulate transcription, but rather affect the availability or stability, affecting transcriptional regulation or simple positive or negative expression of that particular molecule.

LncRNAs can also be destabilized to limit their function as in the case of lncRNA HULC. IGF2BP1 binds and destabilizes lncRNA HULC through the recruitment of CNOT1 protein, the scaffold of the CCR4-NOT deadenylase complex, which is important for the cytoplasmic RNA decay machinery (Hammerle et al., 2013). In this example, the lncRNA is not the decoy, but rather the protein is the decoy titrating away the lncRNA limiting its biological function to impact gene regulation.

LncRNAs in liver have complex regulation schemes. LncRNA-ATB is regulated by TGF- ß and then lncRNA-ATB itself regulates multiple signaling processes. Acting as a decoy, in this case against miRNA instead of protein, lncRNA-ATB titrates away the miR-200 family (Sun and Wong, 2015). miR-200s act to repress the epithelial-mesenchymal transition (EMT)

inducing transcription factors, ZEB1 and ZEB2, at the mRNA level. LncRNA-ATB also regulates the IL-11/STAT3 pathway by stabilizing IL-11 mRNA leading to increased IL-11 protein secretion (Sun and Wong, 2015).

In the previous examples of miRNA regulation, liver lncRNAs require physical binding to miRNAs serving as decoys titrating away these repressors from being biologically active. However, lncRNAs can also regulate miRNA by other mechanisms. LncRNA DANCR blocks miRNA repression by binding CTNNB1 mRNA which is normally repressed by miR-214, miR-320, and miR-199a (Yuan et al., 2016). Here, the lncRNA serves not as a decoy but rather a shield for repression similarly like lncRNA-PRAL that blocks ubiquitination stabilizing p53. In this example, however, the mRNA and not the protein is protected.

1.4.7 LncRNAs as Biomarkers

Early indication of disease can lead to quicker diagnosis and treatment preceding better health outcomes due to the prevention of irreversible precipitous disease progression. Misdiagnosis is also an obstacle in healthcare, because often clinical treatment itself causes death and disease. For example, globally it has been estimated that 141,700 people died in 2013 from the effects of medical treatment (Mortality and Causes of Death, 2015) indicating a need for correct diagnosis preventing unneeded treatments. Finding ways to diagnose that are non-invasive are also more practical, usually more cost-effective, and less harmful to patients. Therefore, correct, quick, and noninvasive diagnosis is an important health concern, and biomarkers have become a more widely used useful tool.

Finding new biomarkers indicating disease using body fluids that are easily obtained as opposed to other methods, such as tissue biopsy, which are invasive and impractical in many

circumstances, can potentially lead to better and faster diagnosis. Many lncRNAs have been found in blood that could potentially serve as indicators of disease. For example, APTR is elevated in serum of patients with liver cirrhosis and research has proposed its role as being potential marker for the disease (Yu et al., 2015b).

Biomarkers for HCC are also in demand due to the need for early diagnosis of cancer for better survival potential. Many lncRNAs have been proposed for this purpose. Not only is MALAT1 expression higher in solid tumors, but elevated levels in the plasma is associated with liver damage and can predict development of HCC (Konishi et al., 2015). Linc00974 is stably expressed in plasma, and using qRT-PCR combination analysis for both linc00974 and CYFRA21-1, an already established tumor marker, high prediction of oncogenesis, tumor growth, and metastasis in HCC patients could be achieved (Tang et al., 2014). Linc00974 upregulates KRT19 by acting as a competitive endogenous RNA for KRT19's inhibitor miR-642. Increasing KRT19 expression activates Notch and TGF-β signaling.

Other lncRNAs have potential for being serum biomarkers, but have yet been tested for this purpose. High expression of lnc-DILC predicts early recurrence and short survival of patients with HCC indicating it may have prognostic value (Wang et al., 2016c), however it has yet to be tested for its prediction of disease after isolation from blood. Further research that discovers positive correlation to lncRNAs in blood with early disease need exploration to aid in early and correct diagnosis for rapid disease treatment.

The contents of extracellular vesicles is gaining new attention emerging as potential biomarkers for disease. Extracellular vesicles are present in many biological fluids including blood, urine, and bile (Patel, 2014). To be effective biomarkers, the contents of extracellular vesicles needs to reflect the contents of the cell of origin, be different between healthy and

disease, and be reliably detected (Mohankumar and Patel, 2015). Characterization of lncRNAs differentially expressed in diseased patients from isolated extracellular vesicles has found promising results. Three potential lncRNA biomarkers, RP11-160H22.5, XLOC_014172, and LOC149086, were recently found to be upregulated in extracellular vesicles found in plasma of patients diagnosed with HCC (Tang et al., 2015). And, lincRNA-ROR, which is also packaged in extracellular vesicles, has the potential to indicate drug sensitivity to chemotherapy against HCC (Takahashi et al., 2014). Linc-ROR is increased in HCC chemoresistance. With sorafenib treatment, linc-ROR increases in both cells and extracellular vesicles excreted by tumor cells. When linc-ROR is increased, there is lower apoptosis and cytotoxicity with drug treatment. Mechanistically, TGF-β enriches linc-ROR within extracellular vesicles (Takahashi et al., 2014).

1.4.8 Targeting lncRNAs

Novel drug targets to treat disease and biomarkers to correctly and quickly diagnose disease have the potential to greatly impact human health. New targets will allow for more effective therapies and reduce the need for drugs with harmful side effects. Due to lncRNAs being highly tissue specific (Derrien et al., 2012), using them as drug targets has the potential to reduce off-target effects and limit unwanted, harmful side effects. The liver is an essential organ. It serves vital functions that are indispensable from the beginning of life before birth to adulthood. Its necessity is emphasized by its capability to regenerate after injury. Research discovering the biological functions of lncRNAs is becoming more and more prevalent as their functions have been found to be more and more abundant and diverse. As more lncRNAs are discovered to be important in normal growth and in diseased states, more targets for therapies and biomarkers will be generated. Deeply understanding the mechanisms that lncRNA utilize to regulate gene function and various other processes will not only give us insights in how our body

normally functions but also how disease progresses. As technology and innovation advances, the discovery of new lncRNAs and how they impact the liver will continue and yield fascinating insights into basic liver physiology and help to better treat liver disease.

1.5. Targeting H19, an Imprinted Long Non-Coding RNA, in Hepatic Functions and Liver Diseases

1.5.1 Abstract

H19 is a long non-coding RNA regulated by genomic imprinting through methylation at the locus between H19 and IGF2. H19 is important in normal liver development, controlling proliferation and impacting genes involved in an important network controlling fetal development. H19 also plays a major role in disease progression, particularly in hepatocellular carcinoma. H19 participates in the epigenetic regulation of many processes impacting diseases, such as activating the miR-200 pathway by histone acetylation to inhibit the epithelial-mesenchymal transition to suppress tumor metastasis. Furthermore, H19's normal regulation is disturbed in diseases, such as hepatocellular carcinoma. In this disease, aberrant epigenetic maintenance results in biallelic expression of IGF2, leading to uncontrolled cellular proliferation. This section aims to aid further research utilizing H19 for drug discovery and the treatment of liver diseases by focusing on both the epigenetic regulation of H19 and how H19 regulates normal liver functions and diseases, particularly by epigenetic mechanisms.

1.5.2 Introduction

H19, a long non-coding RNA (lncRNA), is both epigenetically regulated and utilizes epigenetic mechanisms to regulate liver cell functions. We will first describe the history of H19 and then focus on the regulation of the gene expression of H19. It is uniquely expressed from

one allele by an intricate process called genomic imprinting. Then, we will examine H19's roles and implications in various normal functions in liver development and growth, including the regulation of bile acid homeostasis and xenobiotic metabolism. We will discuss dysregulation of H19 in the progression of liver-related diseases, including steatosis, fibrosis, cirrhosis, diabetes, and hepatocellular carcinoma (HCC) with a particular focus on the epithelial to mesenchymal transition (EMT). Finally, we will explore using H19 in therapies for liver diseases either by targeting H19 or by directly using the *H19* promoter to drive selective toxicity in cancer. The mechanisms of regulation will be highlighted, emphasizing epigenetic mechanisms.

The discovery and characterization of H19, one of the first lncRNAs described, overviews how lncRNAs were first discovered and assumed to have functions despite not coding for protein. In 1984, the Tilghman lab discovered an RNA transcript that was highly expressed in fetal mouse liver, but decreased in adult liver. They screened a fetal liver cDNA library for moderately abundant clones that hybridized only to a fetal liver cDNA probe, but did not hybridize to an adult liver cDNA probe and other controls; the clone was designated H19 based on its position being the 19th clone in row H. In the discussion of their paper, Pachnis et al. state, "The identity of this protein encoded by H19, if indeed one exists, is unknown at this time" (Pachnis et al., 1984). Further characterization by sequencing revealed that *H19* had multiple translation termination signals in all three reading frames, but conversely, was highly conserved. H19 was still transcribed by RNA polymerase II, spliced, and polyadenylated, but puzzlingly did not associate with ribosomes. This dual nature of being similar to protein-coding genes, but incapable of translation led the authors to conclude "…the product of this unusual gene may be an RNA molecule" (Brannan et al., 1990). Since then, genome-wide technologies, such as microarrays, particularly tiling arrays, which allow for characterization of sequenced regions

where function was not known, resulted in a boom of lncRNA discovery. Technology continues to develop at an alarming rate, and the fascinating H19 discovery was only the beginning of a new field in science.

There are numerous reviews discussing the vast amount of research on H19. Prior reviews cover topics as extensive as H19's regulation (Sasaki et al., 2000; Banerjee et al., 2001; Gabory et al., 2006; Nordin et al., 2014), or its broad role in cancer (Raveh et al., 2015; Jing et al., 2016; Matouk et al., 2016). Other reviews are more specific to particular types of cancer (Wake et al., 1998; Matouk et al., 2015; Lin et al., 2016). There is a need for more reviews discussing diseases in a particular organ, especially the liver. When H19 is discussed in the context of HCC, reviews examine other lncRNAs in their analysis without focus on H19. First, will be a description of H19's role in liver diseases, including HCC, with a particular emphasis in any epigenetic regulation where H19 participates. We will also highlight research from other organ systems that need translational examination in the liver. Overall, we aim to provide a resource for future research on H19, so liver diseases may be treated more effectively in the future.

1.5.3 Characterization of H19 and Its Participation in Epigenetic Regulation

1.5.3.1. LncRNAs

LncRNAs are transcripts greater than 200 nucleotides that do not code for proteins. Despite not being translated, approximately 35,000 lncRNAs have been discovered that exhibit characteristics of mRNA, such as being 5′ capped, spliced, and poly-adenylated (Carninci et al., 2005). Furthermore, lncRNAs are generated similarly to proteins, having similar histone-modification profiles, splicing signals, and exon and intron lengths (Derrien et al., 2012).

LncRNAs are abundant, comprising 80% of all transcripts (Kapranov et al., 2007) and their expression levels are highly tissue specific (Derrien et al., 2012).

Molecular functions of lncRNAs are described as signals, decoys, guides, and scaffolds (Wang and Chang, 2011). LncRNAs act effectively as signals due to their specific expression in specific cell types and stages of development as well as their capability to respond to stimuli. Many lncRNAs found in liver currently described in the literature can be classified as signals due to being tissue specific, disease state specific, and/or developmentally specifically expressed. LncRNAs also act as decoys by binding and titrating away proteins. They can be guides capable of binding regulatory molecules, including chromatin remodelers and transcription factors, directing them to specific DNA targets to control gene expression. With different domains capable of binding different molecules, they can also be scaffolds, assembling a complex arrangement of components (Wang and Chang, 2011). Thus, lncRNAs can exhibit many functions and work within the cell to regulate different processes by various molecular mechanisms.

1.5.3.2 H19

The characteristics of H19 are similar to other lncRNAs in both structure and their temporal and tissue-specific expression pattern. Structurally, the *H19* gene contains five exons and four introns, producing a 2.3-kb lncRNA after splicing. The *H19* gene contains shorter introns than most lncRNA genes, each less than 100 base pairs (Kent et al., 2002). It is transcribed from chromosome 7 in the mouse and chromosome 11 in the human. It is adjacent to the protein-coding gene, *insulin-like growth factor 2 (IGF2),* an important fetal growth factor. These two genes share regulatory sequences required for their expression, including two enhancers located 3' downstream of *H19* (Leighton et al., 1995b). Their expression is also

controlled by an *imprinting control region (ICR)* between the two gene loci and exhibits differential methylation (Drewell et al., 2002). Temporally, H19 is expressed in the embryo, but subsequently downregulated in all tissues, excluding skeletal muscle, shortly after birth (Brunkow and Tilghman, 1991). During the fetal and postnatal ages, H19 is abundant in the liver. H19 expression has been shown to correlate with the expression of IGF2, implicating H19's important role in liver development.

H19 has many diverse biological functions. It is known to participate in the regulation of cell proliferation (Yamamoto et al., 2004) and differentiation (Dey et al., 2014), as well as its role in cancer both as an oncogene (Hibi et al., 1996; Barsyte-Lovejoy et al., 2006) and as a tumor suppressor (Yoshimizu et al., 2008). In the developing fetus, H19 regulates a number of important genes within the imprinted gene network (IGN), including *Igf2*, responsible for proper embryonic development (Gabory et al., 2010). Due to participating in many known biological functions and being one of the first lncRNAs discovered and characterized, H19 is one of the most well-studied lncRNAs.

The *H19* locus harbors multiple transcripts (Figure 1.1). The main transcript, H19, functions as the full lncRNA, and it encodes within its first exon two variants of microRNA, miR-675 (Dey et al., 2014). There are also two antisense transcripts produced from the locus, 91H and HOTS. The HOTS transcript can produce a nucleolar protein (Onyango and Feinberg, 2011), while 91H appears to function solely as an lncRNA (Berteaux et al., 2008). A review and discussion on the antisense transcripts is presented in Section 1.5.7.

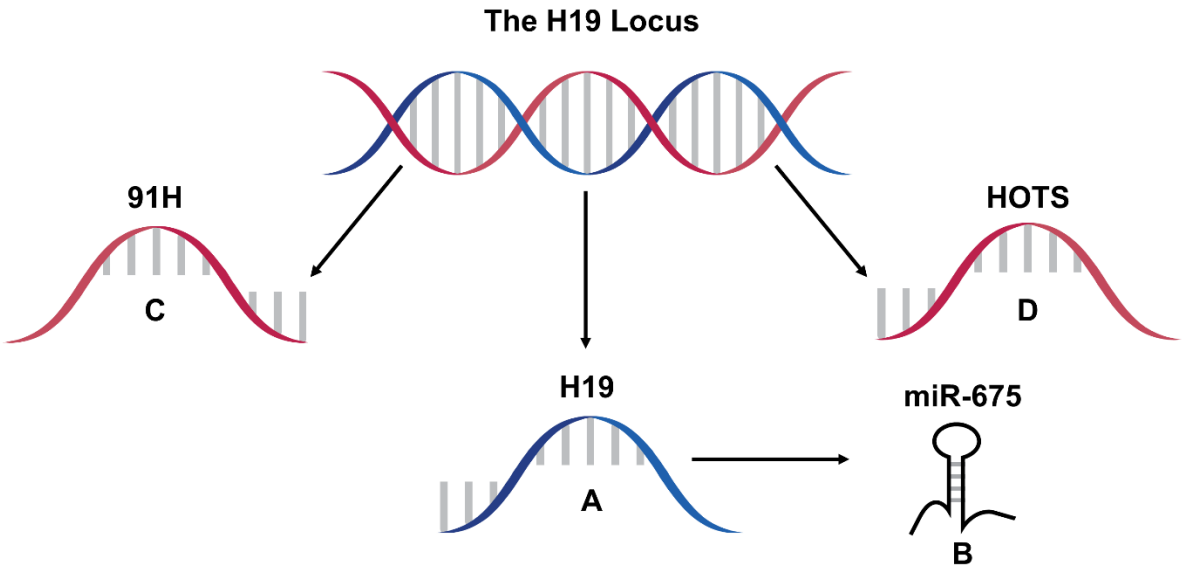

The H19 Locus
91H
C
HOTS
D
H19
A
miR-675
B

Figure 1.1 Transcription at the *H19* locus consists of the well-characterized H19 lncRNA (**A**) and its microRNA miR-675 (**B**) encoded within the first exon of *H19*. Two antisense transcripts are also formed from the locus. 91H RNA (**C**) is described as varying in length, but can potentially be transcribed from the complementary DNA strand entirely encompassing *H19* and other portions of its regulatory sequences. HOTS RNA (**D**) is transcribed from most of the antisense sequence of *H19* and upstream bases. HOTS can be translated to form a nucleolar protein.

1.5.3.3 Regulation of H19 Expression by Epigenetic Mechanisms

H19 is regulated by the epigenetic phenomenon, genomic imprinting. Normally, H19 is only expressed from one parental allele and silenced epigenetically on the reciprocal chromosome. The paternal allele is imprinted, or silenced, due to the highly methylated *ICR* found between *H19* and *IGF2*. The *ICR* is a *differentially methylated region (DMR)* due to having different methylation statuses depending on the chromosomal parental origin. At the maternal allele, H19 is expressed, and on this chromosome, the *ICR* is hypomethylated (Manoharan et al., 2004). *IGF2* is also imprinted, but opposite of *H19*, expression is driven from the paternal allele and the maternal allele is silenced (DeChiara et al., 1991). The same *ICR* also controls *IGF2* imprinting. Located approximately 2 kb upstream of the H19 promoter between *H19* and *IGF2*, this region regulates monoallelic expression of those genes by being differentially methylated depending on the parental origin of the allele. In normal conditions, the *ICR* is hypermethylated at the paternal allele and hypomethylated at the maternal allele. This balance is important in controlling normal expression of these genes. Deleting 10 kb upstream *H19* disrupts this region and causes biallelic IGF2 expression, thereby disrupting imprinting (Ripoche et al., 1997). The mechanism controlling imprinting of *H19* and *IGF2* involves the binding of either MeCP2 or CTCF based on the methylation status of the *ICR*.

