ROLE OF AQUAPORINS DURING HEPATOCELLULAR CARCINOMA INITIATION AND PROGRESSION

by

Matthew Adrian Mattocks

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Approved by:

Dr. Iain H. McKillop

Dr. Mark Clemens

Dr. Didier Dréau

Dr. Laura Schrum

Dr. Charles Lee

ABSTRACT

MATTHEW ADRIAN MATTOCKS. Role of aquaporins during hepatocellular carcinoma initiation and progression. (Under direction of DR. IAIN HUGH MCKILLOP)

Aquaporins (AQPs) are a family of proteinaceous water channels involved in bile production and apoptosis. Aquaporins 8, 9 and 0 are expressed in hepatocytes and cAMP dependent signalling regulates AQP8 expression and localization in normal hepatocytes. Also, AQP9 has promoter binding sites for AP-1, a downstream transcription factor in IL-6 signaling. The role of AQP8 and 9 in hepatocellular carcinoma (HCC) initiation and progression has not been determined. The role of AQP8/9 in HCC initiation and progression was examined in rodent models of HCC as well as human HCC. Further, the role of cAMP and IL6 in regulating AQP8/9 expression and/or localization was examined. To examine the role of AQP8/9 in HCC initiation and progression both a rat, hepatoma cell inoculation model and a diethylnitrosamine (DEN) initiated, hepatocarcinogenic, progressive mouse model, were used. A rat hepatoma cell line was used to examine the role of cAMP and IL6 on AQP8/9 expression and localization. While IL6 significantly affected AQP8 membrane localization, cAMP did not. Further, there was no significant affect on AQP8/9 expression from cAMP pathway modulation or IL6 exposure. However, cAMP pathway modulation did significantly affect cell responsiveness to osmotic challenge. In a mouse model of HCC, AQP8 expression in plasma membrane was significantly higher in mice euthanized 24 weeks after DEN injection compared to control mice. However, AQP8 and 9 membrane localization was significantly lower in mice euthanized 48 weeks after DEN injection compared to control mice. In human HCC, AQP9 membrane localization is significantly decreased in tumor tissue compared to non-tumor tissue. Also, AQP8/9 expression did not correlate with known risk factors for HCC. Lastly, doxycycline controlled expression of AQP8, in a rat model of HCC, significantly inhibited tumor progression *in vivo*. In summary, AQP8 membrane localization is affected by IL6 exposure but cAMP significantly affects cell responsiveness to osmotic stress. Also, AQP9 membrane expression is reduced in rodent models of HCC as well as human HCC. Finally, expression of AQP8 in a rat model HCC, *in vivo,* reduced tumor proliferation.

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Lastly, my beloved wife Kym. This work is dedicated to her.

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LIST OF ABBREVIATIONS

Abbrv.	Meaning
AFB1	Aflatoxin B1
AFP	Alpha Fetoprotein
ATP	Adenosine Triphosphate
AVD	Apoptotic Volume Decrease
C/EBP	CCAAT/Enhancer Binding Protein
cAMP	Cyclic Adenosine 3',5'-Monophosphate
CPI	Complete Protease Inhibitor
CRE	cAMP Response Element
DAPI	4',6-Diamidino-2-phenylindole dihydrochloride
DEN	Diethylnitrosamine
DMEM	Dulbecco's minimum essential media
DMSO	Dimethylsulphoxide
EDTA	Ethylenediaminetetraacetic Acid
EMEM	Eagle's minimum essential media
ER	Endoplasmic Reticulum
EtBr	Ethidium Bromide
EtOH	Ethyl alcohol
FBS	Fetal Bovine Serum
GDP	Guanine Diphosphate
GTP	Guanine Triphosphate
HBV	Hepatitis B Virus
HCC	Hepatocellular Carcinoma
HCV	Hepatitis C Virus
HSC	Hepatic Stellate Cell
ICER	Inducible cAMP Early Repressor
IL-6	Interleukin-6
IL-6Rα	Interleukin-6 α-Receptor
MCS	Multiple Cloning Site
MDL	MDL-12,330A Hydrochloride
MIP	Membrane Intrinsic Protein
NFDM	Non-Fat Dry Milk
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PKA	Protein Kinase A

PKC	Protein Kinase C
rrIL-6	Recombinant Rat Interleukin-6
TEMED	N,N,N`,N`-Tetramethylethylenediamine
TetR	(Bacterial) Tetracycline Repressor
TGFβ	Transforming Growth Factor Beta
TNFα	Tumor Necrosis Factor Alpha
TRAIL	Tumor necrosis factor Related Apoptosis Inducing Ligand
TRE	Tetracycline Response Element

CHAPTER 1:INTRODUCTION

1.1. Hepatocellular Carcinoma

1.1.1. Epidemiology

The World Health Organization (WHO) estimated that in 2002 approximately seven million years of life were lost worldwide due to hepatocellular carcinoma (HCC) (World Health Organization 2002). Hepatocellular carcinoma killed approximately 600,000 people in 2002 and was the 4th leading cause of cancer death (World Health Organization 2002) (Figure 1.1). In the United States alone, approximately 15,000 people were diagnosed with some form of primary liver cancer, in 2004, (predominately HCC or cholangial carcinoma) and in that same year approximately 14,000 people died (U.S. Cancer Statistics Working Group 2007). Only pancreatic cancer has a higher percentage of people killed among the individual cancers tracked by the Centers for Disease Control (CDC) (U.S. Cancer Statistics Working Group 2007). The American Cancer Society, along with the CDC, estimates that by 2030 as many as twenty million people could die of cancer a year in the U.S., which is estimated to account for 12% of all deaths from any cause (Mackay *et al.* 2006).

Hepatocellular carcinoma is a disease that disproportionately affects men (Bosch *et al.* 2005), and is most prevalent in sub-Saharan Africa, China and East Asia (Mackay *et al.* 2006). Globally, men are approximately two-and-half times more likely to develop HCC than women and in southern Africa and in south-east Asia men are 3 times more likely to develop HCC than women (Bosch *et al.* 2005). Even in migrant populations, the relative risk of Asian men

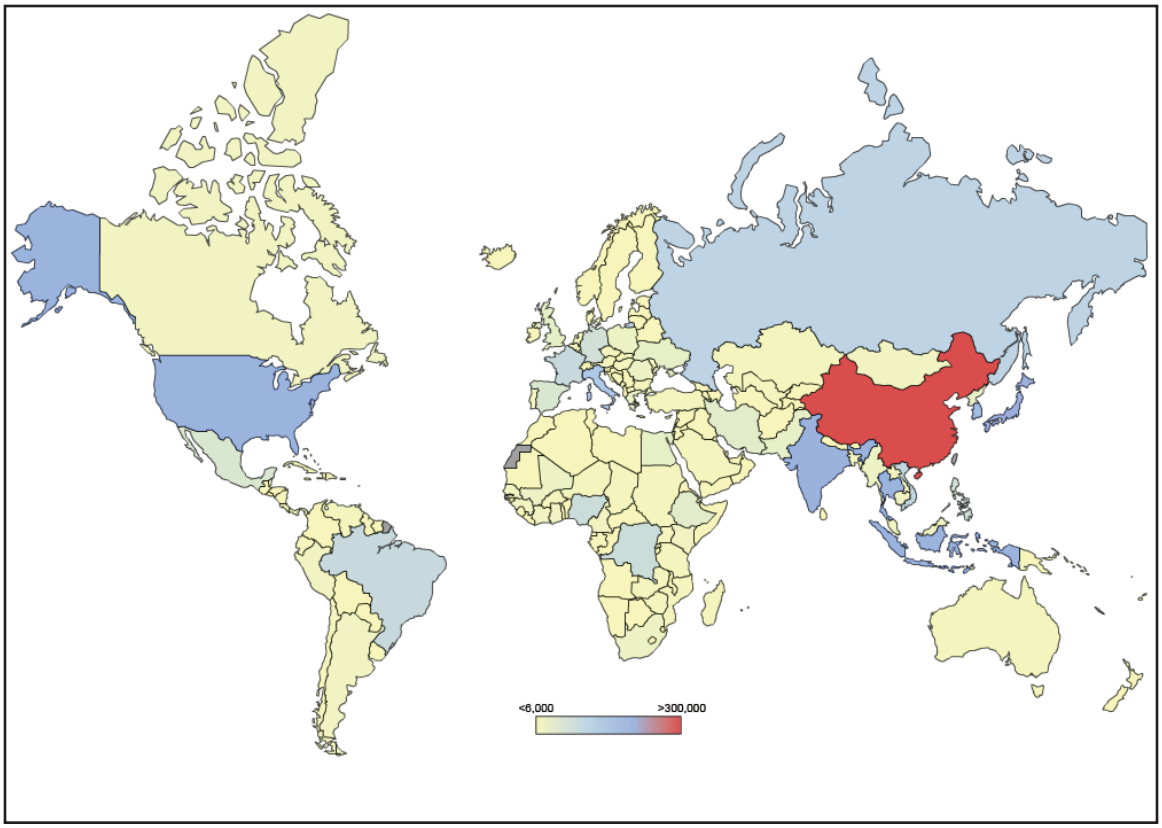


Figure 1.1: Liver Cancer Deaths in 2002. Geographical distribution of reported deaths from hepatocellular carcinoma worldwide (World Health Organization 2002).

in the United States developing HCC is 10.9 (Bosch *et al.* 2005). And for west African men in England and Wales the relative risk of developing HCC is 31.6 (Bosch *et al.* 2005). The etiology of HCC has largely been determined. Up to 80% of HCC cases are related to chronic viral hepatitis B (HBV) and C (HCV) infection, which are known risk factors for the development of HCC (Bosch *et al.* 2005; Sherman 2004; Thomas *et al.* 2005). There is also a correlation between high alcohol consumption and HCC (*via* cirrhosis and fibrosis) (Voigt 2005). Lastly, hepatotoxic agents as well as hormones have been associated with the development of HCC (McKillop *et al.* 2006).