On the hypermethylated *ICR* found on the paternal chromosome, H19 is repressed and IGF2 expressed. H19 expression is silenced through histone deacetylation by MeCP2 binding to methylated CpG dinucleotides. After binding, histone deacetylases (HDACs) interact with MeCP2 to repress H19 (Drewell et al., 2002). Furthermore, in this state of *ICR* methylation, the *ICR* binds another *DMR* located within *IGF2*, *DMR2*. This *ICR* and *DMR2* interaction moves *IGF2* into position to interact with the enhancer region driving IGF2 expression (Kurukuti et al.,

2006). The binding of the insulator CTCF to the *ICR* is disallowed due to the high number of methyl groups (Kurukuti et al., 2006).

Conversely, on the hypomethylated *ICR* found on the maternal chromosome, H19 expression is not repressed by MeCP2 binding and able to interact with its enhancer region. CTCF is allowed to bind, insulating and preventing the expression of IGF2 by two known mechanisms. First, CTCF binding allows the interaction of the *ICR* with *matrix attachment region 3 (MAR3)* and another *DMR*, *DMR1*, which is located in an intergenic region of *IGF2*. These interactions result in a loop around *IGF2* and impediment of expression (Kurukuti et al., 2006), as well as disallowing the enhancers from interacting with *IGF2* (Murrell et al., 2004). Specifically in liver, the binding of CTCF to the *ICR* is mediated by the interaction of CTCF with prohibitin 1 (Ramani et al., 2016). Second, CTCF interacts with the *IGF2* promoter region and recruits polycomb repressive complexes leading to H3K27 methylation causing suppression of IGF2 (Li et al., 2008).

In summary, two states on separate parental alleles exist in normal conditions when H19 and IGF2 are co-expressed. On the paternal allele, the *ICR* is methylated, and the enhancer region is able to interact with *IGF2* to allow expression. On the maternal allele, the *ICR* is not methylated, allowing the same enhancer region to, instead, drive H19 expression. Therefore, this region and its methylation status are critical for both H19 and IGF2 expression, expressed coordinately albeit from opposite alleles.

1.5.3.4 H19 Regulates Gene Expression by Epigenetic Mechanisms

Activating or repressing H19 may be important as an epigenetic therapy for future treatment of epigenetic abnormalities in diseases. In this section, we aim to highlight known

cases where H19 directly participates in either the repression or activation of transcription through direct epigenetic mechanisms. First, we will examine H19 in the repression of genes and, second, in the activation of genes. Finally, we will examine how H19's role as an epigenetic modifier impacts chemotherapy resistance. In these examples, H19 plays a clear role in affecting transcription through chromatin remodeling.

In many examples, H19 recruits epigenetic modifiers, acting as a guide to repress gene expression. Important for embryonic development, H19 controls at least nine imprinted genes (*Igf2, Igf1r, Dlk1, Meg3, Slc38a4, Peg1, Dcn, Cdkn1c,* and *Gnas*) in the mouse IGN (Monnier et al., 2013). The control over some of these genes occurs by H19 interaction with methyl-CpG-binding domain protein 1 (MBD1), a protein in the same family as MeCP2 (discussed earlier as a repressor of H19 expression). MBD1 binds to methylated DNA to recruit HDACs and histone lysine methyltransferase (KMT)-containing complexes, SETDB1 and SUV39H1, silencing genes via H3K9 methylation, inducing chromatin compaction (Figure 1.2 A) (Monnier et al., 2013). In a second example, H19 represses gene expression through the interaction with enhancer of zeste homolog 2 (EZH2), a H3K27 methyltransferase in a part of the polycomb repressive complex 2 (PRC2) in bladder cancer. H19 association with EZH2 results in the activation in Wnt signaling and recruitment of PRC2 to silence E-cadherin (Figure 1.2 B) (Luo et al., 2013). Although, the direct H19 and EZH2 binding has not been determined in liver, EZH2 has been shown to silence tumor suppressor microRNAs in liver cancer and is upregulated in HCC (Au et al., 2012). It would be interesting to examine H19's role in HCC to determine if they are binding partners in this condition.

H19 can also promote gene expression by acting as a guide for epigenetic modifying enzymes. For example, H19 binds hnRNP U, part of a complex with RNA polymerase II and a

histone acetyltransferase, PCAF. Histone acetylation results in the upregulation of genes within the miR-200 family. Activation of the miR-200 family ultimately suppresses metastasis in HCC (Figure 1.3) (Zhang et al., 2013). These examples highlight how H19 can both repress and activate gene expression by epigenetic mechanisms, making context crucial in understanding its functions.

H19's role as an epigenetic modifier may also be important in the study of a barrier in cancer treatment, chemotherapy drug resistance. P-glycoprotein, an efflux transporter, is often upregulated in cancer cells. This prevents cancer drugs from accumulating in cancer cells, thereby reducing their efficacy. H19 may induce P-glycoprotein expression by regulating *MDR1* promoter methylation (Tsang and Kwok, 2007). In a doxorubicin-resistant hepatocellular carcinoma cell line, R-HepG2, it was found that knockdown of H19 resulted in an increase of promoter methylation at the *MDR1* promoter. Establishment of the mechanism, by which H19 regulates the promoter methylation status of efflux transporters in chemotherapy drug resistance, will further define H19's role to influence gene expression as an epigenetic modifier.

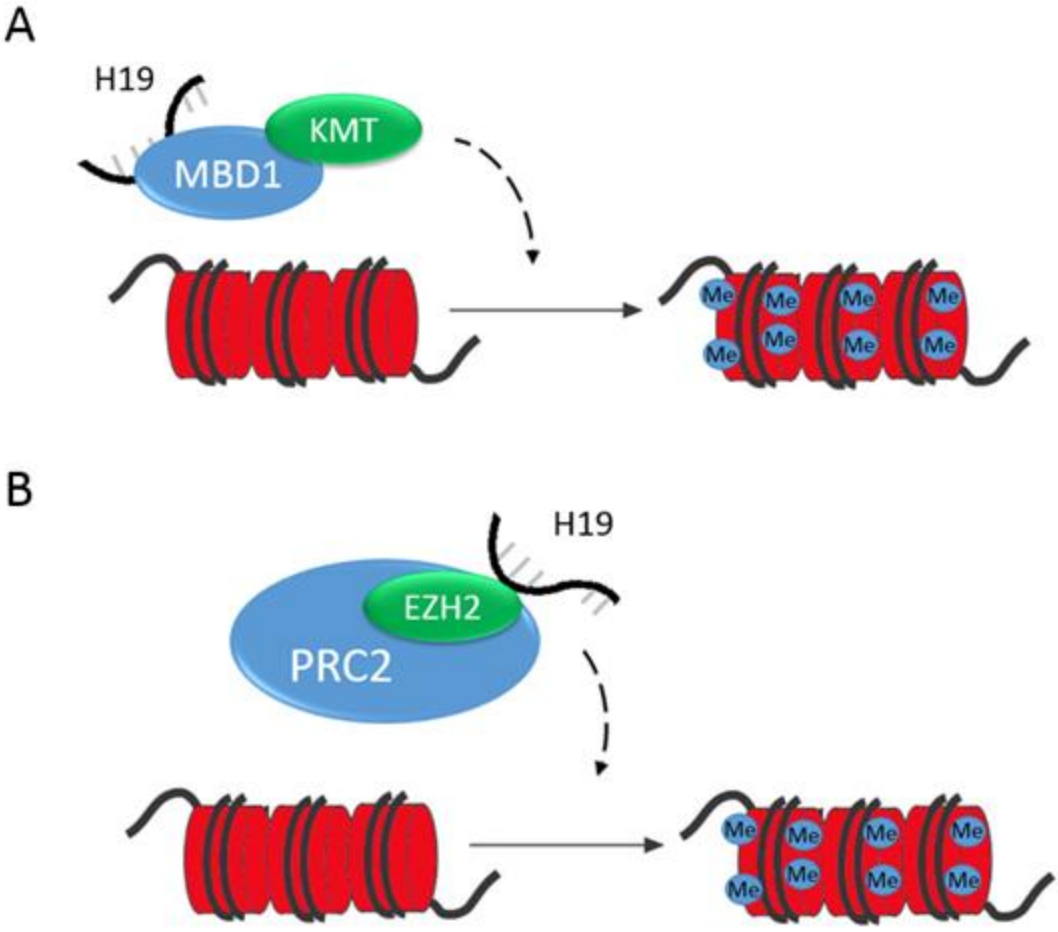

A
H19
KMT
MBD1
Me
B
H19
EZH2
PRC2
Me

Figure 1.2. Epigenetic regulation by H19. H19 acts to repress genes within the imprinted gene network, such as *IGF2*. H19 binds MBD1. MBD1 then binds methylated DNA and then recruits histone lysine methyltransferases (KMTs) to silence these genes by chromatin compaction (**A**). H19 silences E-cadherin. H19 binds EZH2, a H3K27 methyltransferase part of the PRC2, causing downregulation of E-cadherin (**B**).

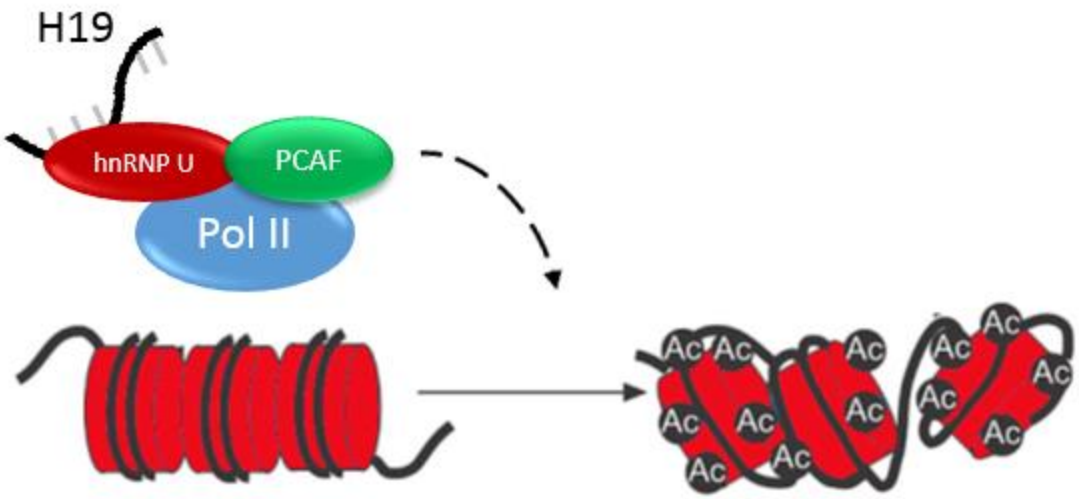

H19
hnRNP U
PCAF
Pol II
Ac
Ac
Ac
Ac
Ac
Ac
Ac
Ac
Ac
Ac
Ac
Ac
Ac

Figure 1.3. H19 can also activate gene transcription. H19 binds hnRNP U, which is a member of the complex with RNA Polymerase II and PCAF, a histone acetyltransferase. H19 binding results in the upregulation of the miR-200 family of microRNAs through histone H3 acetylation.

1.5.4 The Roles of H19 in Normal Liver Functions

1.5.4.1 The Roles of H19 in Liver Development

The liver is an essential organ for life. Hepatocytes, the main parenchyma of the liver, comprise almost 80% of the adult liver's total mass (Kmiec, 2001). The remaining mass includes cholangiocytes, sinusoidal endothelial cells, stromal cells, Kupffer cells, and stellate cells. The liver has many important biological functions, such as detoxification, modification and excretion of exogenous and endogenous substances, the synthesis of cholesterol, bile salts, and phospholipids, and blood glucose regulation (VanPutte et al., 2013). The liver is also the major organ for hematopoiesis in the fetus (Georgiades et al., 2002). We will examine H19's role in some of these major processes, including its role when these processes are not functioning normally in disease.

Many studies have shown that lncRNAs are important regulators of normal liver development. LncRNAs are differentially expressed throughout different stages of development from embryogenesis to adult life. Our lab has previously characterized, using whole transcriptome analysis, the expression patterns of liver lncRNAs by RNA-sequencing in mice before birth to adult (Peng et al., 2014). We have discovered that there are three major oncogenic patterns of differential expression and potentially a functional transition of lncRNAs that occur at the neonatal, adolescent, and adult stages, implying their importance in liver maturation. H19 was found to be the most differentially expressed annotated lncRNA between fetal and adult liver. Mechanistically, H19 expression is drastically silenced at the postnatal age due to transcriptional repression by zinc fingers and homeoboxes 2 (Zhx2) protein (Perincheri et al., 2005), the same protein that regulates alpha-fetoprotein, which led to H19's initial discovery

(Pachnis et al., 1984). This intricate temporal control indicates H19 is a regulator of liver development.

H19 controls liver growth and potentially the decline of the hematopoietic role of the liver after birth through the IGN. The decline of the expression of genes within the IGN coordinates the deceleration of organ growth, including the liver after birth (Lui et al., 2008). As previously described, H19 has been shown to regulate genes within the IGN by epigenetic mechanisms (Monnier et al., 2013), indicating that H19 has a major influence on development. *Igf2* and *Dlk1*, both in the IGN, are also important for hematopoiesis (Zhang and Lodish, 2004; Sakajiri et al., 2005), and the liver is the main site of hematopoiesis during birth. The repression of Igf2 and Dlk1 by H19, which has been shown experimentally, indicates that H19 is potentially responsible for the developmental switch of the liver starting as a hematopoietic organ to an organ that focuses mainly in metabolism. H19's control over the IGN leads to many implications on its role in regulating liver development.

H19 also regulates the proliferation of liver in development by Wnt signaling. The Wnt signaling pathway is critical for postnatal liver growth (Apte et al., 2007). H19 inhibits proliferation in fetal liver by inhibiting the canonical Wnt signaling pathway through inhibition of its major intracellular signal transducer, β-catenin (Wang et al., 2016b). H19 can block the interaction of hnRNP U with actin, leading to transcriptional repression of genes involved in the Wnt signaling pathway (Wang et al., 2016b). Despite these two mechanisms, there is also evidence of H19 activating Wnt signaling, albeit in a different context and cell type: bladder cancer (Luo et al., 2013). These examples show how H19 can act by different mechanisms to affect one master regulation pathway. In the context of liver proliferation in development, H19 appears to be a negative regulator of Wnt signaling.

The microRNA encoded within *H19*, miR-675, is also potentially important for development. The RNA binding protein, HuR, blocks the processing of miR-675 from *H19*, but during gestation, HuR is downregulated, causing miR-675 expression. Overexpression of miR-675 in extraembryonic cell lines causes reduced proliferation. Mechanistically, miR-675 inhibits Igf1r, and this inhibition can limit placental growth (Keniry et al., 2012).

H19 is also implicated in abnormal fetal development, regulated by epigenetic mechanisms. Developmental-specific methylation occurs at different regions around the *H19/IGF2* locus in *DMRs* in promoters for both genes (Li et al., 1998). DNA methylation of imprinted genes is first erased in primordial germ cells and reestablished later in the formation of gametes (Arnaud, 2010). In vitro fertilization (IVF) may disrupt normal embryonic and fetal growth, causing abnormal gene expression in liver. Through disruption of epigenetic regulatory networks, errors on both the maternal and paternal alleles occur at the *H19 DMR* and the *IGF2 DMR2*, respectively. H19 is significantly downregulated, and IGF2 is upregulated in livers of mice at birth and three weeks of age if they were conceived via IVF. At 1.5 years of age, mice conceived via IVF had significantly lower H19 and IGF2 expression in liver. IVF causes hypomethylation at the *H19 DMR*, indicating that early life manipulation affects vulnerability to differential methylation. In humans, growth disorders are higher and birth weights are lower in newborns conceived by IVF (Le et al., 2013). This study highlights the importance of maintaining proper methylation statuses at the *H19/IGF2* locus in development.

1.5.4.2 The Roles of H19 in the Regulation of Xenobiotic Metabolism and Transport

The liver is the most important organ for metabolizing endogenous compounds and xenobiotics, including drugs. The liver contains multiple classes and families of metabolizing enzymes. These enzymes participate in reactions to make xenobiotic compounds more water

soluble and capable of clearance through excretion in the urine or transportation into the bile. For example, the cytochrome P450 enzymes are a class of phase I enzymes. They typically function as monooxygenases that insert oxygen into molecules, making them more water soluble and easier to clear from the body (Werck-Reichhart and Feyereisen, 2000).

Phase II enzymes are drug-metabolizing enzymes capable of conjugation reactions (Jancova et al., 2010). Drug transporters are also important for clearing xenobiotics from the body (Klaassen, 2002). Efflux transporters, such as MDR1, are upregulated in HCC (Bonin et al., 2002), often inhibiting chemotherapy drugs from effectively inhibiting cancer cells. Currently, there is little research on how lncRNAs affect xenobiotic metabolizing enzymes, particularly in mammalian systems, indicating a knowledge gap and a potential area for further study.