1.1.2. Viral Hepatitis B & C

Hepatitis B virus is a known risk factor for HCC and in areas with a high prevalence of HBV it is the primary cause of HCC (Tran *et al.* 2004). Individuals who have chronic HBV are approximately 100 times more likely to develop HCC than those not infected (Sherman 2004) and it is estimated that 60-80% of worldwide cases of HCC are due to chronic HBV infection(Lavanchy 2005). It is estimated that in 2000 there were 22,000 cases of HBV in the United States alone (Tran *et al.* 2004). Further, in Eastern Europe as many as 55% of the population tests positive for antibodies against the Hepatitis B antigen and in southeast Asia, as well as certain parts of China and Africa, as many as 95% of the population tests positive for these antibodies (Tran *et al.* 2004).

Hepatitis C virus is also a known risk factor for HCC and is associated with a higher incidence of HCC than HBV (Bosch *et al.* 2005). Like HBV, HCV is endemic in northern and central Africa and high incidence rates occur in most Asian nations (Bosch *et al.* 2005; Shepard *et al.* 2005). In the United States, rates of chronic liver disease were declining through most of the 1980s but have been rising steadily since the 1990s and the increase in chronic liver disease

is thought to be due, in large part, to the increased prevalence of HCV (Shepard *et al.* 2005). Indeed, while hospitalization rate of HCC associated with HBV infection remained constant through the 1990s, the hospitalization rate of HCC associated with HCV infection more than doubled (Bosch *et al.* 2005).

Among the five known hepatitis virus types (A-E), current data indicates that only hepatitis B and C are associated with the development of HCC (Dash *et al.* 2005). This represents something of an anomaly as HBV and HCV are from different viral families, have different modes of infection (HBV is a DNA virus while HCV is an RNA virus) and different relationships to the host genome (HCV is episomal while HBV is integrated into the host genome)(Dash *et al.* 2005). Despite these differences both viruses result in the same end-stage disease pathology, HCC. Namely, chronic infection by one or both of these viruses can result in liver cirrhosis, which if uncleared, can result in HCC development (Dash *et al.* 2005; Villeneuve 2005). It should be noted though, that a given patient is about half as likely to develop cirrhosis if only infected with HBV compared to HCV (Rehermann et al. 2005).

There are two commercially available vaccines for HBV, Recombivax HB (Merck) and Engerix-B (GSK). Hepatitis B virus vaccines have been reported to have high efficacy, 80-90%, and are well tolerated, though antibody titers decline over time (Hassan *et al.* 2007; Yu *et al.* 2004). Though, older people, males, obesity, smoking and immunocompromised patients are all at statistically significantly higher risk of not responding to HBV vaccination (Yu *et al.* 2004). Further, post HBV exposure vaccination has reported to be 70% effective in preventing the HBV infection (Yu *et al.* 2004). Also, HBV vaccination can be used with patients with mild-to-moderate chronic liver disease although it is contraindicated in advanced cirrhotic patients (Yu *et al.* 2004). Hepatitis B virus vaccination has been reported to reduce viral load in HBV

infected patients (Yu *et al.* 2004).

There are no commercially available vaccines for HCV. This is due in part to the absence of an *in vitro* cell culture system for HCV and lack of a suitable rodent model to study HCV. In a study using chimpanzees as a model, data indicated that immunization against nonstructural proteins of HCV resulted in subclinical HCV infection in previously immunized chimps (Puig *et al.* 2006). That is, a naïve chimp immunized against the same HCV proteins showed highly significant decrease in viral load as well as a robust CD4+ T-cell response (Puig *et al.* 2006). Unfortunately, this effect only persisted for 10 weeks whereby a new viral population dominated and infection set it in, as well as a loss of effective CD4+ T-cell response (Puig *et al.* 2006).

1.1.3. Alcohol & Cirrhosis

In Africa and Asia as high as 80% of HCC cases arise from viral hepatitis (Bosch *et al.* 2005), while in the United States, alcohol consumption is a greater risk factor for HCC (El-Serag *et al.* 2000; Voigt 2005). In 2000, hospitalization rates of patients with HCC, due to alcoholic cirrhosis, were twice that of patients with a HCV pathology and six times greater than HBV patients (El-Serag *et al.* 2000). The distinction between a normal liver and a cirrhotic liver is not discrete. It is a continuum based on levels of extracellular membrane proteins (specifically, collagen, laminin and fibronectin) which results in capillarization of the sinusoids (Poli 2000). There are many factors involved in the development of liver fibrosis. Data indicates that, among the many factors which play a role in the development of liver fibrosis, three are critical, TNFα, TGFβ (Poli 2000) and reactive oxygen species(Parola *et al.* 2001; Poli 2000). Chronic, heavy ethanol consumption results in numerous hepatic and systemic responses. Broadly, chronic ethanol consumption results in the initial activation of pro-inflammatory pathways yet, in mid and late stage alcoholic liver disease anti-inflammatory

pathways become activated (Crews *et al.* 2006). Specifically, the metabolism of ethanol by cytochrome P450 2E1 (CYP2E1) results in the production of reactive oxygen species (ROS) (Neuman 2003). Further, metabolism of ethanol by ADH results in downstream production of TNFα (a Th1/pro-inflammatory cytokine) by Kupffer cells (Neuman 2003). Under chronic exposure ethanol stimulates Kupffer cells to release TGFβ (a Th2/anti-inflammatory cytokine), which acts to activate hepatic stellate cells (HSCs)(Crews *et al.* 2006). It is hypothesized that chronic, heavy ethanol consumption results in a switch from Th1 cytokines to Th2 cytokines and it is, in part, this switch that drives fibrosis and cirrhosis (Crews *et al.* 2006). The exact mechanisms at work, and the critical factors that drive this switch, remain to be determined, as does the mechanism whereby HCC arises from the underlying cirrhotic liver.

The International Agency for Research on Cancer has determined, based on epidemiological data, that ethanol is a carcinogen (Seitz *et al.* 2007). The mechanism proposed establishes acetaldehyde, a product of alcohol metabolism, as the primary driver of carcinogenesis (Seitz *et al.* 2007). Acetaldehyde, a toxic product of ethanol metabolism, is carcinogenic (Poschl *et al.* 2004; Seitz *et al.* 2001; Seitz *et al.* 2007; Stickel *et al.* 2002). Previous studies indicate that acetaldehyde acts through two mechanisms to initiate and drive cancer progression. The first is by acting as an enhancer of fibrosis by causing the production of ROS and the activation of hepatic stellate cells (Siegmund *et al.* 2005) and the second is as a direct mutagen by disrupting DNA synthesis and repair (Poschl *et al.* 2004). Acetaldehyde is known to covalently bond to DNA froming DNA adducts both *in vitro* and *in vivo* (Brooks et al. 2005). *In vitro* high concentrations of borohydride compounds are required for adduct formation but *in vivo,* no enzyme has been decisively identified. Although vitamin C and glutathione have been reported to catalize adduct formation *in vitro* (Brooks et al. 2005). Further, acetaldehyde

inhibits O6 methyl-guanyltranferase, which repairs adducts resulting from exposure to alkylating agents (Poschl *et al.* 2004). It should be noted though, that the exact mechanism by which acetaldehyde results in HCC remains to be fully elucidated.

1.1.4. Aflatoxins

Aflatoxins are fungal products which are known to be hepatotoxic and carcinogenic (Stickel *et al.* 2002). While aflatoxin exposure is relatively low in the United States (Hoque *et al.* 1999), in Asia (China especially) aflatoxin exposure is endemic (Wang *et al.* 2001; Wang *et al.* 1996).