H19 affects drug transporter and phase II conjugation, resulting in alteration of the metabolism and disposition of drugs. H19 is overexpressed in a number of drug-resistant cell lines, including doxorubicin-resistant liver cancer cells (Tsang and Kwok, 2007) and cisplatin-resistant ovarian cancer A2780-DR cells (Zheng et al., 2016). In both studies, knockdown of H19 expression results in restored chemotherapeutic sensitivity. The doxorubicin-resistant liver cancer cells exhibit an overexpression of the transporter MDR1. Sensitivity is restored by methylation of the *MDR1* promoter, causing its repression and inability to efflux the drug (Tsang and Kwok, 2007). In the cisplatin-resistant ovarian cancer cells, H19 knockdown coincided with a reduction of glutathione S-transferase P1 (GSTP1), responsible for cisplatin inactivation (Zheng et al., 2016). Examination of changes in phase II glutathione conjugation and in drug transporters after H19 knockdown in liver cancer cell lines should further be explored, as these enzymes are important for clearing drugs and for the accumulation of drugs in cancer cells.

Although H19 has not been directly linked to the regulation of phase I metabolism enzymes, current research may support H19 having a role. Gene expression profiles of P450 enzymes change in the developing liver, and normal adult P450 expression is not established in mice until a specific postnatal age (Hart et al., 2009). This change corresponds inversely with the expression pattern of H19 in liver. ZHX2, described earlier as the repressor that targets H19 for silencing in postnatal liver, also regulates sexually dimorphic, developmentally-regulated P450 genes in liver, including *Cyp2a4*, *Cyp2b13*, and *Cyp4a12* (Townsend Creasy et al., 2016). Human *CYP2A6* has the highest homology to mouse *Cyp2a4*, and CYP2A6 is responsible for metabolizing nicotine, carcinogens, and several pharmaceuticals. Although there is no direct link of H19's role in regulating these enzymes, its direct repressor has been described as important. Further research is needed to determine if H19 regulates phase I metabolism and its implication on the metabolism of drugs, especially in the fetus and in postnatal liver. This research will aid our understanding of how the fetus, neonates, and children handle drugs while they express H19 compared to adults without H19 expression in liver.

1.5.5 The Roles of H19 in the Progression of Liver Diseases

1.5.5.1 The Roles of H19 in the Development of Steatosis, Fibrosis, and Cirrhosis

Targeting H19 in cholestatic liver fibrosis may reduce liver injury. Accumulation of bile acid leads to cell injury, causing inflammation and fibrosis (Zollner and Trauner, 2009). One cause of cholestasis is the buildup of cytotoxic bile acids due to bile acid synthesis and accumulation from blocked uptake into hepatocytes and inhibited renal excretion via NTCP (Zhang et al., 2016). Although H19 does not seem to play a role in bile acid synthesis, its direct repressor, SHP, represses CYP7A1 and CYP8B1 after activation by FXR. The antiapoptotic protein, BCL2, appears to have an overarching control over SHP and H19. Mechanistically,

BCL2 overexpression results in a drastic 47-fold increase in H19 due to its degradation of the SHP repressor. The molecular basis, by which H19 regulates liver fibrosis, has yet to be completely determined. Despite not directly controlling P450s or FXR expression, the knockdown of H19 still partially rescues BCL2-induced liver injury (Zhang et al., 2016). This partial rescue of injury may be an important observation for the treatment of cholestatic liver fibrosis pointing to H19 as a target for potential therapy.

Another condition, non-alcoholic fatty liver disease (NAFLD), occurs when fat is deposited in liver, steatosis. LncRNAs, including H19 and its co-regulated protein-coding partner, IGF2, may play important roles in this disease progression. One study found that approximately 500 lncRNAs were upregulated and 1,200 lncRNAs were downregulated in human livers of patients with NAFLD compared to healthy livers (Sun et al., 2015). Another study links H19 to steatosis through PLIN2, a member of the lipid droplet protein family that is markedly increased in fatty liver. When PLIN2 was inhibited, a 548-fold increase in H19 was observed with a significant decrease in liver triglycerides (Imai et al., 2012). In another study, p62, a binding protein of IGF2 mRNA, was highly expressed in diseased liver. When overexpressed in mice, p62 can induce the steatotic phenotype (Tybl et al., 2011), including a two-fold increase in triglycerides compared to wild type (Laggai et al., 2013). It was first found that p62 expression did not induce inflammation, showing correlation to NAFLD, but not progressing fully to NASH. However, other researchers have reported p62 overexpression resulting in the activation of NF-κB, suggesting an increased inflammatory response and progression to NASH (Simon et al., 2014).

NAFLD can progress to its most extreme form, non-alcoholic steatohepatitis (NASH). NASH is the major cause of cirrhosis of the liver. Cirrhosis, a disease where the liver does not

function properly due to long-term damage, resulted in 1.2 million deaths in 2013 (Mortality and Causes of Death, 2015). Cirrhosis is usually caused by alcohol or hepatitis B or C. Fibrosis, or scarring of the liver, and steatosis, fatty liver, often precedes cirrhosis. Hepatic stellate cells are the main extracellular matrix producing cells and upon activation can promote fibrosis and cirrhosis. H19 is known to sequester miR-874 in the intestinal barrier (Su et al., 2016b), and this microRNA is upregulated in hepatic stellate cells upon activation, potentially implicating a mechanism that needs further study (Kitano and Bloomston, 2016). Furthermore, whole transcriptome RNA-sequencing analysis was performed in cirrhotic livers compared to normal healthy tissue, and H19 was discovered to be upregulated in cirrhotic liver tissue (Esposti et al., 2016). Only correlation of H19 to this disease has been reported, and these studies do not examine mechanism, again indicating a need for further research. It is clear, however, that lncRNAs, including H19, are involved in NAFLD and potentially to its progression to NASH.

1.5.5.2 The Roles of H19 in the Regulation of Diabetes

The body needs to maintain blood sugar at precise levels and the liver is a major site of glucose regulation. In times of low blood sugar, the alpha cells of the pancreas secrete glucagon, stimulating the liver to release glucose stores and induce the production of more glucose through gluconeogenesis. Conversely, glycogenesis, the process of generating glycogen from glucose, is stimulated by insulin generated in the beta cells of the pancreas when blood glucose levels are high. Insulin promotes the liver and muscle to take up blood glucose, effectively dropping blood glucose levels. Diabetes mellitus (DM) is a disease caused by prolonged increased blood sugar levels due to dysregulation of these processes. DM is caused by either the pancreas not producing enough insulin (type I DM) or the body, including the liver, not properly responding to insulin (type II DM) (Roder et al., 2016).

There is superficial evidence for H19's significance in type II DM. Smooth muscle cells cultured in the presence of insulin express a five-fold increase in H19 than when cultured in media alone (Han et al., 1996). This indicates that H19, at the very least, responds to changes in insulin. Mice with a whole-body knockout of H19 expression (through targeted deletion at the maternal allele) are 27% heavier (Leighton et al., 1995a) due to this mutation, which also results in biallelic expression of IGF2 (Ripoche et al., 1997) and improper growth after birth, predisposing them to type II DM (Eriksson et al., 2003). These observations begin the story, but more convincing mechanistic studies examining changes in the epigenetics profiles of *H19* and *IGF2* are needed to fully determine H19's role.

Epigenetic phenomenon in the liver at the *ICR* between *H19* and *IGF2*, and H19 expression are proposed to play a role in type II DM. In a study examining differential mRNA expression levels and patterns of DNA methylation in liver tissue, significant differences were observed between normal patients and patients with type II DM, such as an increase in H19 expression and the degree of methylation at its gene locus (Nilsson et al., 2015). Another study reported a significant degree of hypomethylation at the *H19* locus in an insulin-resistant female (Murphy et al., 2012). Currently, there is little research on the mechanism of insulin resistance in the liver regarding H19; only associations regarding expression and methylation status have been characterized. Because the liver is a major site of insulin resistance in type II DM, there is the need for further research in liver, specifically. There is also significant evidence that H19 is important in other tissues, such as insulin resistance in muscle and insulin production in the pancreas due to glucose intolerance.

H19 plays a role in both humans and mice with type II DM in the muscle. H19 was shown to be significantly decreased and to act as a decoy for microRNA let-7 (Gao et al., 2014).

H19 acts as a sponge that sequesters let-7, which targets genes, such as *Insr* and *Lpl*, to inhibit the insulin-PI3K-mTOR pathway. Downregulation of H19 in diabetic muscle limits let-7 sequestration, subsequently increasing inhibition of the pathway and promoting insulin resistance. Let-7 has also been reported to destabilize and downregulate H19 as a protective mechanism in hyperinsulinemic conditions in non-diabetic muscle cells via a KSRP-dependent, insulin/PI3K/AKT signaling system. Chronic downregulation of H19 limits glycogenesis and interferes with normal glucose metabolism (Gao et al., 2014). Examining how H19 interacts with let-7 in liver may yield parallel mechanisms. Let-7 is expressed in liver, and as stated earlier, H19 expression was shown to increase in patients with type II DM. Another example of H19 sequestering microRNAs affecting important signaling pathways important for glucose regulation was studied in tendon-derived stem cells. Here, H19 directly targets miR-29b-3p (Lu et al., 2016a). This microRNA has also been shown to lead to dysregulation of insulin/PI3K-AKT signaling in both the livers and pancreases in a mouse model that develops hyperglycemia (Kwon et al., 2014). Translational research is needed to further elucidate H19's role on these targets in liver.

The pancreas is another site of differential methylation of the *H19 ICR* in mice exposed to high intrauterine glucose, resulting in diabetes. Researchers induced diabetes in pregnant females. As a result, their pups received a high exposure to glucose, leading to low expression levels of H19 and IGF2. Low expression was due to hypermethylation of the *ICR* in the pups' pancreatic islets. Low expression of H19 and IGF2 was also observed in the sperm of these mice. This indicates high intrauterine glucose exposure can cause glucose intolerance, and this disease state can be passed down further to future offspring (Ding et al., 2012). This study

provides a compelling mechanism for the inheritance of childhood diabetes with H19 as a significant factor.

H19 may act differently in different tissues impacting diabetes in a multifaceted fashion. Diabetes linked to H19 in liver has been severely understudied, despite being an important organ in insulin resistance. H19 has been shown to regulate glucose intolerance and insulin resistance, but studies have mainly focused on muscle and the pancreas. Discovering how H19 participates in this disorder in the liver will allow for a more systemic understanding of the problem.

1.5.5.3 The Roles of H19 in Hepatocellular Carcinoma

Hepatocellular carcinoma (HCC) is a serious disease with few treatment options, needing more targeted therapies for better treatment. HCC is the fifth most common cancer (Parkin et al., 2005) and the most common liver cancer, accounting for 75% of all primary cases (Ahmed and Lobo, 2009). HCC has many known risk factors, including hepatitis B and C (Tanaka et al., 2011). Treatment includes liver transplantation with a survival rate ranging from 67% to 91% from studies performed in the late 2000s (Vitale et al., 2007), pharmacological intervention with a tyrosine kinase inhibitor, sorafenib, for inhibiting tumor-cell proliferation (Llovet et al., 2008) or surgical resection (Ang et al., 2015). HCC is the most studied liver disease, and there have been many newly-discovered roles that various lncRNAs play in its progression, paving the way for new drug targets.

H19's importance has been widely implicated in HCC; however, reports are inconsistent in its role of promoting cancer as an oncogene or acting as a tumor suppressor. Raveh *et al.* recently published an excellent extensive review regarding H19's role in cancer initiation, progression, and metastasis, where they aimed to resolve conflicting literature (Raveh et al.,

2015). There are many differences in HCC compared to other cancers, so the need to concentrate attention on mechanisms specific to liver is important. For instance, there is a difference in how p53, the tumor suppressor regarded as guardian of the genome, behaves in different cancers. It appears p53 does not control the reemergence of H19 expression in HCC as it functions in other cell types. In HeLa cells derived from cervical cancer, repression of H19 by p53 was observed at the *H19* promoter (Dugimont et al., 1998). In the thymus, p53 also suppresses H19 by regulating DNA methyltransferase expression profiles. This causes methylation at the *H19 ICR*, leading to changes in H19 and IGF2 expression (Park et al., 2005). In the liver, however, p53 does not contribute to the methylation status of the *ICR*, and insignificant changes in H19 and IGF2 expression were observed after knockout of p53 (Park et al., 2005). This example highlights how H19 is regulated differently between different cancers and cell types, as well as the need to focus on H19 in the context of HCC.

Many examples discuss H19 promoting cancer. The reemergence of H19 and IGF2 expression in HCC alone implicates them as positive regulators. After H19 and IGF2 are downregulated at the postnatal ages, they are both reactivated to be expressed in adult livers with HCC (Cariani et al., 1988; Ariel et al., 1997; Kim and Lee, 1997). Both genes exhibit biallelic expression due to a dysregulation of their imprinting (Kim and Lee, 1997). H19 re-expression might be explained by repression of *Zhx2*, the gene that silences H19 at the postnatal age, in HCC, through promoter methylation (Lv et al., 2006). Targeting H19 expression may be a potential therapy, as H19 knockdown in cancer cell lines, such as Hep3B, has been shown to decrease tumor weight and tumor volume (Matouk et al., 2007).

HCC tumorigenesis promotion is also regulated by miR-675 through various mechanisms. First, miR-675 directly increases proliferation in HCC by affecting cell cycle

regulation through the inhibition of retinoblastoma protein (Hernandez et al., 2013). Second, miR-675 upregulates H19 expression. Molecularly, miR-675 inhibits HP1α causing histone modifications (reduced H3K9 trimethylation, reduced H3K27 trimethylation and increased H3K27 acetylation) at the *EGR1* promoter, enhancing its transcription. EGR1, in turn, upregulates H19, activating PKM2. Ultimately, this results in tumor formation and the promotion of angiogenesis (Li et al., 2015a).

Tumors transitioning from benign to malignant undergo angiogenesis, and H19 impacts this area of tumorigenesis, as well. To stimulate angiogenesis, H19 has been found to be released from the exosomes of CD90+ liver cells to endothelial cells. H19 then induces the expression of various transcripts, such as VEGF, known to stimulate angiogenesis in endothelial cells (Conigliaro et al., 2015). Angiogenesis inhibitors are used in the treatment of cancer pointing to H19 as a potential target in this example. Previously mentioned sorafenib displays antiangiogenic activity outside of its main mechanism to suppress tumor growth (Gotink and Verheul, 2010). Suppressing H19 may also help to treat HCC through inhibition of angiogenesis.

Other lncRNAs regulate liver cancer in tandem with H19. Recent studies have suggested that initiation of HCC can start with progenitor cells of the liver rather than the parenchyma. The lncRNA CUDR accelerates liver cancer stem cell growth by binding cyclin D1 and, as a complex, binding both the promoters of *H19* and *c-Myc*. Increased expression of H19 promotes excessive TERT enhancing telomerase activity, and c-Myc increases liver cancer stem cell proliferation (Pu et al., 2015). These well-defined mechanisms support the role of H19 being an oncogene. However, H19 can also assume a role in contrary to the promotion of HCC. There is

increasing evidence that supports H19 as being a tumor suppressor acting to inhibit metastasis (Hernandez et al., 2013; Zhang et al., 2013).

1.5.5.4 The Roles of H19 in the Epithelial-to-Mesenchymal Transition

Tumor metastasis requires the ability of cancer cells from a primary site to invade a secondary site. In order for this to occur, the cancer must transition from its originating cell type to a cell type capable of differentiating into various other cell types. EMT occurs when epithelial cells become mesenchymal cells with this capability. An original cell loses its polarity and cell-to-cell adhesion, gaining migratory and invasive properties. This gives the cell the ability to differentiate back into a cell type of the tissue it has invaded.

Epigenetic changes are involved in cancer metastasis in HCC, and H19 has been shown to impact pathways, resulting in epigenetic changes leading to tumor suppression. The next example was previously discussed in a prior section examining H19 regulation by epigenetic mechanisms. H19 suppresses metastasis by repressing markers for EMT through the regulation of the miR-200 family. The markers for EMT examined (E-cadherin, cytokeratin-8, cytokeratin-19, and claudin 1) were increased following H19 knockdown, indicating H19 inhibits EMT. H19 accomplishes this via histone acetylation to activate the miR-200 pathway after complexing with hnRNP U/PCAF/RNA Pol II (Zhang et al., 2013). The miR-200 family was found to enhance E-cadherin expression in two different HCC cell lines (Hung et al., 2013). The miR-200 family achieved this enhancement by directly targeting E-cadherin's transcriptional repressors, ZEB1 and ZEB2, as determined in NMuMG murine mammary epithelial cells (Korpal et al., 2008). E-cadherin expression enhancement hinders EMT in an *in vitro* model of EMT induced by transforming growth factor-β in NmuMG cells. E-cadherin expression also decreases motility in different cell lines derived from cancer, including HepJ5 cells, an HCC cell line (Hung et al.,

2013). Again, the H19 transcript is not fully responsible for its function in EMT, as miR-675 also plays a role. Evasiveness is reduced by inhibiting Twist1, a key mediator of EMT (Hernandez et al., 2013). Despite the previous assumption that H19 promotes HCC by increasing proliferation and angiogenesis, H19 opposes metastasis. Understanding this complicated interplay between H19 acting as an oncogene and a tumor suppressor results in a more complex, but clearer picture of how H19 participates in each role.