The role of aflatoxins (specifically aflatoxin B1—AFB1) in the initiation and progression of HCC has been the subject of a great deal of research. Similar to ethanol, AFB1 is not directly carcinogenic, but when converted into exo-8,9-epoxide by cytochrome p450, DNA adducts result (Zhang *et al.* 2006). The carcinogenic effect of chronic aflatoxin exposure results from these DNA adduct lesions and direct mutation of p53 (Kensler *et al.* 2004; Stickel *et al.* 2002). Further, high AFB1 exposure promotes DNA hypermethylation which is proposed to play a role in hepatocyte DNA damage and p53 mutation (Zhang *et al.* 2003). Previous studies reported aflatoxin mutates p53 by guanine to thymine transversions on codon 249 (Bosch *et al.* 2005; Lasky *et al.* 1997). Also, AFB1 appears to act synergistically with HBV and HCV infections in HCC progression (Bosch *et al.* 2005). Lastly, AFB1 is metabolized by CYP2E1, which is induced by ethanol consumption as such, it is hypothesized that alcohol consumption can exacerbate the gene damage caused by AFB1 exposure (Voigt 2005). Indeed, previous studies have reported that even moderate ethanol consumption (24 grams per day, which is equivalent to having two coffees and Kahlua) concomitant with AFB1 exposure results in a thirty-five fold increase in risk of developing HCC (Voigt 2005).

1.2. Guanine Nucleotide-Binding Proteins & Related Pathways

1.2.1. Structure & Function

Guanine nucleotide-binding proteins (G-proteins) are heterotrimeric proteins, consisting of three subunits: α, β and γ. At the time of writing (2006), sixteen different α subunits (Bünemann *et al.* 2003), five different β subunits (Watson *et al.* 1994) and eleven different γ subunits have been identified in mammals (Morishita *et al.* 1995). Canonically, G-protein dependent pathways are activated by a receptor mediated conformational change in the α subunit resulting in the displacement of guanine diphosphate (GDP) and the binding of guanine triphosphate (GTP)(Miller *et al.* 1988). The exchanging of GDP with GTP is catalyzed by guanine exchange factors (GEFs). While α subunits have inherent GTPase activity, this activity is typically slow (as long as hours), but hydrolysis is enhanced by GTPaseactivating proteins (GAPs) (Sprang 1997). The presence of GAPs can reduce the hydrolysis rate by as much as 100-fold (Sprang 1997). The exchange of GDP with GTP activates the α subunit and causes the dissociation of the α subunit from the βγ heterodimer (Forse 2000). However, studies indicate that activation of the α and βγ subunits may rearrange instead of complete dissociation (Bünemann *et al.* 2003; Cherfils *et al.* 2003; Robishaw *et al.* 2004). While the exact mechanism by which heterotrimeric G-proteins are activated remains, in part, uncertain, there are convincing data that indicates that in response to Gα-receptor activation, the α subunit initiates the production of cyclic adenosine 3',5'-monophosphate (cAMP) by activating adenylate cyclase (Dessauer *et al.* 1998; Simonds 1999; Sunahara *et al.* 1997; Whisnant *et al.* 1996; Yan *et al.* 1997).

1.2.2. Adenylate Cyclase & cAMP

Adenylate cyclase (AC) is an enzyme which catalyzes the conversion of ATP to cAMP.

Because cAMP is an essential second messenger and because adenylate cyclase is tethered to the plasma membrane, and is responsive to hormones, it plays a critical role in transmitting signals into the cell (Mayer *et al.* 1998). Adenylate cyclase activity is significantly reduced in HCC compared to normal hepatocytes but is significantly higher when the tumor is exposed to glucagon (Mayer *et al.* 1998).

First described in 1958 by Rall and Sutherland, cAMP is a ubiquitous, intracellular signaling molecule (Rall *et al.* 1958; Zhang *et al.* 2005). Cyclic-AMP is formed by the bonding of C3 oxygen to the C5 phosphate and is catalyzed by adenylate cyclase. Cyclic-AMP functions as a second messenger in a wide array of signaling pathways, from development, to liver regeneration, to apoptosis (Fimia *et al.* 2001; Servillo *et al.* 2002). Of particular interest regarding the work in this dissertation, is the role that cAMP plays in the translocation of proteins and tissue and cellular polarization. While a great deal of work remains to be done, preliminary reports suggest cAMP plays a role in determinating central nervous system polarity *via* modulation of protein expression. For example, sonic hedgehog plays a critical role in the correct polarization of the dorsal/ventral aspects of the development of the central nervous system (Epstein *et al.* 1996). Further, cAMP-dependent protein kinase A (PKA) is a negative regulator of Sonic hedgehog activity and removal of PKA activity is sufficient to activate Sonic hedgehog (Epstein *et al.* 1996). In addition to tissue polarization, cAMP also regulates cell polarity. In HepG2 cells (a human derived HCC cell line), glucosylceramide and sphingomyelin (both sphingolipids) are preferentially located in the apical and basolateral membranes, respectively. However, following cAMP treatment, this arrangement is disturbed and sphingomyelin remains in the apical membrane (van Ijzendoorn *et al.* 1997). Further, glucosylceramide and sphingomyelin are, in part, responsible for the creation of membrane

domains in the apical and basolateral membrane and current data supports that it is to these domains that aquaporins, as well as other proteins, are targeted (Tietz *et al.* 2005; van IJzendoorn *et al.* 1999).

In the liver, cAMP is involved in liver regeneration (Diehl *et al.* 1992),bile production (*via* glucose) and cell proliferation where cAMP is either anti- or pro-proliferative depending on the concentration of cAMP and the presence or absence of other factors) (Diehl *et al.* 1992; LeSage *et al.* 2001). In HCC, cAMP inhibits HCC cell proliferation *in vitro* (Kovach *et al.* 2006; Liu *et al.* 2005) but the exact mechanism by which this inhibition occurs remains to be elucidated. Currently data suggest cAMP may inhibit HCC cell proliferation by acting on TGFβ (Schiller *et al.* 2003), by modulation of the regulatory subunits of PKA (Cho-Chung *et al.* 2002), by modulating Akt (Liu *et al.* 2005), and by induction of inducible cAMP early repressor (ICER) (Servillo *et al.* 2002).

1.2.3. Protein Kinase A

Canonically, PKA is activated by the cooperative binding of two cAMP molecules to each of the two regulatory subunits which repress PKA activity (Taylor *et al.* 1999). Binding of cAMP to the regulatory subunits initiates their dissociation from the two catalytic subunits, which are the effector subunits of PKA (Taylor *et al.* 1999). There are many downstream targets for PKA, in fact most of the cell signaling which occurs in response to cAMP production is due to the activity of PKA (Mayr *et al.* 2001; Montminy 1997; Taskén *et al.* 2004). Currently, there appears to be no independent role (i.e. one that is not in response to cAMP stimulation) for PKA in hepatocellular carcinoma (Figure 1.2).

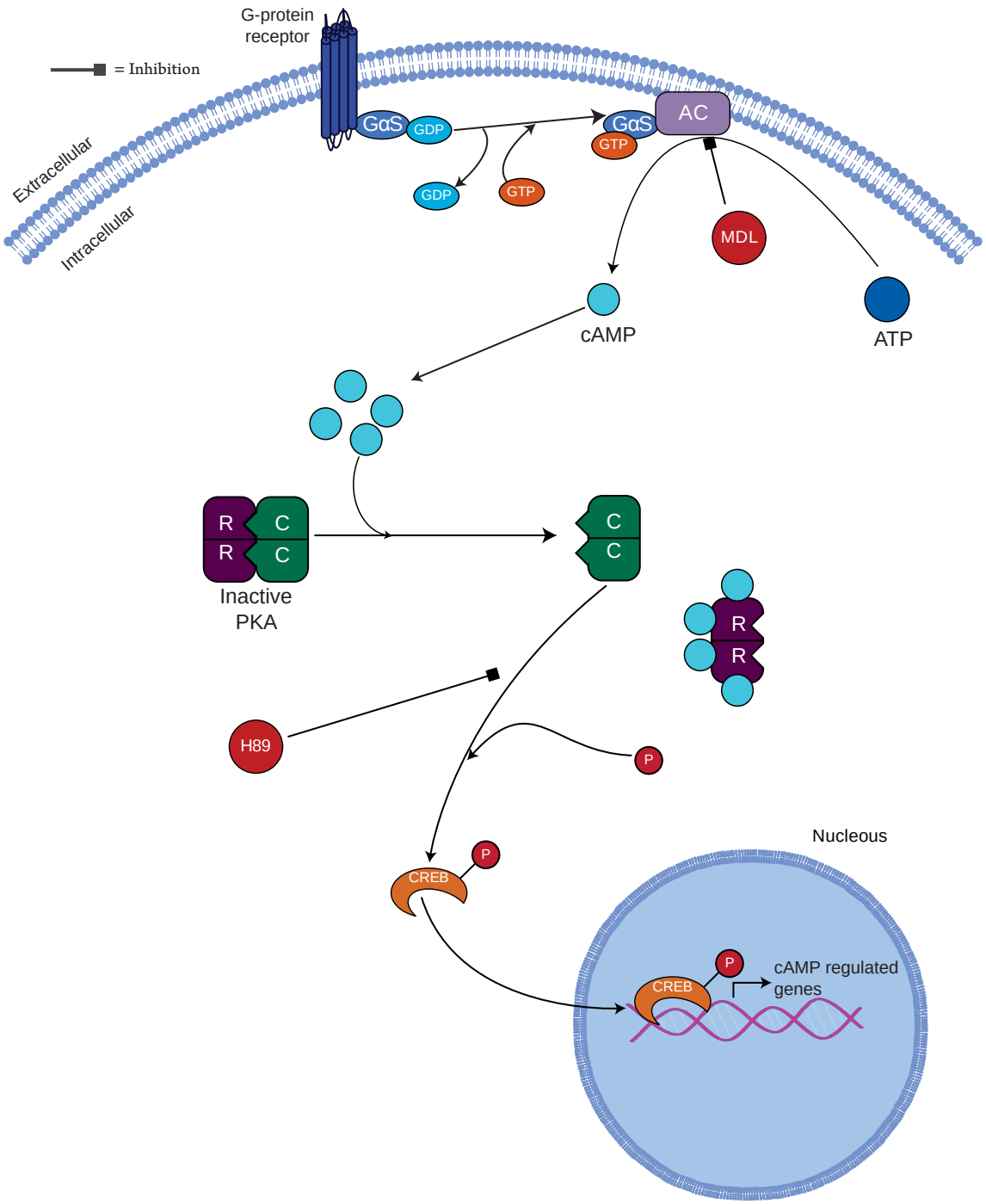


Figure 1.2: Cyclic-AMP Signaling Pathway. In response to G-protein coupled receptor activation, GDP bound to $G_{\alpha}S$ is displaced by GTP, activating $G_{\alpha}S$. Activated $G_{\alpha}S$, stimulates adenylate cyclase (AC) to convert ATP to cAMP. Cyclic-AMP activates protein kinase A (PKA) by dissociation of regulatory subunits bound to the catalytic subunits. This in turn catalyzes the phosphorylation and translocation of CREB to the nucleus and regulates cAMP dependent gene transcription. MDL inhibits the conversion of ATP to cAMP. H89 inhibits CREB phosphorylation by PKA.

1.3. Cell Death

1.3.1. Necrosis

There are two processes by which a cell dies; necrosis or apoptosis (Table 1.1). Necrosis was once thought to be a passive, unregulated process which occurs when the cell is exposed to severe physical or metabolic challenge resulting in acute disruption of the plasma membrane, followed by the cellular contents being released into the extracellular environment (Lemasters 2005). The release of cellular constituents results in an increase in local inflammation (Henke *et al.* 2005). This localized immune response is exacerbated by the fact that it is rare for a single cell to undergo necrosis. Rather, large numbers of contiguous cells will be stimulated to undergo apoptosis following a dramatic insult (Malhi *et al.* 2006). With this being said, more recent data indicates that necrosis may not be as passive or as unregulated as previously thought (Harwood *et al.* 2005; Majno *et al.* 1995). It has been reported that under severe chemical or metabolic challenge the cell will undergo necrosis *via* ATP depletion resulting in cell swelling and if left unchecked the plasma membrane will be compromised resulting in the cell rupturing (Malhi *et al.* 2006). While there are mechanistic pathways involved in this process, necrosis still results in significant damage to neighboring cells and neighboring tissues (Harwood *et al.* 2005; Henke *et al.* 2005). Typically, the inflammatory response is local but necrosis can cause widespread tissue damage and compromise organ function (Henke *et al.* 2005). Because of the large scale damage resulting from necrosis, a more controlled process has evolved. A process by which a single cell can be removed from a population of cells in such a way that damage to neighboring cells is reduced, if not ameliorated, and that does not initiate an inflammatory response by the body; the process which evolved is apoptosis.

	Apoptosis	Necrosis
Initiation	Through receptors or mitochondria.	Severe chemical or mechanical insult. Rapid ATP withdrawl.
Regulation	Highly regulated through a large network of proteins.	Largely unregulated.
Immune Response	None. Released cellular constiutients are confined within vessicals.	High. Cell rupture results in release antigenic proteins and inflammation.
DNA Breakdown	DNA is cleaved following PARP deactivation. Complete DNA destruction results.	Little. The rapidity of necrosis largely prevents any orderly breakdown of DNA.
Water Movement	Water flows out of the cell resulting in cell shrinkage.	Water flows into the cell resulting in cell swelling and eventual rupture.
Speed	Slow. Can take hours.	Fast. Can occur in minutes.

Table 1.1: Comparison of apoptosis and necrosis.

1.3.2. Initiation & Regulation Of Apoptosis

Apoptosis can be initiated either extrinsically or intrinsically (Figure 1.3). As the name implies, extrinsic apoptosis is initiated when a ligand binds to an extracellular receptor (Guicciardi *et al.* 2005). There are a limited number of ligands which activate the extrinsic apoptotic pathway, the principal ones being Fas ligand (FasL), Tumor necrosis factor-α (TNFα), tumor necrosis factor related apoptosis inducing ligand (TRAIL) and granzyme B (Guicciardi *et al.* 2005). Binding of these ligands (with the exception of Granzyme B) to their respective receptors results in the binding of adaptor proteins to form the death inducing signaling complex (DISC), which results in the activation of caspase 8 (Lemasters 2005). Granzyme B is an enzyme secreted by cytotoxic T lymphocytes during T cell mediated killing of a cell (Bleackley 2005) and should be noted, that the discovery of caspases was due to work done involving granzyme B (Bleackley 2005). In contrast to other apoptotic signals, granzyme B bypasses most of the regulatory pathways of apoptosis and directly activates caspase 3 (Bleackley 2005).

There are many regulatory points, and a great deal of cross-talk, in both extrinsic and intrinsic apoptotic pathways. In the extrinsic pathway, DISC formation results in the activation of caspase 8 (Harwood *et al.* 2005). This is followed by cleavage activation of BH3 domain-only death agonist (Bid) to truncated Bid (tBid) (Harwood *et al.* 2005). Truncated Bid then translocates to the mitochondria whereby Bax and Bak are recruted, resulting in Bax/Bak dimerization and insertion into the mitochondrial membrane (Lemasters 2005). Insertion of Bax/Bak results in the membrane permeability transition (MPT), which permits the release of cytochrome c into the cytoplasm (Lemasters 2005). Cytochrome c, ATP and apoptotic protease activating factor-1 (Apaf-1) form the apoptosome, which in turn activates caspase