1.5.6 Targeting H19 for Development of Therapeutic Approaches for Liver Diseases

New therapies to treat liver diseases are needed and more research will need to follow before effectively utilizing H19. Currently, there is very little research connecting H19 to diseases, such as type II DM or NAFLD and NASH, and more studies are needed before therapies can be designed utilizing H19 as a target. However, there is exciting research using *H19* to treat HCC, which is greatly needed. Currently, there is only one approved pharmacological intervention to treat HCC, sorafenib. In 2008, it was found that survival is extended only three months for patients with advanced HCC after treatment with sorafenib (Llovet et al., 2008). H19 has been suggested as a candidate tumor marker for HCC (Ariel et al., 1998). Although diagnosis is an important step to treatment, it is more powerful to think of directly targeting or using *H19* in a gene therapy approach. DTA-H19 is a plasmid containing a diphtheria toxin 'A' chain and expression is driven by the *H19* promoter. Toxicity is highly selective, because cancerous cells have been shown to activate the *H19* promoter and normal healthy cells do not. Current research has displayed a delay in tumor growth and tumor regression of colon adenocarcinoma metastases in the livers of rat (Sorin et al., 2011), as well as clinical trials for bladder and ovarian cancer by the company BioCancell (Jerusalem, Israel)

(http://www.biocancell.com/lead-program/bc-819/). This indicates the potential for novel gene therapies using lncRNAs, such as H19, promising new drug modalities.

Drug resistance is a major problem in chemotherapy. In the two examples regarding chemotherapy drug metabolism and transport presented earlier, there is promise in targeting H19. Often, MDR1 is overexpressed, leading to increased efflux of cancer drugs and inefficacy. Targeting H19 in cancer therapy may reduce MDR1-associated drug resistance, as H19 knockdown has been shown to suppress MDR1 expression. Suppression is through increasing *MDR1* promoter methylation, leading to increased accumulation and efficacy/toxicity of doxorubicin in doxorubicin-resistant R-HepG2 cells (Tsang and Kwok, 2007). The cisplatin-resistant cell line A2780-DR cells also become chemosensitive with knockdown of H19 (Zheng et al., 2016). These two examples are potentially important in the fight against drug resistance and treating cancers.

1.5.7 Further Considerations at the H19 Locus, 91H and HOTS

Genes overlapping the *H19* gene sequence with transcriptional activity have been discovered other than *H19's* well-studied microRNA, *miR-675* (Figure 1.1). *91H* and *H19 opposite tumor suppressor (HOTS)* are both antisense to *H19* and have been discussed in the context of disease. Their roles in liver have yet to be determined, leaving the door open for further research. Due to their implication in other diseases, their sharing of sequence, and either their similar regulation with or regulation of H19, it is important to discuss them here.

91H in human is a potentially 120 kb long transcript coded antisense to *H19*. At full-length, it overlaps the entire *H19* gene, the *ICR* between *H19* and *IGF2*, and the previously discussed enhancers that drive expression of H19 and IGF2 (Berteaux et al., 2008). Like H19,

91H is also upregulated in a number of cancers (Berteaux et al., 2008; Deng et al., 2014; Gao et al., 2015; Xia et al., 2016), and mirrors studies examining H19's role as a tumor suppressor and an oncogene with no clear consensus of the overall general mechanism. 91H is also important for the regulation of IGF2 expression. Knockdown studies have shown that 91H contributes to IGF2 expression at the paternal allele (Berteaux et al., 2008). 91H is also responsible for maintaining *H19/IGF2* imprinting and preventing DNA methylation at the *H19/IGF2* locus (*ICR* and *H19* promoter) on the maternal allele, potentially by binding and masking these sites, driving H19 and IGF2 expression (Vennin et al., 2016). Outside of controlling imprinting, 91H can also directly activate a promoter of *IGF2*. This activation can be counteracted by H19 (Tran et al., 2012). In order to develop future targets for therapies, it may be important to understand these mechanisms of 91H that have a direct effect on H19 expression, as well as 91H's role in normal liver function, including its potential to cause liver disease.

HOTS is another gene transcribed from the *H19* locus that overlaps all but the first exon, first intron, and most of the second exon of *H19* in human. HOTS is maternally expressed and imprinted like H19, but unlike H19, it codes for a protein. When overexpressed, HOTS inhibits various tumor types, and knockdown of HOTS results in tumor growth. In samples of Wilms tumors, it was observed that a loss of imprinting at the locus that results in biallelic expression of IGF2 also silences of HOTS. This led the authors to conclude that HOTS is a tumor suppressor (Onyango and Feinberg, 2011). With little research on this transcript and its protein, it is difficult to assume that it is a useful target in therapy. However, targeted overexpression or gene therapy using HOTS may be useful to suppress cancer. As more information was discerned over the years studying H19, its role in disease was discovered to be immensely complex. The same could be true in further investigations of both 91H and HOTS.

1.5.8 Conclusions

LncRNA research is a relatively new focus. Since H19 was one of the first lncRNAs to be identified and characterized, there is an abundant amount of research depicting its various roles. The epigenetic regulation of *H19* is unique and complex, being an imprinted gene containing a regulatory region that is differentially methylated depending on the parental origin. H19 participates in normal liver functions, such as development, and its dysregulation occurs in many liver disease, including HCC, type II DM, NAFLD, NASH, and cholestatic liver fibrosis. LncRNAs, including H19, have unique regulatory abilities, capable of binding proteins. They are also capable of participating in epigenetic modification of genes to affect gene expression in numerous signaling pathways or are direct effectors of processes in normal function and disease. Many mechanisms have been presented, indicating H19 is a complex actor with roles in many organ systems, times of development, and in many disease states. Its complex role in HCC has shown H19 to act as an oncogene regulating proliferation and angiogenesis while unusually also acting as a tumor suppressor inhibiting metastasis. As the science progresses and gives us new insights into how lncRNAs and H19 control normal liver function and disease, therapies to treat disorders will follow eventually to improve overall human health.

Chapter 2: The Role of H19, a Long Non-coding RNA, in Mouse Liver Postnatal Growth

2.1 Introduction

Liver development requires both cell proliferation and differentiation. Cell proliferation allows the liver to achieve its proper size in the body and cell differentiation allows the liver to attain proper functions. Both of these events coincide concurrently during postnatal liver

maturation. During this important, but understudied, phase of development, the liver undergoes a switch in functions. In mouse, the liver becomes the major hematopoietic organ in the fetus between embryonic days 10 to 15 (Zorn, 2008). After birth it matures into an organ primarily functioning in metabolism (Hata et al., 2007). Throughout ontogenesis until the liver is fully mature, changes occur in the expression profiles of many protein-coding genes involved in important liver functions (Li et al., 2009a; Si-Tayeb et al., 2010), including the regulation of energy metabolism (Renaud et al., 2014) and drug metabolism and transport (Collardeau-Frachon and Scoazec, 2008; Cui et al., 2012; Peng et al., 2012; Lu et al., 2013; Peng et al., 2013), which are responsible for important physiological functions of mature liver. However, it is not fully understood what initiates this switch in functions.

Proteins involved in signaling pathways and regulation of splicing are implicated in playing important roles in cell growth and differentiation during postnatal liver maturation. Knockout of mouse liver β-catenin, the intracellular transducer in Wnt signaling, results in a decrease in cell proliferation and a decrease in postnatal liver size (Apte et al., 2007). Yes-associated protein, the downstream effector of Hippo Kinase signaling, affects cell proliferation and regulates genes controlling hepatic functions including metabolism of bile acids and retinoic acids in mouse (Septer et al., 2012). Two splicing factors are also known to affect postnatal liver maturation. Epithelial splicing regulatory protein 2 is induced in hepatocytes during the postnatal age and controls the neonatal-to-adult switch of splice isoforms in gene transcripts involved in proliferation and differentiation that control hepatocyte maturation in both mouse and human (Bhate et al., 2015). Serine/arginine-rich splicing factor 3 (SRSF3) alters splicing of genes that regulate glucose and lipid metabolism. Knockout of *Srsf3* in mice results in a decline in postnatal liver growth by a prolonged expression of fetal markers including persistence of

hepatic hematopoiesis (Sen et al., 2013). Clearly, protein-coding genes are important for the functional switch in developing liver, however, the role of non-coding genes has largely been unexplored.

Long non-coding RNAs (lncRNAs) also change in identifiable expression patterns throughout ontogenesis and potentially regulate liver development. Recently, our laboratory examined the alterations of gene expression in lncRNAs during the liver's functional transition through the prenatal stage to adult life in mice (Peng et al., 2014). Three major ontogenic expression patterns were found with specific lncRNAs enriched at the neonatal, adolescent, and adult ages, indicating lncRNAs are developmentally regulated. Expression of lncRNAs with their neighboring protein-coding genes was found to be correlated, indicating lncRNAs may regulate the expression of nearby protein-coding genes or share regulatory regions within their gene loci that coordinate their expression. LncRNA H19 was found to be most differentially expressed lncRNA comparing prenatal expression to adult expression, and its *cis* protein-coding partner, IGF2, an important fetal growth factor, exhibits a similar temporal expression pattern. Despite being one of the most studied lncRNAs, H19's role in liver development has not been fully determined.

H19 is involved in normal liver physiology and is implicated in liver diseases (Pope et al., 2017). H19 is expressed in liver during periods of increased cell proliferation, including liver regeneration after injury (Pachnis et al., 1984) and hepatocellular carcinoma (Ariel et al., 1997; Kim and Lee, 1997; Matouk et al., 2007). H19 is also highly expressed in fetal liver during organogenesis. In a human fetal liver cell line, H19 expression inhibits cell proliferation through Wnt signaling potentially to prevent overgrowth of fetal liver tissue (Wang et al., 2016a). We

aim to test whether H19 plays a similar role in postnatal liver development, including both liver growth and liver maturation, using an *H19* knockout mouse model (Ripoche et al., 1997).

To test the effect of H19 on postnatal liver development, we used three genetically different mouse groups. *H19* is an imprinted gene uniquely expressed only from the maternal allele (Bartolomei et al., 1991). Therefore, we carefully bred *H19* knockout mice to generate mice heterozygous for the null mutation strictly controlling which parental allele *H19* is fully intact or fully removed. Using wild type mice ($H19^{+/+}$), *H19* maternal allele knockouts ($H19^{M-/P+}$), and *H19* paternal allele knockouts ($H19^{M+/P-}$), we were able to assess the role of H19 in postnatal liver development when H19 is expressed or not expressed from a specific allele.

2.2 Materials and Methods

2.2.1 Animals

Eight-week-old C57BL/6 mouse breeding pairs were purchased from Jackson Laboratory (Bar Harbor, ME). A permission for the use of previously characterized *H19* knockout mice (Ripoche et al., 1997) was received from Dr. Luisa Dandolo. A pair of *H19* heterozygous male knockout mice (male-$H19^{+/-}$) were received from Dr. Linheng Li's laboratory at the Stowers Institute for Medical Research (Kansas City, MO, USA). Mice were housed according to the animal care guidelines provided by the American Association for Animal Laboratory Sciences and were bred under standard conditions in the Laboratory Animal Resources Facility at the University of Connecticut (Protocol Number: A15-040). The use of these mice was approved by the Institutional Animal Care and Use Committee, Office of Research Compliance. A breeding scheme to generate paternal and maternal *H19* knockout mice is illustrated in Figure 2.1. Male heterozygous *H19* knockout ($H19^{+/-}$) mice (F-0) carrying the $H19^{-}$ on one allele with

undetermined parental origin were initially bred with wild type ($H19^{+/+}$) mice of the same C57BL/6 background to generate F-1 male-$H19^{+/-}$ and female-$H19^{+/-}$ heterozygous. The F-1 female $H19^{+/-}$ were further bred with wild type mice to generate F-2 male and female maternal *H19* knockout ($H19^{M-/P+}$) offspring and the F-1 male-$H19^{+/-}$ were further bred with wild type mice to generate F-2 male and female paternal *H19* knockout ($H19^{M+/P-}$) offspring. F-2 wild type ($H19^{M+/P+}$) mice generated were used as controls. Liver samples were collected at the following ages: day 5, 10 (neonatal), 15, 20, 30 (adolescent), and 60 (adult) after birth. Livers from both males and females were collected for days 30 and 60 only, and livers from animals collected 30 days before birth were not considered sexually mature and not separated by sex. The livers were immediately frozen in liquid nitrogen and stored at -80°C or fixed in formalin.

2.2.2 Human Liver Samples

Human liver samples were procured and RNA-sequencing performed by Dr. J. Steven Leeder at Children's Mercy Hospital (Kansas City, MO, USA). A total of 60 livers ranging in age from before birth to 18 years after birth were analyzed for H19 and IGF2 expression.

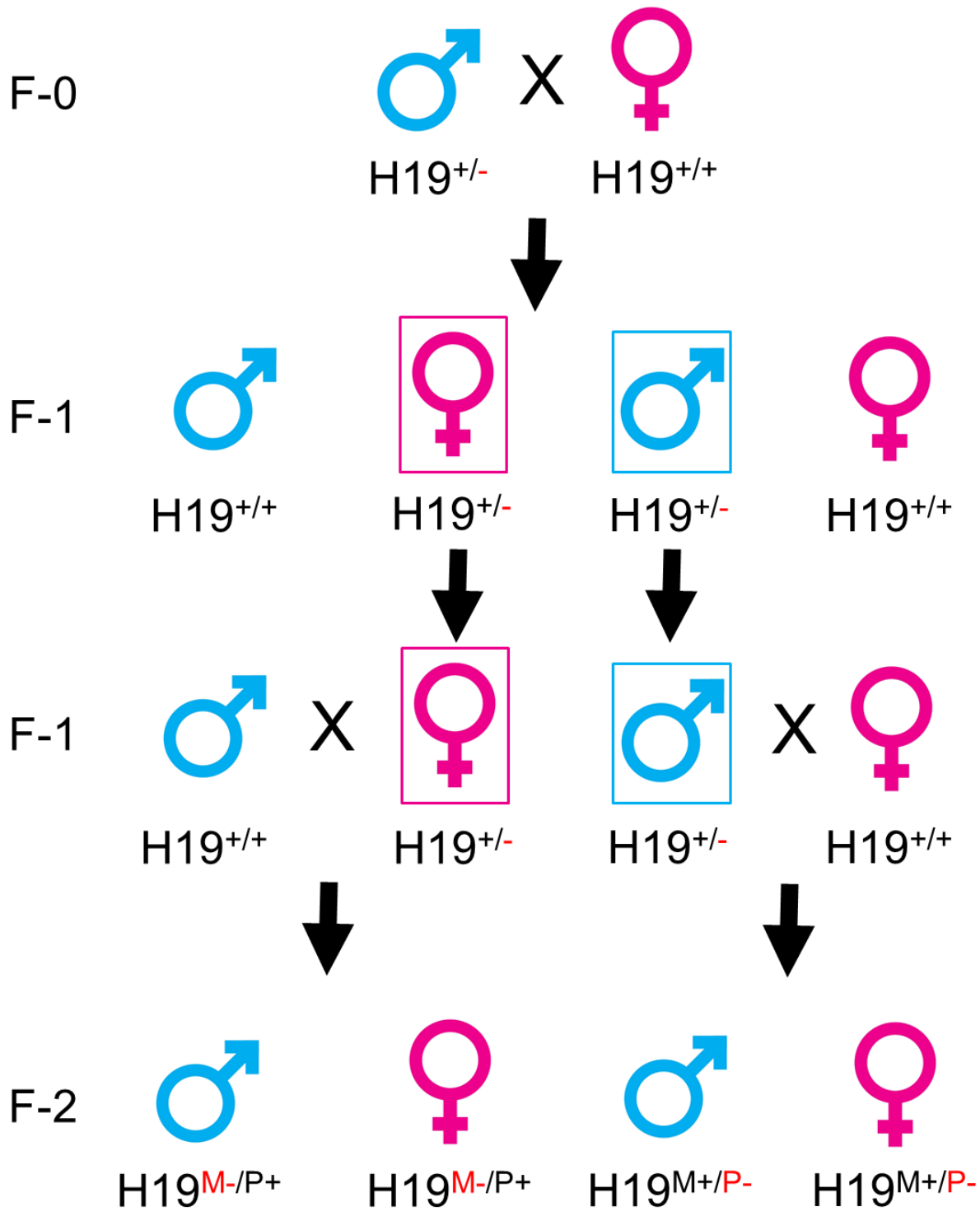

F-0
X
H19+/-
H19+/+
F-1
H19+/+
H19+/-
H19+/-
H19+/+
F-1
X
X
H19+/+
H19+/-
H19+/-
H19+/+
F-2
H19M-/P+
H19M-/P+
H19M+/P-
H19M+/P-

Figure 2.1. Breeding scheme diagram used to generate mice heterozygous for maternal and paternal *H19* knockout.

2.2.3 Genotyping

Ear or tail snips were collected from mice for DNA extraction. PCR reactions were performed for identification of H19 deletion using a REDExtract-N-Amp Tissue PCR Kit purchased from Sigma-Aldrich (St. Louis, MO, USA). Primers were designed using a PrimerQuest Tool from Integrated DNA Technologies (Coralville, IA, USA). Sequences for the primers are forward 5'-CTGTTCATACTCCGTGGGATAG-3', forward CAGACATTCATCCCGGTTACTT-3', reverse 5'-CCTACCCATTACGAGCCTTAC-3', and reverse 5'-GGGACCCATCTGTGTCTTGT-3'.

2.2.4 Reverse transcription polymerase chain reaction (RT-PCR)

Total RNAs were isolated from livers without gallbladders using TRIzol reagent from Life Technologies (Guilford, CT, USA) according to the manufacturer's protocol. RNA concentrations were determined using a Nano Drop spectrophotometer from Nano Drop Technologies (Wilmington, DE, USA) at a wavelength of 260 nm. The integrity of the total RNAs was evaluated on an Agilent 2200 Tape Station from Agilent Technologies (Santa Clara, CA, USA). Gene expression at the RNA level of H19, miR-675-3p, miR-675-5p, IGF2, IGF1, IGF1R, β-catenin, cyclin D1, CYP3A16, CYP3A11, CYP2B10, CYP2C29, and GAPDH was determined by TaqMan assays from Life Technologies (Carlsbad, CA, USA) according to the manufacturer's protocol. Data were analyzed by generation of cycling time (Ct) and delta Ct (ΔCt) values for all genes against GAPDH.

2.2.5 Immunohistochemistry

Fresh liver tissues were fixed in 10% buffered formalin, embedded in paraffin, and sections were prepared and stained by the Connecticut Veterinary Medical Diagnostic Laboratory at the University of Connecticut (Storrs, CT, USA). Immunohistochemistry was performed using antibodies against Ki-67 (rabbit polyclonal, Cat. No. ab15580) and proliferating cell nuclear antigen (PCNA) (rabbit polyclonal, Cat. No. ab18197) from Abcam (Cambridge, UK). A secondary antibody conjugated to peroxidase allowed for color precipitation using a VECTOR NovaRED Peroxidase (HRP) Substrate Kit from Vector Laboratories (Burlingame, CA, USA). Images were captured using an EVOS XL Core Cell Imaging System from Thermo Fisher Scientific (Waltham, MA, USA). Positively stained nuclei were identified by using the ImageJ image processing program, version 1.50i, from National Institutes of Health (Bethesda, MD, USA) using a color brightness threshold with a signal intensity greater than or equal to 200.

2.2.6 Western blotting

Total proteins in liver lysates were isolated in RIPA Buffer and protein concentrations were determined by using a Qubit 2.0 Fluorometer by Invitrogen (Carlsbad, CA, USA) and the Lowry protein assay from Bio-Rad (Hercules, CA, USA) with absorbance at 750 nm using a Spectra MAX 190 from Molecular Devices (Sunnyvale, CA, USA). Proteins were run on polyacrylamide gels using the Mini-PROTEAN Tetra System by Bio-Rad (Hercules, CA, USA) and transferred onto PVDF membranes. Primary antibodies purchased from Cell Signaling Technology (Danvers, MA, USA) include IGF1R (1:1000, rabbit polyclonal, Cat. No. 3027), active β-catenin (1:1000, rabbit monoclonal, Cat. No. 8814), and total β-catenin (1:1000, rabbit monoclonal, Cat. No. 8480), and albumin (1:1000, rabbit polyclonal, Cat. No. 4929). Primary antibodies against Wnt6 (1:1000, rabbit polyclonal, Cat. No. ab50030) was purchased from

Abcam (Cambridge, UK), α-fetoprotein (1:100, mouse monoclonal, Cat. No. MA5-12754) was purchased from Thermo Fisher Scientific (Waltham, MA, USA), and GAPDH (1:3000, rabbit polyclonal, Cat. No. G9545) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Secondary antibodies, anti-rabbit IgG (1:1000, goat, Cat. No. 7074) and anti-mouse IgG (1:2000, horse, Cat. No. 7076), were purchased from Cell Signaling Technology (Danvers, MA, USA). Primary antibodies were conjugated to secondary antibodies conjugated to Horseradish peroxidase for detection using a ChemiDoc MP Imaging System from Bio-Rad (Hercules, CA, USA).

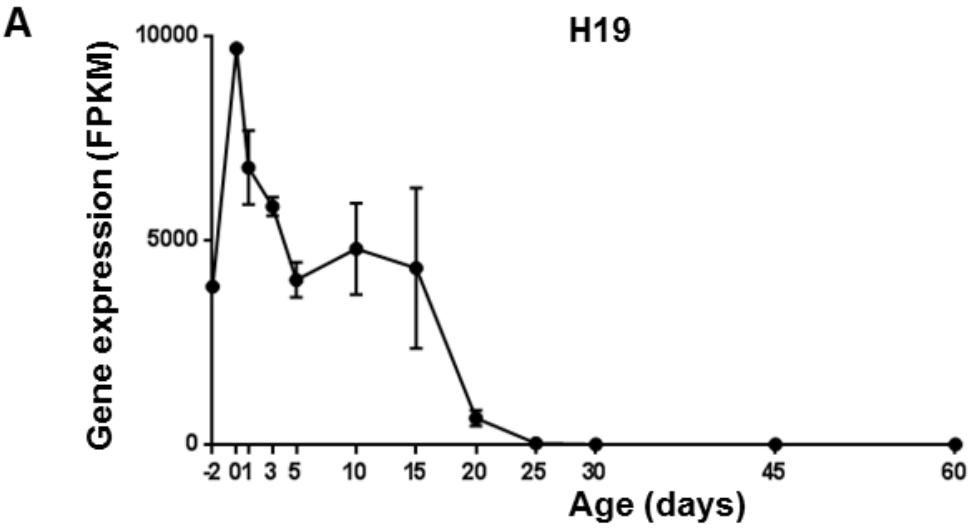
2.2.7 Statistical analysis

The data are shown as the mean ± standard deviation. The significance of differences between means was determined using two-tailed unpaired Student's *t* tests. Statistical analyses were performed using Prism7, version 7.01 from GraphPad Software, Inc. (La Jolla, CA, USA). Differences were considered to be significant if $p<0.05$.

2.3 Results

2.3.1 Ontogenic expression of H19 and IGF2 in liver during postnatal maturation

Previously generated RNA-sequencing (RNA-Seq) data (Peng et al., 2014) were used to examine H19 and IGF2 expression in wild type mouse liver (n = 36) at fetal, postnatal, and adult ages (Figure 2.2). In mouse liver, H19 and IGF2 are highly expressed before birth and rise to their highest level of expression around the time of birth. Expression levels precipitously decline at a postnatal age (approximately 20 days after birth) to nearly undetectable levels. Adult mouse liver does not express H19 or IGF2.


A
H19
Gene expression (FPKM)
10000
5000
0
-2 01 3 5 10 15 20 25 30 45 60
Age (days)

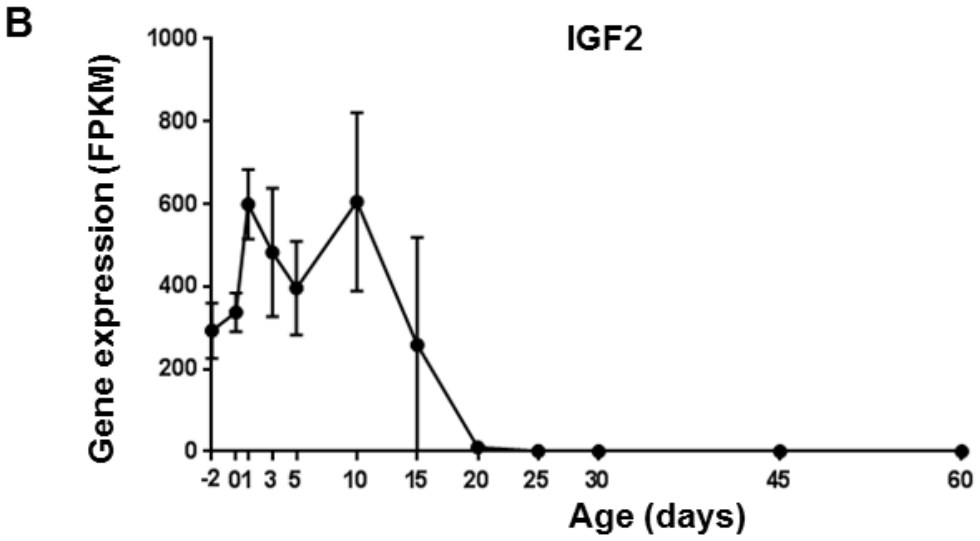

B
IGF2
Gene expression (FPKM)
1000
800
600
400
200
0
-2 01 3 5 10 15 20 25 30 45 60
Age (days)

Figure 2.2. Ontogenic expression of H19 and IGF2 in mice during postnatal maturation in liver. Expression of H19 (A) and IGF2 (B) was determined by RNA-Seq in mouse livers at ages -2, 0, 3, 5, 10, 15, 20, 30, 45, and 60 days after birth (n = 3 per group). Expression levels were measured as FPKM (Fragments Per Kilobase of transcript per Million mapped reads). Values are represented as mean ± S.D.

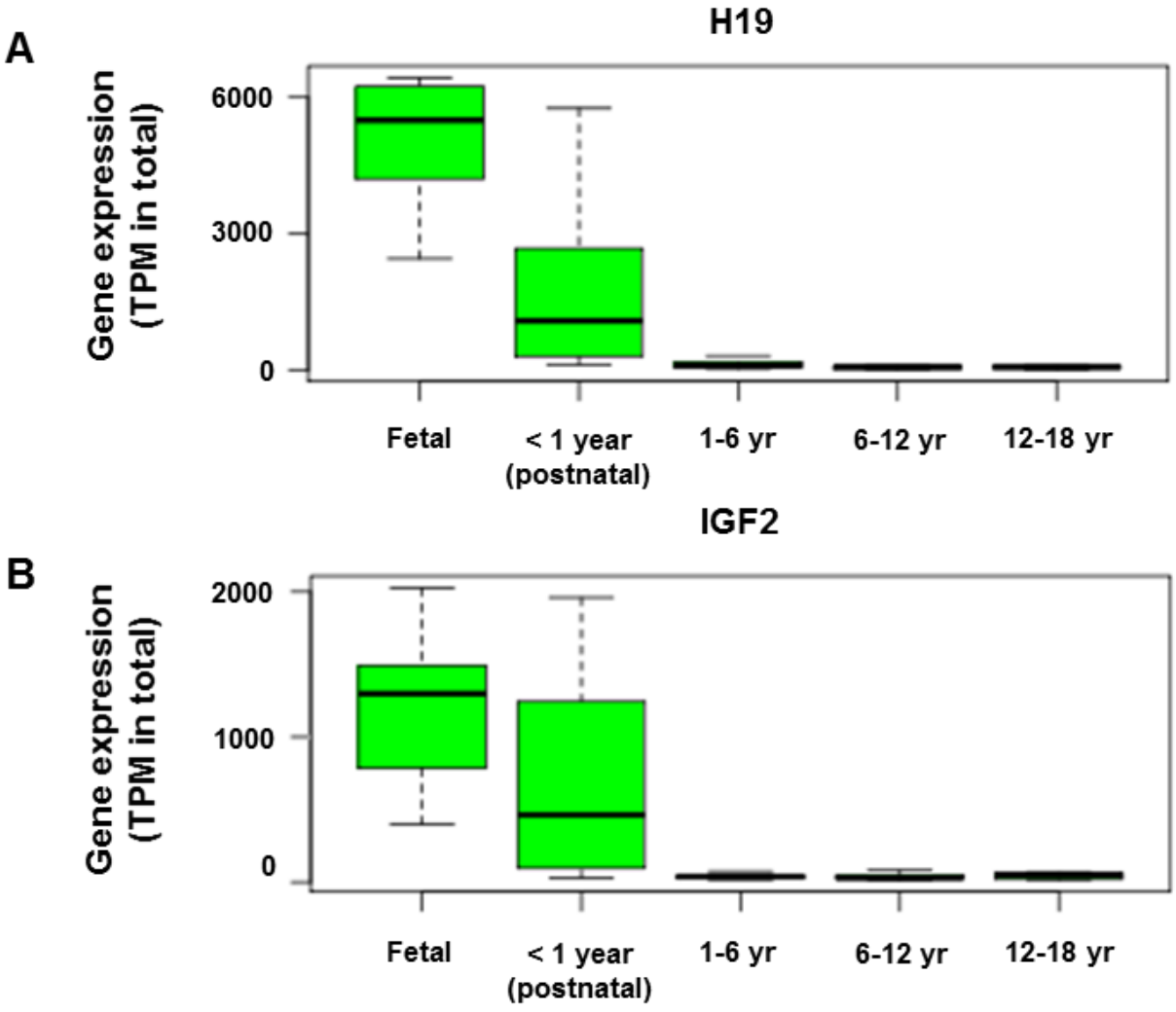

A
H19
Gene expression
(TPM in total)
6000
3000
0
Fetal
< 1 year
(postnatal)
1-6 yr
6-12 yr
12-18 yr
B
IGF2
Gene expression
(TPM in total)
2000
1000
0
Fetal
< 1 year
(postnatal)
1-6 yr
6-12 yr
12-18 yr

Figure 2.3. Ontogenic expression of H19 and IGF2 in human during postnatal maturation in liver. Expression of H19 (A) and IGF2 (B) was determined by RNA-Seq in human livers at ages before birth, less than 1 year, 1 to 6 years, 6 to 12 years, and 12 to 18 years (n = 60). Expression levels were measured as TPM (Transcripts Per Million). Values are represented as mean ± S.D.

2.3.2 Abolishment of expression of H19 and miR-675 in H19 knockout mice

Expression of H19 in livers of wild type, maternal *H19* knockout, and paternal *H19* knockout mice was determined by RT-PCR at ages of 5, 10, 15, 20, 30, and 60 days after birth (Figure 2.4 A). In all ages examined, H19 was not expressed in mice when the gene knockout was on the maternal allele despite the mice possessing an intact paternal allele ($H19^{M-/P+}$). Wild type mice ($H19^{+/+}$) and heterozygous mice with *H19* knockout on the paternal allele ($H19^{M+/P-}$) exhibited similar expression of H19 at all ages.

Two different conserved microRNAs, miR-675-3p and miR-675-5p, are produced from the first exon of *H19* (Dey et al., 2014). Using two different sets of primers directed against each miR-675 variant, their expression was determined by RT-PCR (Figure 2.4 B). The pattern of means for both miR-675-3p and miR-675-5p expression followed H19 expression. In most wild type individuals, expression was high at early ages until approximately 20 days after birth when levels precipitously decline. Certain wild type individuals, despite normally expressing H19 at early ages, showed very little miR-675 expression, indicating large interindividual variation. Similar to H19, miR-675 was only expressed when *H19* was intact at the maternal allele ($H19^{M+/P+}$ and $H19^{M+/P-}$) while knockout on the paternal allele ($H19^{M+/P-}$) was inconsequential to miR-675 expression. Essentially, *H19* maternal allele knockout mice ($H19^{M-/P+}$) are also miR-675 knockout mice.

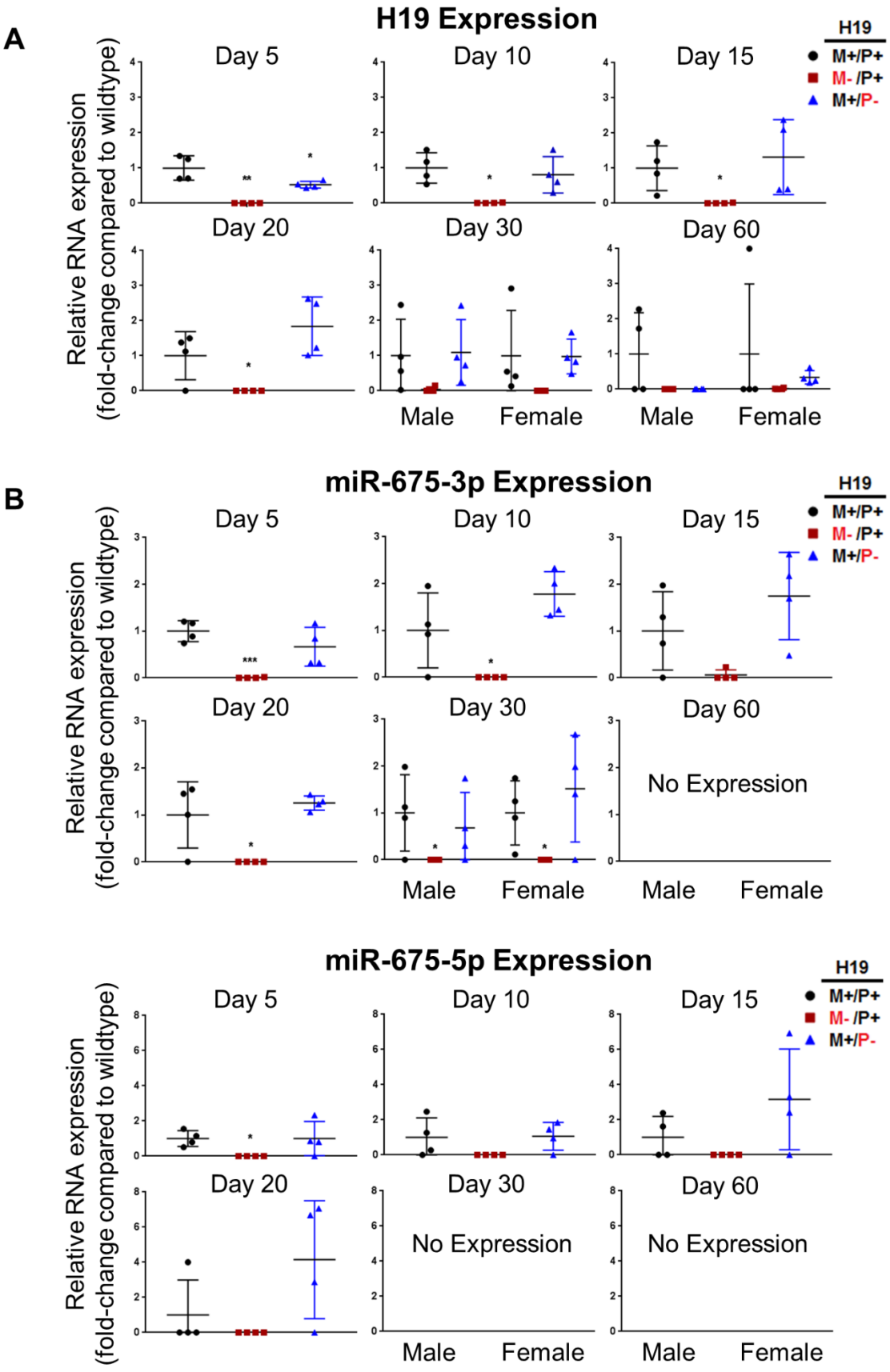

A
H19 Expression
Relative RNA expression
(fold-change compared to wildtype)
Day 5
Day 10
Day 15
Day 20
Day 30
Day 60
Male
Female
H19
M+/P+
M- /P+
M+/P-
B
miR-675-3p Expression
No Expression
miR-675-5p Expression
No Expression
No Expression

Figure 2.4. Expression of H19 and miR-675 RNA in mouse livers with *H19* knockout on different parental alleles. RNA expression of H19 (A) and miR-675-3p and miR-675-5p (B) in mouse livers at ages 5, 10, 15, 20, 30, and 60 days after birth (n = 4 per group) was determined by RT-PCR in wild type ($H19^{M+/P+}$), maternal *H19* knockout ($H19^{M-/P+}$), and paternal *H19* knockout ($H19^{M+/P-}$) mice measured as fold-changes compared to the wild type. Values are represented as mean ± S.D. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

2.3.3 Changes of liver and body weights in the absence of H19 expression during postnatal maturation

H19 impacts liver weight in the developing and adult male livers (Figure 2.5 A). Liver weights are significantly higher only immediately after birth and in male adult mice not expressing H19. Liver weights were significantly higher 5 days after birth in mice without H19 expression ($p<0.01$). No significant changes in liver weight were observed between wild type mice and mice not expressing H19 for ages 10, 15, 20, or 30 days after birth. Adult males, but not adult females, have significantly higher ($p<0.001$) liver weights when measured 60 days after birth with *H19* knockout on the maternal allele ($H19^{M-/P+}$).