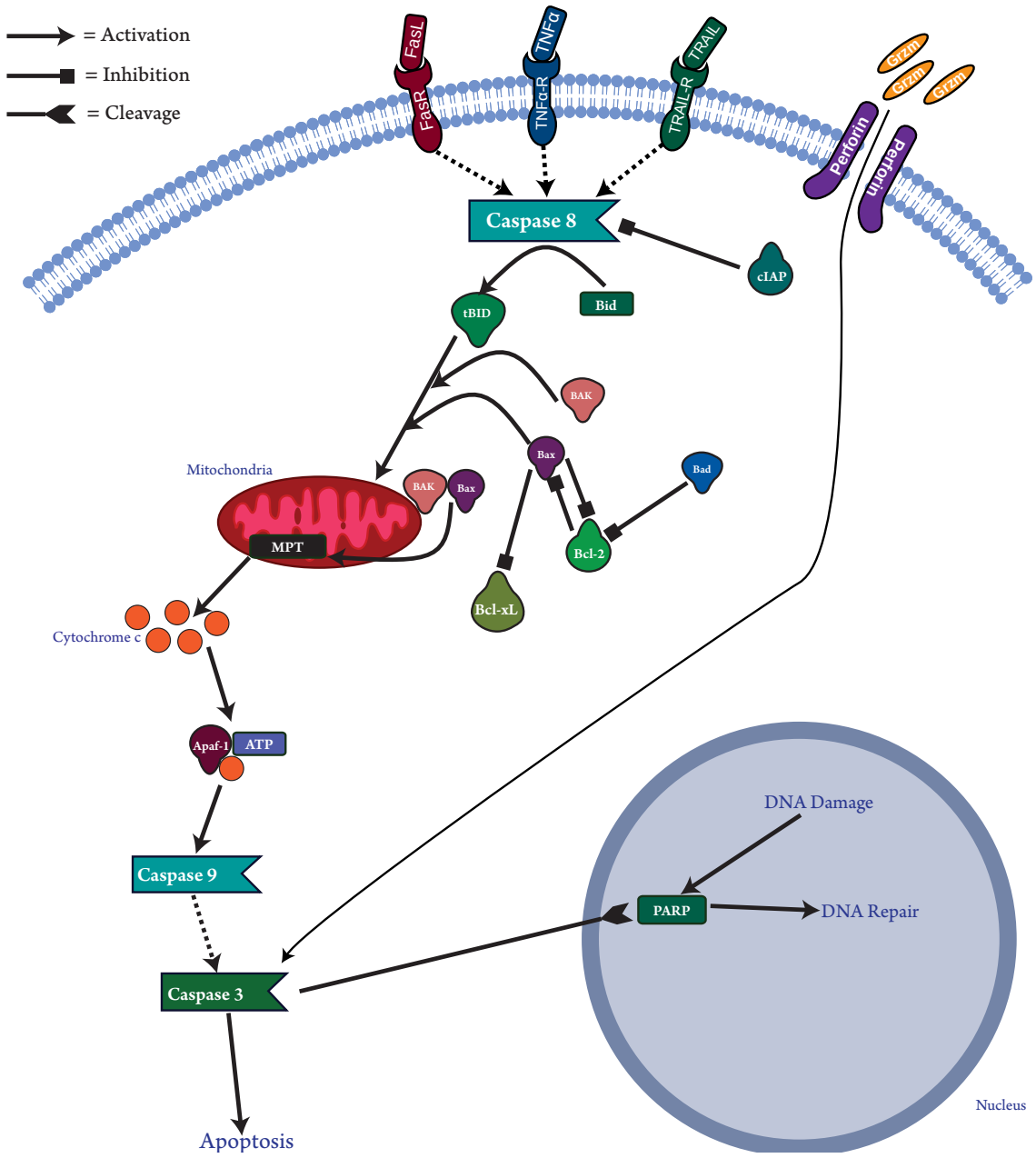


Figure 1.3: Extrinsic apoptotic pathway: Following activation of a death receptor (Fas, TNFα or TRAIL), Caspase 8 is activated and cleaves BH3 interacting domain death agonist (Bid) to truncated-bid (tBid). Truncated-Bid translocates to the mitochondria and recruits Bcl-2 associated X protein (Bax) and Bcl-2 agonist killer (Bak). There, Bax and Bak form a heterodimer resulting in mitochondrial membrane permeability transition (MPT). MPT results in the release of cytochrome c which forms a heteromeric protein complex with apoptotic protease activating factor 1 (Apaf-1) and ATP. This complex initiates a caspase cascade resulting in activation of caspase 3, the inactivation of polyadenosine ribose polymerase (PARP) and DNA destruction. Alternatively, Granzyme-B (Grzm) enters into the cell by perforin pores and it then directly activates caspase 3. Apoptosis can be inhibited by a number of proteins, such as B-cell CLL/lymphoma 2 (Bcl-2) and Bcl2-like 1 (Bcl-xL).

9 by cleaving procaspase 9 (Harwood *et al.* 2005). Activated caspase 9 cleaves procaspase 3 resulting in caspase 3 (the activated isoform of the enzyme), which is a central control point for apoptotic pathways (Guicciardi *et al.* 2005; Harwood *et al.* 2005; Lemasters 2005). Caspase 3 is the last regulatory point in both extrinsic and intrinsic apoptosis and, once activation of caspase 3 occurs, there are no further regulatory checkpoints and a chain reaction of caspase activation proceeds, culminating with cleavage inhibition of polyadenosine ribose polymerase (PARP)and preventing DNA repair mechanisms from being activated (Lemasters 2005). The activation of PARP results in DNA fragmentation and cell death (Guicciardi *et al.* 2005).

Intrinsic apoptosis usually occurs in response to severe DNA damage which cannot be repaired (typically due to chemical or γ-radiation exposure) (Guicciardi *et al.* 2005). This type of DNA damage results in phosphorylation of p53 which in turn activates Bax (Hanahan *et al.* 2000). Along with its role in the MPT, Bax also functions as a repressor of anti-apoptotic proteins Bcl-2 and Bcl-xL (Hanahan *et al.* 2000). Mitochondria are the convergence points between extrinsic and intrinsic apoptotic pathways. Whether apoptosis is initiated by an extracellular ligand or catastrophic DNA damage, activated pathways that result in cell death merge at the mitochondria with the MPT (Cheng *et al.* 2001; Donovan *et al.* 2004; Guicciardi *et al.* 2005). Following the MPT, the caspase cascade and the subsequent cellular events are the same regardless of the event which initiated apoptosis (Donovan *et al.* 2004; Guicciardi *et al.* 2005; Lemasters 2005). It should be noted that even at late stages of apoptosis, a cell can switch to a necrotic process if a second cellular insult occurs (in the case of extrinsic initiation), or if DNA damage worsens (Majno *et al.* 1995).

1.3.3. Cellular Events

The purpose of apoptosis, as opposed to necrosis, is to selectively remove a cell with

minimal damage to surrounding cells. Necrosis is characterized by a depletion of ATP, thereby halting Na^+ /K^+ ion pumps, which results in water rapidly entering the cells and rupture of the plasma membrane. In contrast, apoptosis is a highly regulated process resulting in radically different morphological changes. Caspase activation requires a decrease in intracellular K^+ from 145mM to less than 50mM (Hughes *et al.* 1997). To effectuate this change water exits the cell *via* aquaporins (Bortner *et al.* 2002). This results in characteristic cell shrinkage observed in all apoptotic cells. Further, the nuclear membrane is broken down and DNA is cleaved by nonspecific nucleases (Lemasters 2005). The last morphological event observed is cell blebbing (Majno *et al.* 1995). To prevent the inflammation response that accompanies necrosis, cellular constituents are broken down while the plasma membrane is intact, and then exocytosed in compartments (called cell bodies) formed from the plasma membrane (Malhi *et al.* 2006). This permits phagocytosis of these cell bodies by neighboring cells (Malhi *et al.* 2006).

1.4. Aquaporins

1.4.1. History & Structure

As early as the 1950's it was hypothesized that a channel must exist to permit the volume and rate of water movement that was observed in red blood cells (Engel *et al.* 2000). By 1983 it was becoming clear, though still controversial, that this channel existed, but it had yet to be characterized (Lewis 1983). In 1986, a seminal paper by Benga, *et.al.* provided a clear demonstration of the presence of water channels in red blood cells (Benga 2003; Benga *et al.* 1986). Later, in what has now resulted in controversy, Peter Agre's group published a article demonstrating the sequence of a water channel (later claiming it to be the discovery of a water channel) by injecting mRNA from then called CHIP28 (now referred to as aquaporin 1) into oocytes and examining swelling changes in the oocyte in response to osmotic challenge

(Preston *et al.* 1992).

Thirteen known mammalian members of the membrane intrinsic protein (MIP) family (AQP0-AQP12) have now been described. Aquaporin's 0-12 proteins demonstrate a high degree of conservation between mammalian species. Though there is very little similarity (19-52%) between specific aquaporin isoforms within the same species (Verkman *et al.* 2000). In hepatocytes three aquaporin isoforms have been detected: AQP8, AQP9 and AQP0 (Matsuzaki *et al.* 2004; Portincasa *et al.* 2003). While AQP8 and AQP9 are regulated and respond to stimuli, AQP0 is constitutively expressed in the cytoplasm and has not been reported to respond to any stimuli tested to date (Huebert *et al.* 2002). For mammals the crystal structure has only been resolved for AQP0, AQP1 and AQP9, yet it is currently believed that all aquaporin family members will have similar structural characteristics despite the primary structure differences (Verkman *et al.* 2000). This belief is predicated on the notion that given the similar function for aquaporin members, the structure of each will be similar. While all aquaporin members permit the passage of water, recent data indicates that certain aquaporin proteins (AQP3, AQP7, AQP9 and AQP10) also permit passage of other water soluble molecules including glycerol, ammonium, urea, and other uncharged, small molecules (Carbrey *et al.* 2003; King *et al.* 2001; Saparov *et al.* 2006). These aquaporin proteins are termed aquaglyceroporins (it should be noted that "aquaporin" is still used as a general term to refer to *all* aquaporins regardless of what is able to pass through the pore).

Generally, aquaporins consist of six alpha helices each of which span the membrane into which they are inserted (Engel *et al.* 2000). Despite the differences in the sequences of aquaporin proteins, several conserved residues (the NPA motifs) exist that distinguish aquaporins from aquaglyceroporins . These residues act to provide passage specificity to the

pore (Engel *et al.* 2000).

1.4.2. Prevalence & Function

Of the thirteen currently identified mammalian aquaporin family members, at least one is found in every cell type and organ in the body (Badaut *et al.* 2004; Elkjaer *et al.* 2000; Ishibashi *et al.* 1998; Knüpfer *et al.* 2007; Labbozzetta *et al.* 2006; Matsuzaki *et al.* 2004; McConnell *et al.* 2002). Beyond mammals, aquaporins are also found in bacteria (Gonen *et al.* 2006) and play a critical role in plant physiology (Tritto *et al.* 2007).

Most simply, aquaporins permit the passage of water (and in some instances small, uncharged molecules) through the plasma membrane (Gonen *et al.* 2006). This process does not require ATP and water movement occurs along a solute concentration gradient (Preston *et al.* 1992). Aquaporin function is dictated by form. Specific residues within the aquaporin channel itself determine what is capable of passing through the pore (Engel *et al.* 2000; Verkman *et al.* 2000). One of the first questions that arose following the discovery of aquaporins was how do they permit the passage of water but exclude protons? The answer was found by observing the *E. coli.* aquaglyceroporin, GlpF(Gonen *et al.* 2006). These studies revealed that water has to act as a proton donor in a hydrogen bonding mechanism which requires an opposite orientation (both in terms of the water molecule and the residues which comprise the pore) between water molecules entering the pore and those leaving it (Gonen *et al.* 2006). In contrast, proton passage would require uniform orientation and therefore protons are precluded from passing through the aquaporin channel (Gonen *et al.* 2006).

Some aquaporins are sensitive to mercury chloride ($HgCl_2$) and exposure to $HgCl_2$ will inhibit movement of any molecule through the pore (Verkman *et al.* 2000). Mercury chloride acts by binding of Hg^{2+} to cysteine 189 in the pore thereby physically blocking the channel

(Preston *et al.* 1993). The primary function of aquaporins is water and glycerol movement and thus they are involved in numerous water and glycerol mediated physiological activities (Sambrook *et al.* 2001). Aquaporins play a critical role in the production of urine (Verkman 2005). Previous work has reported that humans with an AQP1 deletion develop a rare condition resulting in the person being unable to form concentrated urine (King *et al.* 2001). Further, a mutation in AQP2 results in a hereditary form of diabetes (van Lieburg *et al.* 1994). Aquaporins also play a critical role in the clearance of brain edema. Work with AQP4-null mice showed that these mice were highly resistant to brain swelling following edema and had significantly reduced edema following ischemic stroke (Manley *et al.* 2000). Interestingly, aquaporins also seem to play a role in tumor angiogenesis and metastatsis. Recent data reports that aquaporins may play a role in tumor neoangiogenesis (Saadoun *et al.* 2005). Aquaporin 1-null mice showed a significantly lower rate of vessel formation and a significantly higher survival rate compared to wild-type control (Saadoun *et al.* 2005). Also, migration and wound healing experiments reported that AQP1 knockout endothelial cells exhibit significantly inhibited migration compared to normal endothelial cells (Saadoun *et al.* 2005). Lastly, one of the questions that arose when it was discovered that some aquaporins allow the passage of glycerol was 'why'? The answer, in part, lay in the fact that glycerol plays a role in adipocyte regulation (Hara-Chikuma *et al.* 2005). Data using AQP7-null (an aquaglyceroporin) mice reported that the lack of AQP7 resulted in significantly higher adipocyte hypertrophy, fat mass accumulation and significantly higher triglyceride production and free fatty acid release (Hara-Chikuma *et al.* 2005).

1.4.3. Regulation of Aquaporins

Current data indicates that aquaporin regulation occurs post-translationaly. While there

is some transcriptional regulation, the majority of the regulatory framework of aquaporins occurs by controlling aquaporin location within the cell. For example, in the kidney, AQP2 is responsive to vasopressin exposure via a cAMP/PKA signaling pathway (Noda *et al.* 2005). Following vasopressin binding to its receptor, Gs protein activation occurs, thereby activating AC resulting in increased intracellular cAMP (Noda *et al.* 2005). Cyclic-AMP then activates PKA which in turn stimulates AQP2 translocation from intracellular vesicles to the apical membrane of renal principal cells (Noda *et al.* 2005). In the brain, a similar regulatory scheme to that of kidneys exists. There are three aquaporins expressed in the brain, AQP1, AQP4, AQP9 (Gunnarson *et al.* 2004). AQP1 and AQP4 appear to be regulated by phosphorylation, both in terms of expression and localization (Gunnarson *et al.* 2004). Further, AQP4 activity is regulated by arginine vasopressin (Gunnarson *et al.* 2004). Previous work with cultured astrocytes reports that arginine vasopressin exposure resulted in a significantly higher cell swelling rate compared to control (Sarfaraz *et al.* 1999). Arginine vasopressin regulates AQP4 activity by modulating the cAMP/PKA pathway (Gunnarson *et al.* 2004). Lastly in the brain, AQP1 is regulated by ubiquitination (Leitch *et al.* 2001).

In hepatocytes three aquaporins have been detected, AQP0, AQP8 and AQP9 (Elkjaer *et al.* 2000; Huebert *et al.* 2002; Koyama *et al.* 1997). Current research suggests that AQP0 plays no role in the formation of bile since it remains intracellular (Huebert *et al.* 2002). Further, AQP0 localization is not responsive to cAMP and remains localized to intracellular vesicles (Huebert *et al.* 2002). Similarly, AQP9 localization is also unresponsive to cAMP exposure (Huebert *et al.* 2002). Unlike AQP0, AQP9 is localized to the basolateral membrane and plays a critical role in bile formation (Huebert *et al.* 2002). Neither AQP0 nor AQP9 protein expression is increased by exposure to cAMP (Huebert *et al.* 2002). In contrast, AQP8 protein

expression is increased in response to cAMP exposure (Huebert *et al.* 2002). Further, AQP8, which normally resides in intracellular vesicles, translocates to the canalicular membrane in response to glucagon exposure (Gradilone *et al.* 2003) (Figure 1.4).

1.4.4. Aquaporins in Liver Physiology and Apoptosis

Compliment production, red blood cell removal, blood detoxification and bile production are some of the numerous physiological functions performed by the liver. Bile is critical for fat and vitamin absorption. Bile is approximately 95% water and this requires considerable water transport through the hepatocytes. Bile acid is produced by degradation of cholesterol and occurs *via* two pathways, the classical pathway and the alternate pathway (Chiang 1998). Both pathways are enzymatically catalyzed and these enzymes represent regulatory focal points during bile production (Chiang 1998). These enzymes are regulated by hormones, diurnal rhythms and bile itself (Chiang 1998). Hepatocytes actively transport bile acids into the bile canaliculus (Anwer 2004). Along with the transport of bile acids, water must be passaged as well. In humans, bile production can reach up to 600mL a day, and studies report that passive diffusion would occur too slowly for this production level to be maintained (Ma *et al.* 1999). Therefore, aquaporins are critical for transporting water from the sinusoid into the bile canaliculus *via* the hepatocytes (Ma *et al.* 1999; Marinelli *et al.* 1997) (Figure 1.4).

Apoptosis is a the primary means by which a single cell can be eliminated with minimal damage to surrounding cells. The process of apoptosis is highly regulated and a predetermined sequence of events must occur for progression (Lemasters 2005). Aquaporins play a critical role in apoptotic progression(Jablonski *et al.* 2007; Jablonski *et al.* 2004; Seitz *et al.* 2007). After apoptosis is initiated (either intrinsically or extrinsically), a cellular volume decrease is observed, termed the apoptotic volume decrease (AVD) (Bortner *et al.* 2002). Before the AVD

occurs, the cell depolarizes by inhibiting potassium ion movement into the cell, by an influx of sodium ions into the cell and by an deactivation of the Na^+/K^+-ATPase pump, which is largely responsible for maintaining normal osmotic balance in the cell (Beauvais *et al.* 1995; Bortner *et al.* 1997; Nobel *et al.* 2000). While K^+ movement into the cell is inhibited, K^+ movement out of the cell is not and this reduction of K^+ concentration is critical for apoptotic progression (Bortner *et al.* 2002; Bortner *et al.* 1997). In fact, caspase activation is inhibited by intracellular K^+ concentrations higher than 50mM (Hughes *et al.* 1997). This lowering of K^+ concentration drives water movement *via* aquaporins (Jablonski *et al.* 2004). Once the cell has completed the AVD, then aquaporin activity is inhibited, ion loss ends and apoptotic progression occurs (Jablonski *et al.* 2004). In HCC, previous work reports that if aquaporin mediated water movement is inhibited then apoptosis is also inhibited (Jablonski *et al.* 2007).