H19 impacts total body weight in developing and adult mice in both males and females (Figure 2.5 B). Despite H19 expression not significantly affecting liver weight in adolescent or adult female liver, body weights are significantly altered at both 30 ($p<0.01$) and 60 ($p<0.001$) days after birth, affecting also the liver/body weight ratio significantly at 30 days after birth ($p<0.05$). Male liver/body weight ratios are not significantly affected by H19 expression (Figure 2.5 C). H19 also controls body weights when the liver is developing. Body weights are significantly higher with no H19 expression at 5 days after birth ($p<0.001$) and 20 days after birth ($p<0.05$). Both males and females had significant ($p<0.001$) increases in body weight at 60 days after birth when H19 was not expressed. Females, but not males, were significantly heavier at 30 days after birth ($p<0.01$) with no H19 expression through life.

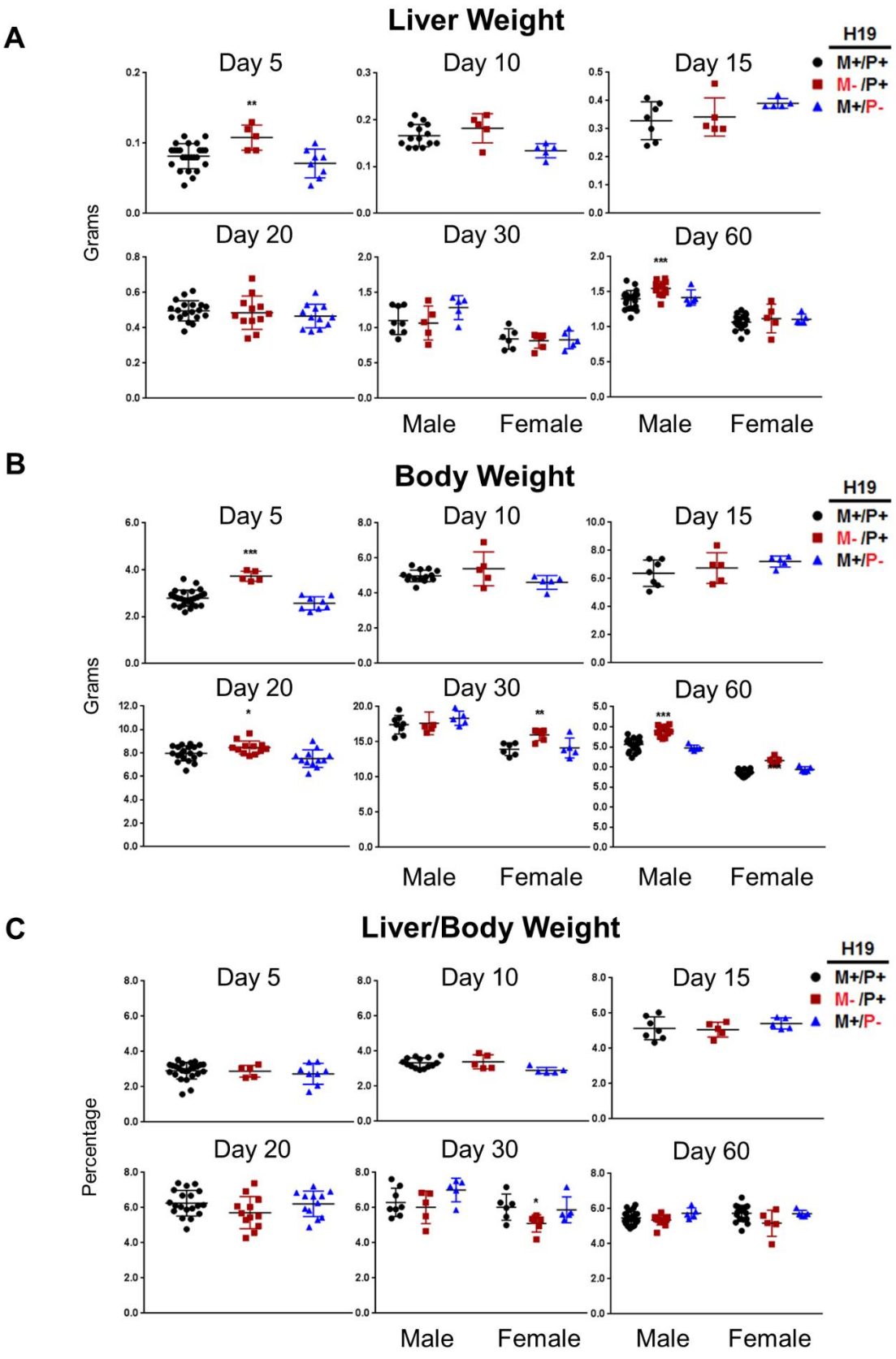

A
Liver Weight
Day 5
Day 10
Day 15
Day 20
Day 30
Day 60
Grams
H19
M+/P+
M- /P+
M+/P-
Male
Female
Male
Female
B
Body Weight
Day 5
Day 10
Day 15
Day 20
Day 30
Day 60
Grams
H19
M+/P+
M- /P+
M+/P-
Male
Female
Male
Female
C
Liver/Body Weight
Day 5
Day 10
Day 15
Day 20
Day 30
Day 60
Percentage
H19
M+/P+
M- /P+
M+/P-
Male
Female
Male
Female

Figure 2.5. Liver and body weights for mice with *H19* knockout on different paternal alleles. Liver weights (A), body weights (B), and liver weight as a percentage of total body weight (C) were measured at the time of liver harvest from mice ages 5, 10, 15, 20, 30, and 60 days after birth (n = 5-25). Values are represented as mean ± S.D. *p<0.05, **p <0.01, ***p<0.001.

2.3.4 Changes in liver cell proliferation in the absence of H19 expression during postnatal maturation

Corresponding data indicates liver weights are increased due to increases in cell proliferation throughout mouse development when H19 is not expressed. Two markers of cell proliferation, Ki-67 and PCNA, were used to measure proliferating cells in the tissue sections (Figure 2.6). When stained for Ki-67, livers from *H19* maternal allele knockouts ($H19^{M-/P+}$) show significantly more Ki-67 positive nuclei in livers of mice at ages 5 ($p<0.001$), 10 ($p<0.001$), 20 ($p<0.001$), 30 in both males and females ($p<0.001$), and 60 only in males ($p<0.001$) days after birth. PCNA was also used to measure cell proliferation in an independent experiment. These data closely resemble Ki-67 staining results, indicating livers without H19 expression proliferate more rapidly during development when compared to wild type. In the maternal allele knockout mice, there were significantly more cells stained positive for PCNA at ages 5 ($p<0.001$), 10 ($p<0.001$), and 20 ($p<0.001$) days after birth. Similar to Ki-67 results, significant differences were observed through postnatal liver development until age 15 days after birth, with significant differences again observed for age 20 days after birth. Discordant measurements were observed between the two assays at ages 30 and 60 days after birth with Ki-67 indicating significant changes for *H19* maternal allele knockout mice ($H19^{M-/P+}$) while PCNA did not stain significantly different at either of these ages. Measurements for heterozygous paternal allele *H19* mutants ($H19^{M+/P-}$) resemble wild type ($H19^{+/+}$).

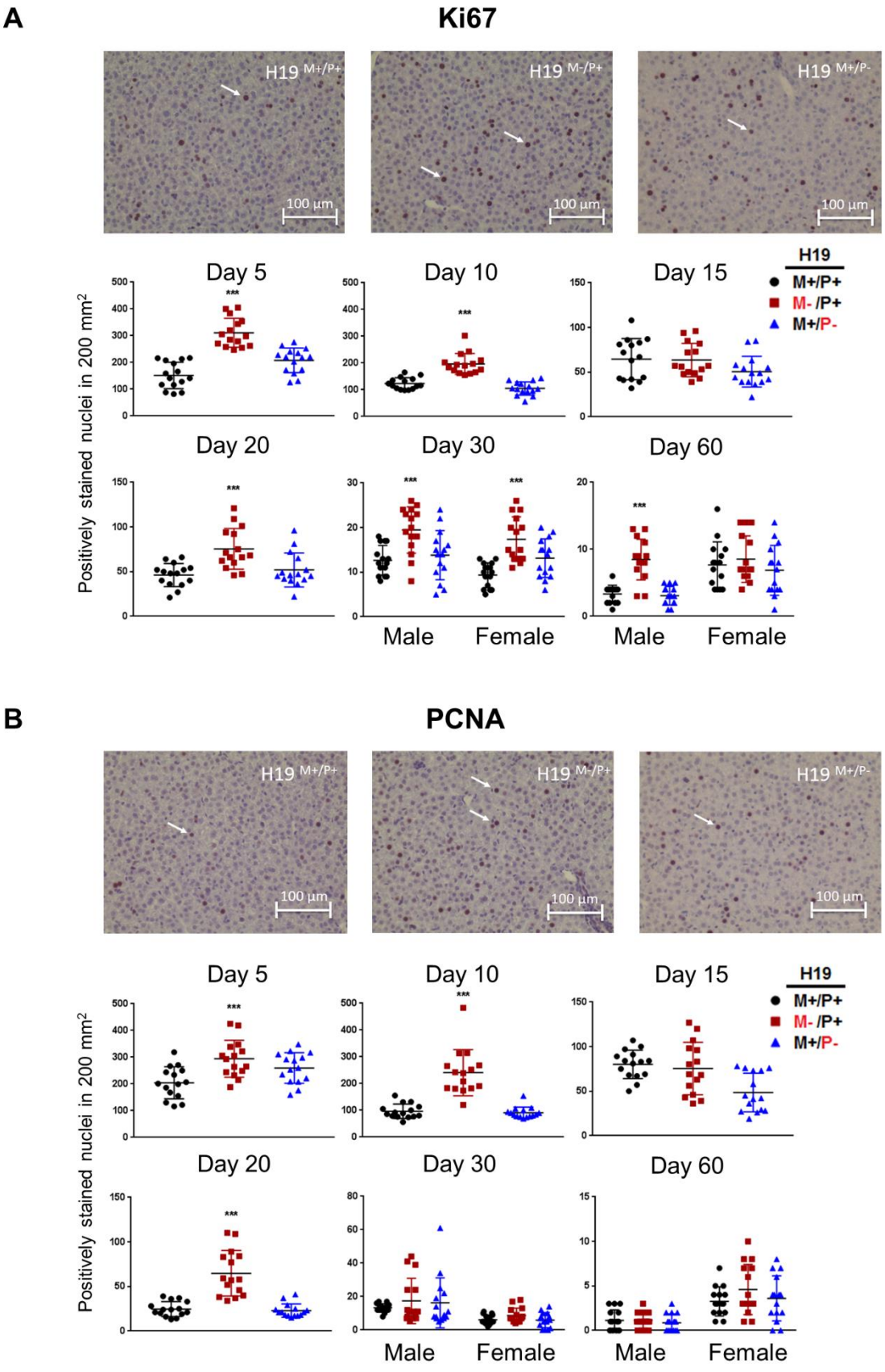
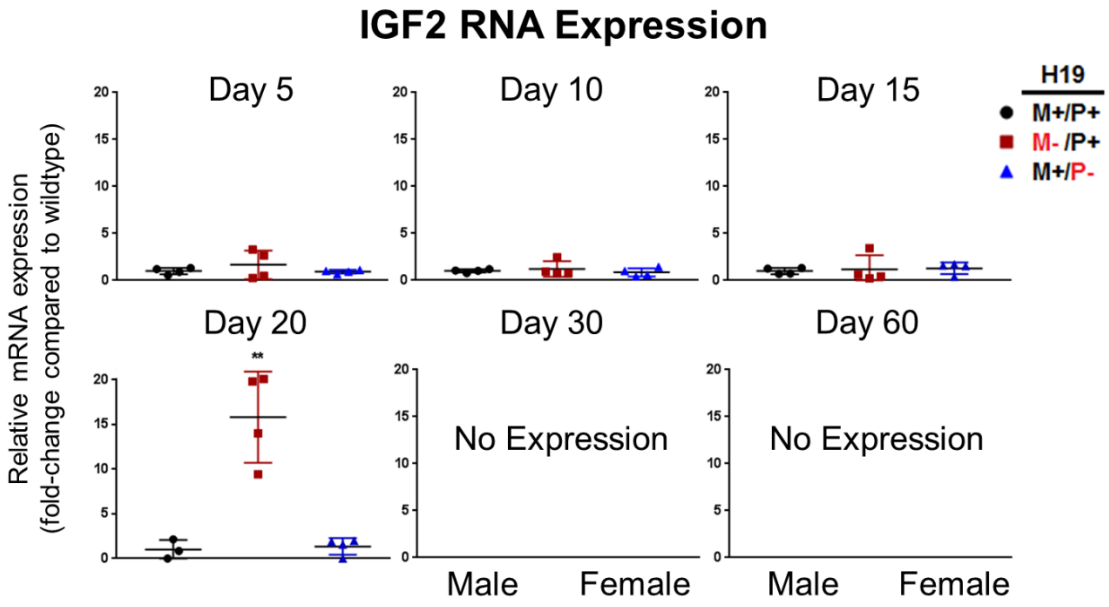

A
Ki67
H19 M+/P+
H19 M-/P+
H19 M+/P-
100 µm
H19
M+/P+
M- /P+
M+/P-
Day 5
Day 10
Day 15
Day 20
Day 30
Day 60
Positively stained nuclei in 200 mm2
Male
Female
B
PCNA

Figure 2.6. Immunohistochemistry analysis for identification of proliferating cells in livers of mice with *H19* knockout on different paternal alleles. Livers were fixed in formalin, embedded in paraffin, sectioned, and stained for (A) Ki67 or (B) PCNA for mice at ages 5, 10, 15, 20, 30, and 60 days after birth (n = 3 per group). A representative image was taken from mice at age 20 days after birth. Arrows indicate cells stained positive for (A) Ki67 and (B) PCNA. ImageJ software was used to count five sections per mouse. Values are represented as mean ± S.D. ***$p<0.001$.

2.3.5 Expression of IGF signaling and Wnt signaling genes in the absence of H19 expression during postnatal maturation

Gene expression patterns for IGF2 and IGF1 were determined using RT-PCR for mice at ages 5, 10, 15, 20, 30, and 60 days after birth (Figures 2.7 A and B, respectively). Both H19 and IGF2 were highly expressed at early ages until 20 days after birth when expression precipitously declines to undetectable levels and no expression persists through adult life. IGF1 exhibits the opposite expression pattern, starting with low expression early in life, steadily increasing as the liver develops. A significant increase ($p<0.01$) in IGF2 mRNA was found in *H19* maternal allele knockout mice ($H19^{M-/P+}$) compared to wild type ($H19^{+/+}$) at 20 days after birth, indicating H19 affects IGF2 expression. However, at all other ages measured, no significant differences were found. IGF1 mRNA was not impacted by the absence of H19 expression.