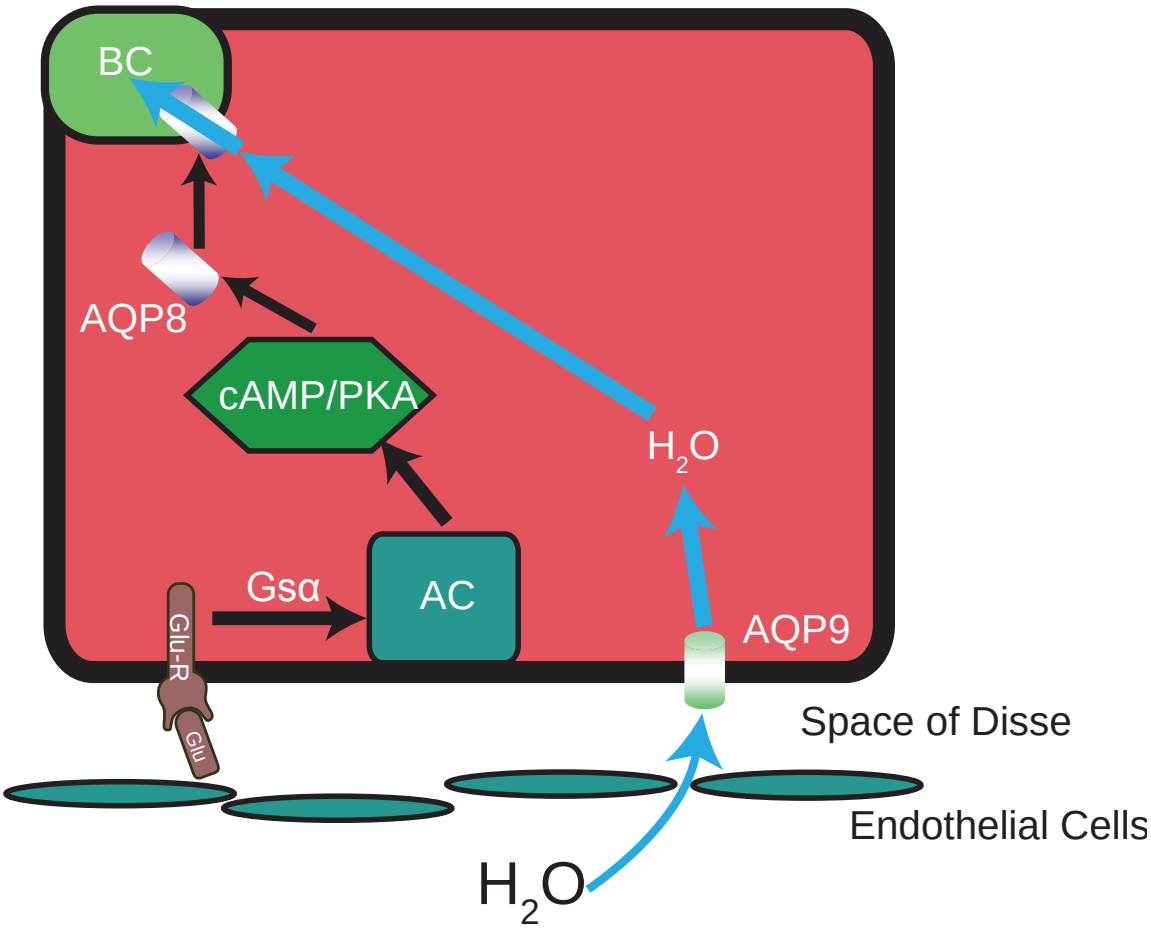


Figure 1.4: AQP8 and 9 regulation and function in normal hepatocytes. In the resting hepatocyte AQP9 is basolaterally expressed and AQP8 is (predominantly) cytoplasmic. Following enteric stimulation (e.g. glucagon; Glu) G stimulatory α ($G_s\alpha$) activates adenylate cyclase (AC) and the resulting increase in cAMP/PKA activity stimulates AQP8 translocation to the canalicular membrane. This effectively creates a conduit from the sinusoid to the bile canaliculus from which water moves along the concentration gradient created by bile salt synthesis and secretion.

Summary

Hepatocellular carcinoma is the 4th leading cause of cancer death. HCC rarely arises spontaneously, with most cases occurring due to chronic viral hepatitis B or C infection, chronic ethanol abuse and/or exposure to aflatoxins. G-proteins are important intracellular signaling pathways by which cells transduce extracellular, ligand mediated signaling and G-protein expression is altered in HCC. One of the primary downstream effects of Gs-protein signaling is the activation of adenylate cyclase resulting in the production of cAMP. Cyclic-AMP is a ubiquitous second messenger which activates PKA by initiating the dissociation of regulatory subunits from PKA resulting in the release of the catalytic subunits.

Aquaporins are a class of proteins which permit the rapid passage of water across the plasma membrane along concentration gradient. The regulatory mechanisms that govern aquaporin expression and localization in the liver remains unclear. Some aquaporins permit passage of small, non-polar solutes, such as glycerol. In hepatocytes, AQP8 is initially localized to intracellular vesicles, while AQP9 is predominantly constitutively expressed in the basolateral membrane. In response to glucagon initiated activation of cAMP/PKA signaling, AQP8 translocates to the canalicular membrane thereby forming a conduit for rapid water movement between sinusoid and bile canaliculis. Glucagon does not affect AQP9 location in normal hepatocytes.

Apoptosis is a highly regulated process by which cells are stimulated to die in such a way as to minimize damage to neighboring cells. Apoptosis is characterized by the apoptotic volume decrease (AVD) simultaneous with ion concentration changes followed by caspase activation, cytochrome c release, DNA fragmentation and finally cell blebbing. In order for the AVD to occur, as well as changes in ion concentration, water must rapidly efflux from the cell to maintain changes in ion concentrations. This efflux is mediated by aquaporins. Previous work in Dr. McKillop's laboratory has reported that AQP8 and AQP9 expression in a rat model of HCC is significantly decreased, and that these tumor cells are resistant to apoptosis. But following *in vitro* culturing AQP8 and AQP9 expression is significantly upregulated and these cells are once again sensitized to apoptotic stimuli. These observations led to an examination of the mechanisms by which this change occurs.

Hypothesis

Changes in aquaporin-8 and aquaporin-9 expression and/or localization are critical in mediating inherent resistance to apoptotic progression in HCC.

Objective 1:

Examine the role of the cAMP/PKA pathway in regulating AQP8 and AQP9 expression, localization and function in HCC cells *in vitro*.

Objective 2:

Examine the role of AQP8 and AQP9 in tumor formation and progression *in vivo*.

CHAPTER 2:GENERAL METHODS

2.1. Assurances

All animals were housed in a vivarium approved by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) and protocols were approved by either the Carolinas Medical Center or University of North Carolina at Charlotte, Institutional Animal Care and Use Committee (IACUC).

2.2. Cell Culture

2.2.1. Materials

Eagle's minimum essential medium (EMEM), Dulbecco's minimum essential medium (DMEM), Hank's F12 medium, phosphate buffered saline (PBS), fetal bovine serum (FBS), fungizone, penicillin/streptomycin (Pen/Strep), sodium bicarbonate (7.5% *w/v*), trypsin-ethylenediaminetetraacetic acid (EDTA; 0.25% *w/v*), and gentamicin sulfate were purchased from Invitrogen (Carlsbad, CA). Trypan blue, dimethylsulphoxide (DMSO), sucrose, sodium chloride (NaCl) and calcium phosphate were purchased from Sigma-Aldrich (St. Louis, MO). 500mL filtering system with 0.22μm filter, 50mL filtering tubes with 0.22μm filter, a "Mr. Frosty" Cryo 1°C freezing container (Nalgene, Rochester, NY), sterile tissue culture flasks (Nunc, Rochester, NY), plates and dishes (BD Falcon, Franklin Lakes, NJ) were purchased from VWR (West Chester, PA). A Bright Line hemocytometer was purchased from Hausser Scientific (Horsham, PA).

2.2.2. Medium Preparation

Medium was prepared in a sterile, laminar flow hood. Medium constituents were combined in the filter container of a 500mL filtering flask and then filtered through a 0.22μm filter. H4IIE complete medium was comprised of: 433mL EMEM, 50mL FBS, 10mL sodium bicarbonate, 5mL Pen/Strep and 2mL fungizone (10% FBS medium--*v/v*). A low serum variant of this medium was also used and comprised the same constituents with the expection of FBS—500μL was used instead of 50mL (0.1% FBS medium--*v/v*). Medium was stored at 4°C, in darkness. When the possibility of cell culture contamination was deemed especially high, gentamicin (1 μL/mL medium) was added. Freezing medium was prepared by combining 55.5% FBS, 33.3% complete medium, 11.1% DMSO (*v/v*) and filtering this solution with a 50mL filtering tube with 0.22μm filter.

2.2.3. Cell Lines

H4IIE, a rat HCC cell line derived from rat hepatomas, was purchased from ATCC (Manassas, VA). H4IIE cells are tumorogenic and P0 stock of these can be replenished by direct injection of these cells into the flanks of ACI rats . The method for this is as follows: $1x10^7$H4IIE cells were injected into the rear flanks of male ACI rats (175-225g), purchased from Harlan Laboratories (Indianapolis, IN). After two weeks, flank tumors were resected and mechanically disrupted (in 10% FBS *v/v*, EMEM medium), and cells cultured in flasks using 10% FBS (*v/v)* H4IIE medium, until 95% confluency. The cells were recovered by trypsinization and frozen in liquid nitrogen as P0 stock (McKillop et al. 1998).