A


IGF2 RNA Expression
Day 5
Day 10
Day 15
Day 20
Day 30
Day 60
No Expression
No Expression
Relative mRNA expression
(fold-change compared to wildtype)
Male
Female
Male
Female
H19
M+/P+
M- /P+
M+/P-

B

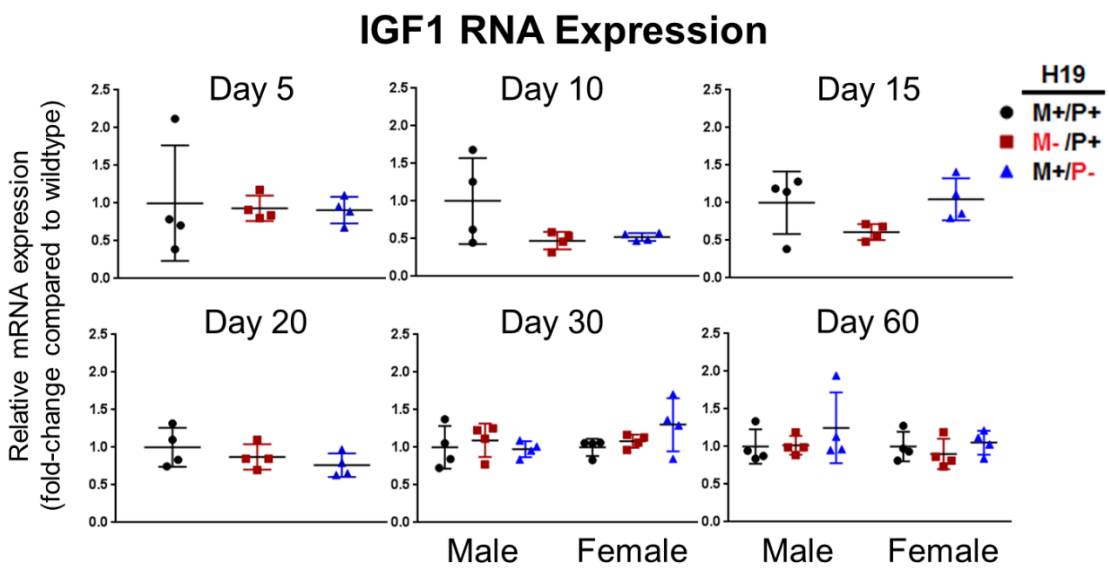

IGF1 RNA Expression
Day 5
Day 10
Day 15
Day 20
Day 30
Day 60
Relative mRNA expression
(fold-change compared to wildtype)
Male
Female
Male
Female
H19
M+/P+
M- /P+
M+/P-

Figure 2.7. Expression of the fetal form IGF2 and adult form IGF1 RNA in mouse liver with *H19* knockout on different parental alleles. RNA expression was determined by RT-PCR for (A) IGF2 and (B) IGF1 in mouse livers at ages 5, 10, 15, 20, 30, and 60 days after birth (n = 4 per group). Values are represented as mean ± S.D. $^{**}p<0.01$.

IGF1R expression was found to increase only at the protein level, but not at the mRNA level in *H19* maternal allele knockouts ($H19^{M-/P+}$) compared to wild type ($H19^{+/+}$). Although no significant differences were observed in mRNA expression at all ages measured (Figure 2.8 A), there was an increase in IGF1R protein expression when H19 was not expressed for each time point measured (Figure 2.8 B). Levels of IGF1R protein expression were highly variable between individuals at each age and experimental groups.

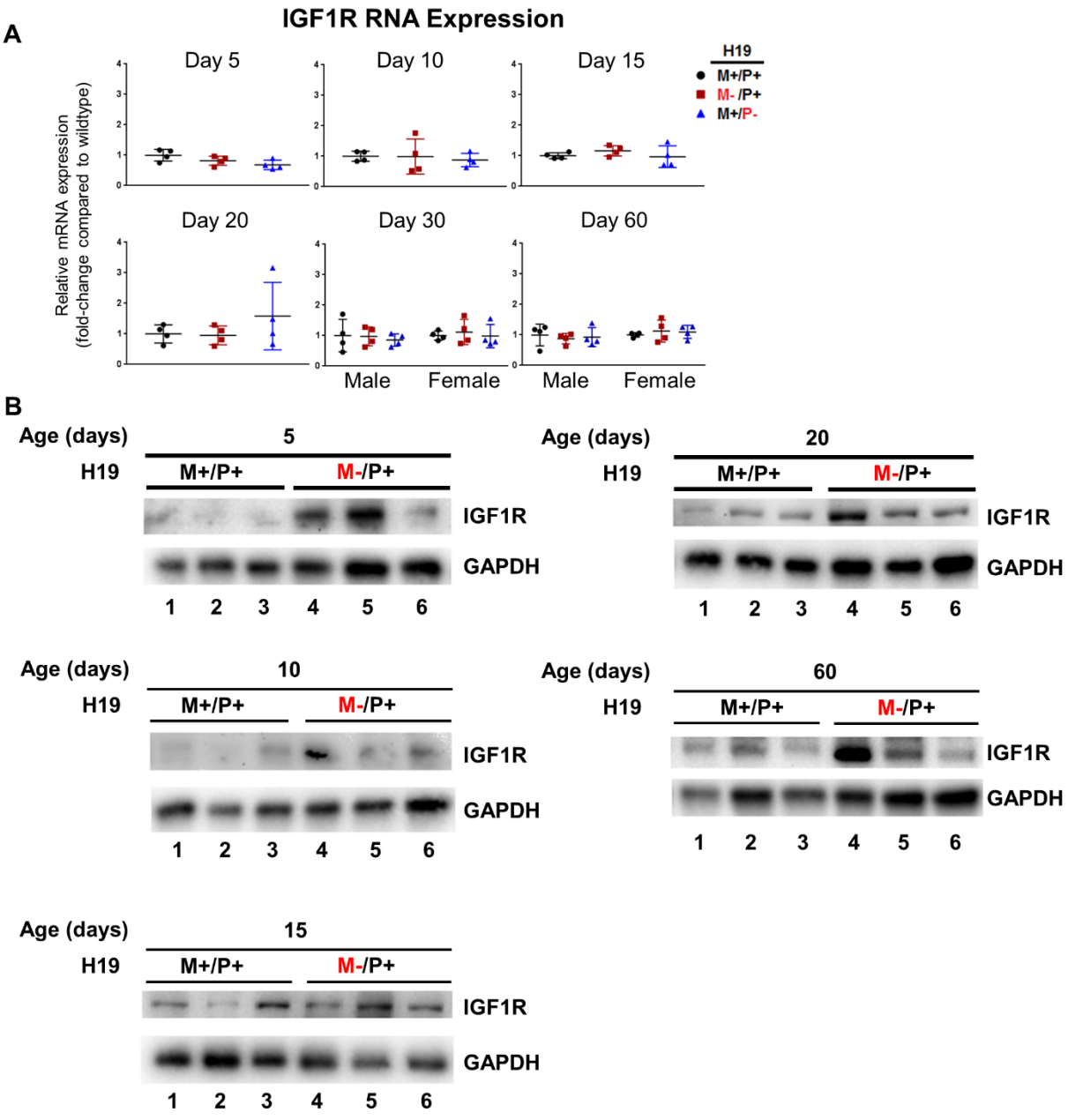

A
IGF1R RNA Expression
Day 5
Day 10
Day 15
Day 20
Day 30
Day 60
H19
M+/P+
M- /P+
M+/P-
Relative mRNA expression
(fold-change compared to wildtype)
Male
Female
Male
Female
B
Age (days)
5
H19
M+/P+
M-/P+
IGF1R
GAPDH
1 2 3 4 5 6
Age (days)
20
H19
M+/P+
M-/P+
IGF1R
GAPDH
1 2 3 4 5 6
Age (days)
10
H19
M+/P+
M-/P+
IGF1R
GAPDH
1 2 3 4 5 6
Age (days)
60
H19
M+/P+
M-/P+
IGF1R
GAPDH
1 2 3 4 5 6
Age (days)
15
H19
M+/P+
M-/P+
IGF1R
GAPDH
1 2 3 4 5 6

Figure 2.8. Expression of IGF1R in mouse livers with *H19* knockout on different parental alleles. (A) RNA expression was determined by RT-PCR in mouse livers at ages 5, 10, 15, 20, 30, and 60 days after birth (n = 4 per group). (B) Protein expression was determined by Western blot for IGF1R for mice at ages 5, 10, 15, 20, and 60 days after birth. Lanes 1-3 indicate wild type ($H19^{+/+}$) individuals and lanes 4-6 indicate $H19^{M-/P+}$ individuals. Values are represented as mean ± S.D.

Wnt signaling was similarly only slightly impacted by the absence of H19 in mice. No significant changes were observed at the mRNA level at all ages tested for either β-catenin or cyclin D1 (Figures 2.9 A and B, respectively). Significant increases ($p<0.05$) were observed at the protein level for active β-catenin in mice at age 5 days after birth and in total β-catenin in mice at age 10 days after birth (Figure 2.9 C). Protein expression was also analyzed for the ligand Wnt6, but no changes were observed.

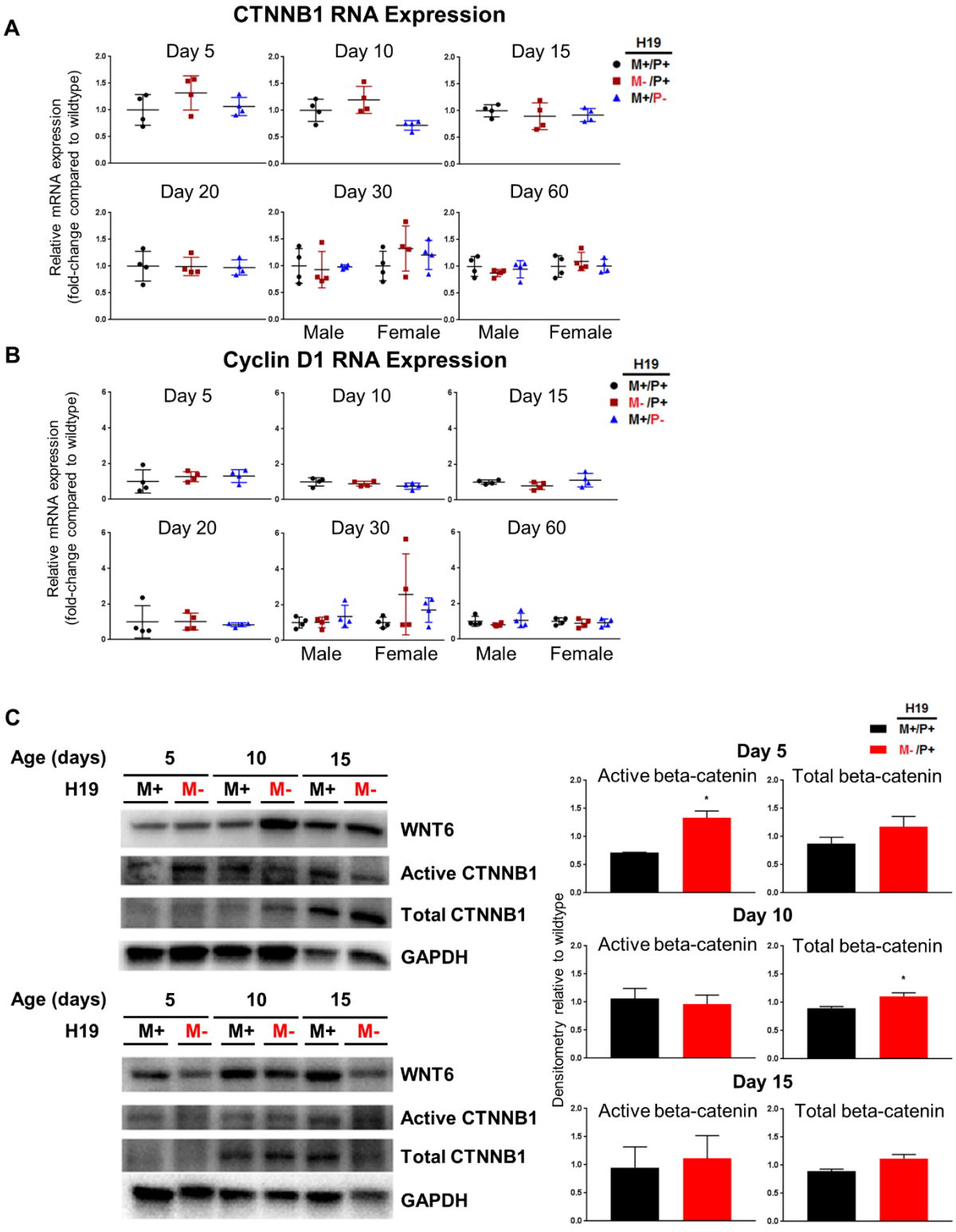

A
CTNNB1 RNA Expression
Day 5
Day 10
Day 15
Day 20
Day 30
Day 60
Relative mRNA expression (fold-change compared to wildtype)
Male
Female
H19
M+/P+
M- /P+
M+/P-
B
Cyclin D1 RNA Expression
C
Age (days)
H19
WNT6
Active CTNNB1
Total CTNNB1
GAPDH
Active beta-catenin
Total beta-catenin
Densitometry relative to wildtype

Figure 2.9. Expression of Wnt signaling in mouse livers with *H19* knockout on different parental alleles. RNA expression was determined by RT-PCR for (A) β-catenin and (B) cyclin D1 in mouse livers at ages 5, 10, 15, 20, 30, and 60 days after birth (n = 4 per group). (C) Protein expression was determined by western blot for Wnt6, active β-catenin, and total β-catenin for mice at ages 5, 10, and 15 days after birth (n = 2 per group). Values are represented as mean ± S.D. *$p<0.05$.

Chapter 3: The Role of H19, a Long Non-coding RNA, in Mouse Liver Postnatal Maturation

3.1 Expression of P450 drug metabolizing enzymes in the absence of H19 expression during postnatal maturation

Gene expression was determined for P450 enzymes in *H19* paternal allele ($H19^{M+/P-}$), *H19* maternal allele knockouts ($H19^{M-/P+}$), and wild type mice ($H19^{+/+}$). There were no significant differences in RNA expression for CYP3A16 (Figure 3.1 A) or CYP3A11 (Figure 3.1 B). A significant increase ($p<0.05$) was found in CYP2B10 in *H19* maternal allele mutants ($H19^{M-/P+}$) compared to wild type ($H19^{+/+}$) for male mice aged 30 days (Figure 3.1 C). Significance was not found in females aged 30 days or at any other ages measured for CYP2B10. CYP2C29 expression is delayed in mice when H19 expression is not present early in life (Figure 3.1 D). A significant decrease ($p<0.05$) was detected at 15 days after birth in *H19* maternal allele mutants ($H19^{M-/P+}$). Normally, as seen in wild type ($H19^{+/+}$) and in paternal *H19* allele knockouts ($H19^{M+/P-}$), CYP2C29 begins to be slightly expressed at 15 days after birth. However, when H19 is not expressed, CYP2C29 is not expressed until later in development and resumes its full normal expression at 20 days after birth.

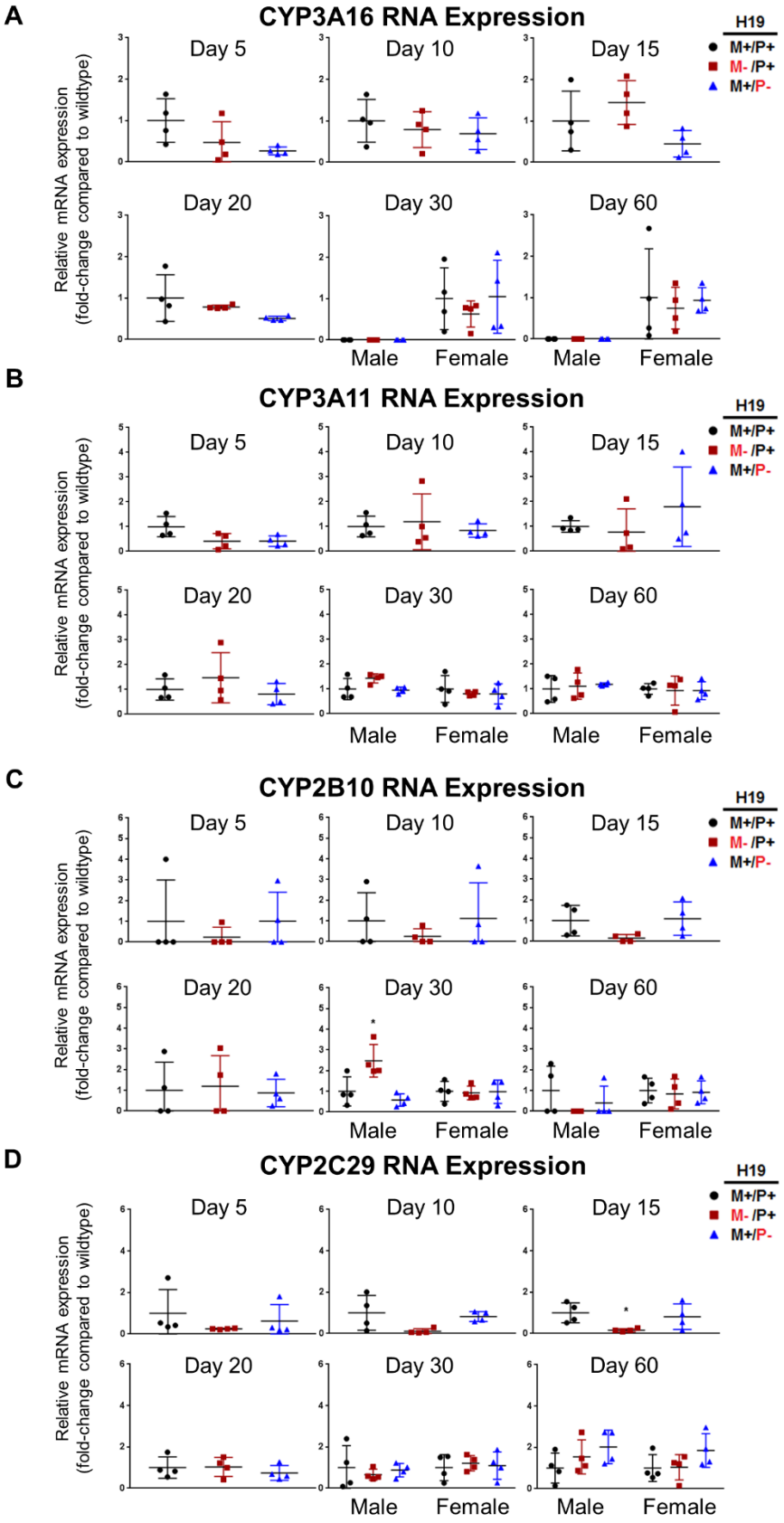

A
CYP3A16 RNA Expression
B
CYP3A11 RNA Expression
C
CYP2B10 RNA Expression
D
CYP2C29 RNA Expression
Day 5
Day 10
Day 15
Day 20
Day 30
Day 60
Male
Female
Relative mRNA expression
(fold-change compared to wildtype)
H19
M+/P+
M-/P+
M+/P-

Figure 3.1. Expression of P450 drug metabolizing enzymes in mouse livers with *H19* knockout on different parental alleles. RNA expression was determined by RT-PCR for (A) CYP3A16, (B) CYP3A11, (C) CYP2B10, and (D) CYP2C29 RNA in mouse livers at ages 5, 10, 15, 20, 30, and 60 days after birth (n = 4 per group). Values are represented as mean ± S.D. *p<0.05.

3.2 Expression of α-fetoprotein and albumin protein in the absence of H19 expression during postnatal maturation

Liver postnatal protein expression for α-fetoprotein was examined in mice at ages corresponding to the decline of expression of H19 in normal wild type mice. No significant differences in α-fetoprotein were observed between *H19* maternal allele knockouts ($H19^{M-/P+}$) and wild type ($H19^{+/+}$) (Figure 3.2 A). In mice aged 20 days after birth, no α-fetoprotein protein expression was found in liver for either test group. As observed in IGF1R protein expression, large interindividual differences were found between the three mice tested in each group.

Liver postnatal protein expression for albumin was examined in mice at the early ages of development. Western blots indicate no significant difference in protein expression of albumin in livers of mice at ages 5 or 10 days after birth (Figure 3.2 B) for either test group. As with α-fetoprotein, there were large interindividual differences.

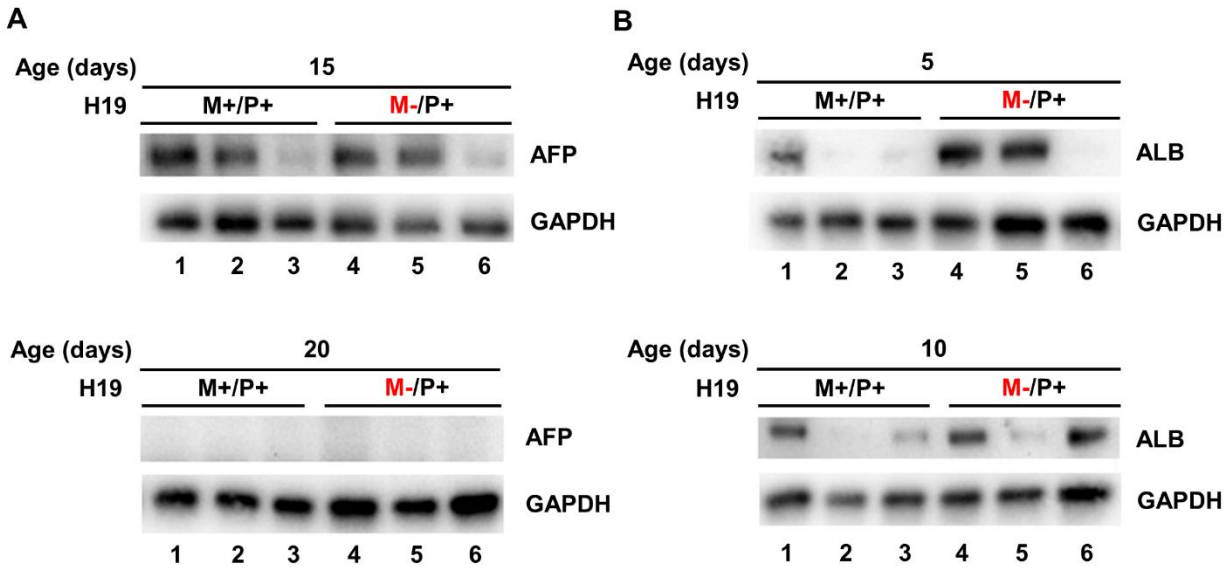

A
Age (days)
15
H19
M+/P+
M-/P+
AFP
GAPDH
1 2 3 4 5 6
Age (days)
20
H19
M+/P+
M-/P+
AFP
GAPDH
1 2 3 4 5 6
B
Age (days)
5
H19
M+/P+
M-/P+
ALB
GAPDH
1 2 3 4 5 6
Age (days)
10
H19
M+/P+
M-/P+
ALB
GAPDH
1 2 3 4 5 6

Figure 3.2. Protein expression of α-fetoprotein and albumin in mouse livers with *H19* knockout on the maternal allele. Protein expression was determined by Western blot for (A) α-fetoprotein in mouse livers ages 15 and 20 days after birth, and (B) albumin in mouse livers ages 5 and 10 days after birth. Lanes 1-3 indicate wild type ($H19^{+/+}$) individuals and lanes 4-6 indicate $H19^{M-/P+}$ individuals. Values are represented as mean ± S.D.

Chapter 4: Summary

4.1 Discussion

Research on H19 in normal liver development has primarily focused on fetal development with little regard to H19's role after birth despite this being a time when the liver is continually growing and is dynamically changing its function from being a hematopoietic organ to a metabolic organ. When discovered, Pachnis *et al.* initially characterized H19 expression in mouse from before birth through the postnatal age until expression is terminated, by their observation, around 28 days after birth (Pachnis et al., 1984). We have recapitulated their initial temporal expression pattern using modern techniques and expanded upon their seminal observations with a focus on H19's role in postnatal liver maturation, including both liver growth and changes in the liver's ability to achieve adult liver function.

Liver growth is accelerated early in postnatal liver development when H19 is not expressed. The overgrowth phenotype has been previously characterized for mice inheriting the *H19*-null allele from their mother (Leighton et al., 1995a). Mice used in this study that were heterozygous containing a functional maternal allele but with a mutated paternal allele resemble homozygous wild type mice throughout our observations, as the paternal allele was inconsequential to H19 expression. We observed the overgrowth phenotype by measuring both the total body and liver weights through the postnatal ages. Early in life, liver weights are significantly affected when H19 is not expressed (Figure 2.5 A). Coordinately, we also measured the level of cell proliferation in liver and found significant changes in positively stained nuclei for two different cell proliferation markers, indicating enhanced proliferation that persists through many measured postnatal ages (Figure 2.6). Ki-67 is preferentially expressed during late G_1, S, G_2, and M phases of the cell cycle, but not in the resting G_0 phase (Scholzen

and Gerdes, 2000). PCNA is an accessory protein for DNA polymerase alpha required for DNA synthesis, and is elevated during the G1/S phase of the cell cycle (Kelman, 1997). Both markers indicate that the absence of H19 expression enhances cell proliferation in the developing mouse livers.

Increases in cell proliferation do not directly result in increases in liver mass. There is a discordance with observations in significant liver weight increases and increases in the markers indicating cell proliferation in *H19* maternal allele knockout mice. Liver weights are only significantly increased immediately in early life and in adult males while increases in proliferation are observed throughout most of liver development at postnatal ages. Interestingly, both markers indicate no significant increases in cell proliferation at 15 days after birth despite showing significant increases before and after this age in the absence of H19 expression, suggesting H19 does not influence proliferation at this specific time of development.

Loss-of-function of *H19* in developing mice induces changes in liver that persist even at ages when H19 is not normally expressed (ages 30 and 60 days after birth). An increase in adult male liver weight can be explained by the accumulation of organ mass at earlier ages too subtle to have been detected at prior ages. Increases in proliferation as observed by Ki-67 at ages 30 and 60 days after birth in *H19* maternal allele knockout mice may be the result of more complex pathway changes that are initiated at the time of normal H19 expression and persist after H19 is no longer expressed. These late age changes were not observed in the PCNA stain (Figure 2.6 B).

We then sought to determine how the loss of H19 expression results in an increase in liver mass and an increase in proliferating cells throughout postnatal development by first examining IGF signaling. There is a strong correlation in expression patterns between lncRNAs

and protein coding genes within the same loci, suggesting *cis* regulation with the lncRNA potentially influencing its protein coding partner (Peng et al., 2014). Others have shown that regulation is not always the lncRNA acting directly on the protein coding gene, but rather its promoter or nearby regulatory regions that influence gene expression of nearby genes (Engreitz et al., 2016). *H19* and *Igf2* reside next to each other on chromosome 7 in the mouse and have similar temporal expression patterns in postnatal liver. Different *H19*-null mouse models have been used to determine the function of not only H19 RNA, but also regulatory sequences surrounding its locus. Some mouse models have deletions that span into the *Imprinting Control Region (ICR)* between *H19* and *Igf2* resulting in disruption of imprinting of *Igf2,* which authors have concluded leads to biallelic expression of IGF2 and the overgrowth phenotype (Leighton et al., 1995a). However, our model has only *H19* and a portion of its promoter containing an Sp1 site and TATA box removed, leaving the *ICR* intact allowing us to study only the effects of H19 on postnatal liver development (Ripoche et al., 1997). Our results demonstrate that deletion of the *ICR*, causing bialleleic expression of IGF2, is not needed to induce the overgrowth phenotype.

Our measurements indicate H19 has a minimal effect on IGF2 expression, with significant increases in IGF2 expression observed only at 20 days after birth (Figure 2.7 A). However, at 5 days after birth, the liver overgrowth phenotype is observed (Figure 2.5 A), and cellular proliferation is significantly increased (Figure 2.6) throughout most postnatal ages when H19 is not expressed. This indicates other pathways may be affected by the *H19* loss-of-function.

The Wnt signaling pathway has been shown to be important in the development of many different tissue types and organs. The canonical intracellular transducer, β-catenin, is activated

after the Wnt ligand binds to a Frizzled family receptor. This activation causes β-catenin accumulation in the cytoplasm and its eventual localization into the nucleus where it acts as a coactivator of transcription factors, affecting gene transcription (Rao and Kuhl, 2010). Cyclin D1, a cell cycle inducer important for the G_1 to S phase transition, is a target for regulation by β-catenin (Tetsu and McCormick, 1999) in many different physiological processes including liver growth (Monga, 2014). Wnt signaling has been shown to be inhibited by H19 in fetal liver leading to inhibition of cell proliferation (Wang et al., 2016b), and Wnt signaling influences proliferation in liver at postnatal ages (Apte et al., 2007). No significant changes were observed at the mRNA level for either β-catenin or a downstream gene target of the pathway important for cellular proliferation, cyclin D1, when H19 is not expressed (Figure 2.9). Protein expression was also analyzed for the Wnt ligand and both activated β-catenin and total β-catenin. Antibody detection against Wnt6 was chosen due to Wnt6 being involved in canonical signaling and its high expression in developing tissues (Zeng et al., 2007). No significant changes were observed in Wnt6 expression, indicating Wnt signaling was affected only downstream in the pathway. Significant differences were discovered at the protein level in early life (age 5 days after birth) for active β-catenin, and total β-catenin is also significantly higher at 10 days after birth indicating similar results as observed in prior studies examining fetal H19 inhibition of β-catenin protein (Wang et al., 2016b). It is not surprising significant changes were not observed at the RNA level for genes within the canonical pathway. However, mRNA expression for cyclin D1, which is a direct target of Wnt signaling at the transcriptional level, was not found to be significantly altered.

H19 encodes a microRNA, miR-675, within its first exon (Cai and Cullen, 2007). *H19* knockout on the maternal allele abolishes expression of both H19 and miR-675 despite the status

of the paternal allele (Figure 2.4). H19 potentially impacts liver growth and proliferation through the action of miR-675. Prior literature has shown proper processing of miR-675 can slow growth in the placenta, and increases of miR-675 downregulate IGF1R, which causes IGF signaling to be inhibited (Keniry et al., 2012). Consistent with our data, removal of H19 expression only causes a significant increase (Figure 2.7 A) in IGF2 at 20 days after birth despite detection of increased liver weights and cell proliferation at earlier ages. This suggests involvement of another regulator. Although we have found differences in liver development at postnatal ages compared to fetal liver development, inhibition of IGF1R by miR-675 may be the mechanism by which *H19* controls liver growth. IGF1R expression was examined at both the RNA and the protein levels. No significant differences were found, but a trend of an increase in IGF1R expression at the protein level was found for each age measured, suggesting a loss of miR-675 expression may be the cause for the overgrowth phenotype and the increase in cell proliferation.

Developmental P450 expression pattern for Cyp3a is not influenced by H19 expression. The CYP3A family undergoes a switch in dominant isoforms during postnatal liver development. Early in life, CYP3A16 is the dominant isoform in mice. The adult CYP3A isoform switches to predominantly CYP3A11 around 20 days after birth (Li et al., 2009b). Despite this developmental shift pointing to CYP3A potentially being impacted by H19 expression, no significant differences were observed between wild type mice and mice with the maternal *H19* allele knocked out (Figures 3.1 A and B).

However, H19 does affect the expression of P450 enzymes CYP2B10 and CYP2C29 at particular points during mouse liver development. Sex differences in CYP2B10 expression are known between wild type males and females. Wild type adult females are known to display a

higher expression level than adult males in mouse liver (Renaud et al., 2011). Our results also point to a sex difference in H19's role affecting the development of CYP2B10 only significantly in male mice and not female mice at 30 days after birth (Figure 3.1 C). CYP2C29 is not normally expressed until the liver begins to mature. In wild type mice, slight expression is observed at 15 days after birth. In *H19* maternal allele knockouts with no H19 expression, the expression of CYP2C29 only begins to be detected at 20 days after birth, and at 15 days after birth is not expressed and significantly ($p<0.05$) lower compared to wild type mice (Figure 3.1 D). Despite these specific changes, P450s were largely found to be unaffected by H19 expression indicating H19 may not be important for liver maturation.

Insignificant changes in expression patterns of albumin and α-fetoprotein, two developmentally regulated genes, also indicate H19 may not be important for liver maturation. Albumin production is a function and marker of normal mature hepatocytes, but can be detected in nascent hepatic cells (Cascio and Zaret, 1991). Albumin production rises continually throughout liver development and is at maximum in adult liver (Jochheim et al., 2004). Due to this expression pattern, we chose the two earliest ages in our study (5 and 10 days after birth) to examine changes in albumin production in developing liver in mice with and without H19 expression. If H19 affects the maturation of liver, a difference in levels of albumin production might be noticed at different developmental ages. Conversely, α-fetoprotein is a fetal liver gene and the major plasma protein present in the fetus. The RNA expression profile of α-fetoprotein in postnatal liver development resembles H19, with highest expression early in life and a dramatic decline after birth. Expression of α-fetoprotein mRNA declines to undetectable levels around 14 days after birth in mouse (Pachnis et al., 1984). Due to this pattern, we chose to examine protein expression of α-fetoprotein at ages 15 and 20 days after birth between wild type

mice and mice not expressing H19. Protein expression was still detected at 15 days after birth in both groups, but by 20 days after birth, α-fetoprotein protein was not observed for either test groups (Figure 3.2 A). No significant changes were observed in the production of albumin or α-fetoprotein when H19 is not expressed (Figure 3.2) indicating H19 may not control these developmentally regulated genes.

H19's role in postnatal liver maturation appears to be consistent with its role in other contexts. H19 is expressed in highly proliferating tissues including fetal and postnatal livers (Pachnis et al., 1984), and H19 expression can reemerge in adult liver during hepatocellular carcinoma (Kim and Lee, 1997; Matouk et al., 2007), or during regeneration after injury (Pachnis et al., 1984; Yamamoto et al., 2004). Despite its expression in proliferating tissue, our data support the hypothesis that normal expression or reemergence of H19 is to limit cellular proliferation to control overgrowth. H19 may be falsely implicated in promoting cell growth if basic correlations linking proliferation and H19 expression are made without mechanistic examination of H19's actual molecular role.

4.2 Conclusions

H19 affects liver growth controlling proliferation through IGF and Wnt signaling, but may be inconsequential to liver maturation during postnatal development. H19's action is potentially through miR-675. miR-675 has been shown to inhibit IGF1R indicating uninhibited IGF signaling may be the cause of the overgrowth phenotype and increases in cell proliferation. Despite H19's regulation of liver growth, evidence suggests H19 may not play a significant role in postnatal liver maturation. Albumin and α-fetoprotein expression patterns were not significantly altered, and P450 expression pattern changes were only affected at specific ages.

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S Taylor MS Tegner J Teichmann SA Ueda HR van Nimwegen E Verardo R Wei CL Yagi K Yamanishi H Zabarovsky E Zhu S Zimmer A Hide W Bult C Grimmond SM Teasdale RD Liu ET Brusic V Quackenbush J Wahlestedt C Mattick JS Hume DA Kai C Sasaki D Tomaru Y Fukuda S Kanamori-Katayama M Suzuki M Aoki J Arakawa T Iida J Imamura K Itoh M Kato T Kawaji H Kawagashira N Kawashima T Kojima M Kondo S Konno H Nakano K Ninomiya N Nishio T Okada M Plessy C Shibata K Shiraki T Suzuki S Tagami M Waki K Watahiki A Okamura-Oho Y Suzuki H Kawai J Hayashizaki Y Consortium F Group RGER and Genome Science G (2005) The transcriptional landscape of the mammalian genome. *Science* **309:**1559-1563.

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