2.2.4. Cell Propagation

Vials of frozen cells were removed from liquid nitrogen storage, thawed rapidly in a 37°C water bath, and the cell suspension transferred to a 15mL tube and centrifuged at 700 *x g* for

5 minutes at room temperature. The supernatant was aspirated and the pelleted cells were suspended in 37°C 10% FBS H4IIE medium (*v/v*). The cell suspension was instilled into a culture flask with 10% FBS (*v/v*) H4IIE medium (37°C).

2.2.5. Cell Passage and Splitting

Monolayer cell cultures were removed from culture dishes using trypsin-EDTA. Briefly, medium was aspirated from the culture vessel, washed once with PBS and an 10mL/175cm^2 of 0.25% trypsin-EDTA (*v/v*) was instilled into the dishes and incubated at 37°C, 5% CO_2 for 5 minutes. The cell suspension was collected into a 15mL tube and centrifuged at 700 *x g* for 5 minutes at room temperature. The supernatant was aspirated and the pellet was suspended in 10% FBS medium (*v/v*). Fresh, sterile culture dishes were prepared by adding an appropriate volume of medium to each dish and an equal amount of the cell suspension per dish. The dishes were then stored in a 37°C, 5% CO_2, 95% air, 100% humidity incubator.

2.2.6. Cell Storage

Cells were detached using trypsin-EDTA method except, following the centrifugation the cell pellet was suspended in a freezing medium solution and aliquoted into cryo vials (1mL/vial). Vials were placed in a Mr. Frosty Cryo 1°C Freezing Container and stored in a -80°C freezer overnight. Cryo vials were removed from the freezing container and stored long-term in liquid nitrogen.

2.3. *In Vivo* Model of Hepatocellular Carcinoma

2.3.1. Materials

Male, ACI/SegHsd (ACI) rats (175-225g) were purchased from Harlan Laboratories (Indianapolis, IN). General animal care (including food, water, housing and veterinary care) was provided by the Department of Comparative Medicine, Cannon Research Center,

Carolinas Medical Center (Charlotte, NC). H4IIE cells were acquired from one of three sources: 1) ATCC (Manasses, VA); 2) Cells, less than passage 3, drawn from frozen stocks; 3) From H4IIE flank tumors initiated in male, ACI rats (175-225g) which were then cultured, expanded and then frozen in liquid nitrogen. Surgeries to inject H4IIE cells and to resect tumors were carried out in operating facilities with supplies (including anesthesia) provided by the Department of Comparative Medicine, Cannon Research Center, Carolinas Medical Center. All animal surgeries were carried out in accordance with approved IACUC protocols.

2.3.2. Tumor Inoculation in ACI Rats

H4IIE cells (≤P3) were cultured in a T175 flask with 10% FBS H4IIE medium (*v/v*) to 90% confluency. Cells were trypsinized by incubating the flasks with 10mL of trypsin-EDTA at 37°C for 3-5 minutes. 5mL of EMEM was added to the flask and the total volume was transferred to a 15mL tube. Cells were centrifuged at 800 *x g* for 5 minutes at 20°C. Supernatant was aspirated and the pellet was suspended in 10mL of 37°C PBS. The cell suspension was centrifuged at 800 *x g* for 5 minutes at 20°C, the supernatant aspirated, and the resulting pellet suspended in 2mL of warm PBS.

Anesthesia was induced using an initial mixture of oxygen (3L/min) and isoflurane (5%--*v/v*) in an air tight Plexiglas box. Once unconscious, the animals were removed from the box and abdominal fur was sheared and scrubbed with betadine. Animals were placed on a heating plate (37°C) and anaesthesia was continually provided *via* nose cone (oxygen = 3L/min, isoflurane = 2.5%--*v/v*). A mid line incision was performed and the liver exposed. Approximately $1x10^7$ cells were injected into the left hepatic lobe and the incision closed using 3-0 vicryl suture. Animals were then returned to a clean cage for recovery. Buprenorphine (0.03mg/Kg) was provided every 12 hours (i.p.) for 48 hours.

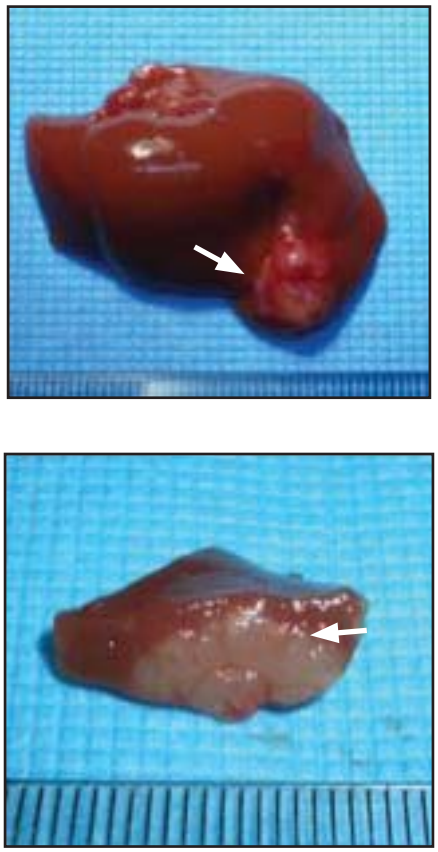


Figure 2.1: Representative images of HCC created by parenchymal H4IIE inoculation. Male, ACI rats (175-225g) were injected with H4IIE cells. Approximately 14 days later, tumors were present in 85% of rats. Top image shows a tumor (white arrow). Bottom image shows a cross-section of the same tumor *in situ* in a resected lobe.

2.3.3. Tumor Resection and Culture

Tumor resection occurred fourteen to sixteen days following surgery (Figure 2.1). Anesthesia was induced with isofluorane and the liver exposed and examined for the presence of a tumor. If no tumor was present the animal was closed and examined again three to five days later. If after this period no gross tumor, the animal was euthanized using Euthasol. If a tumor was present the animal was euthanized by cardiac exsanguination and the liver resected. The tumor was grossly dissected and placed in 0.5% FBS H4IIE medium and homogenized by vortexing, shaking and vigorous pipetting. The tumor homogenate was collected and centrifuged at 800 *x g* for 4 minutes. The supernatant was aspirated, the pellet of cells was suspended in 0.1% FBS H4IIE medium (*v/v*) and the suspension then evenly aliquoted into appropriate tissue culture vessels.

2.4. Cell Swelling Assay

2.4.1. Materials

Mercury chloride ($HgCl_2$) and DMSO were purchased from Sigma-Aldrich (St. Louis, MO). A model FACSCalibur flow cytometer was purchased from Becton Dickinson (Franklin Lakes, NJ). Falcon FACS tubes were purchased from Becton Dickinson (Franklin Lakes, NJ). EDTA free trypsin and PBS were purchased from Invitrogen (Carlsbad, CA).

2.4.2. Methods

Cell monolayers were harvested by EDTA free trypsin, collected, centrifuged at 700 *x g* for 5 minutes (4°C), the trypsin solution was aspirated and cells suspended in PBS (37°C). This cell suspension was centrifuged at 700 *x g* for 5 minutes at 4°C, the PBS aspirated and cells suspended in minimum medium (37°C).

To measure cell size, a series of analyzes were conducted for each sample: 1) 350μL of

cell suspension was mixed with 150μL of room temperature PBS. 2) 350μL of cell suspension was mixed with 150μL of ultrapure water for 1 minute to adjust the osmolality to 210mOsm. 3) 350μL of cell suspension was mixed with 10mM $HgCl_2$ (in ultrapure water) to yield the indicated final $HgCl_2$ concentration for an indicated time period 4) 350μL of cell suspension was mixed with 10mM $HgCl_2$ for the indicated period of time then ultrapure water was added to make the final volume of 500μL for 1 minute.

2.5. Reverse Transcription-Polymerase Chain Reaction

2.5.1. Materials

RNeasy kits and Shedder kits were purchased from Qiagen (Valencia, CA). Isopropyl alcohol, ethyl alcohol, glycerol, sodium acetate, 100x Tris(hydroxymethyl)aminomethane (Tris)–Ethylenediaminetetraacetic acid (EDTA) solution, ethidium bromide solution and diethyl pyrocarbonate (DEPC) were purchased from Sigma-Aldrich (St. Louis, MO). Model 5402, model 5415 centrifuges and model 5331 thermocycler were purchased from Eppendorf (Hauppauge, NY). Primers against AQP8, 9 and β-actin were purchased from IDT (Coralville, IA) (Table 2.2). Reverse transcription kits and GoTaq Green polymerase chain reaction (PCR) kits were purchased from Promega (Madison, WI). Agarose, power packs, gel boxes and DNA loading dye were purchased from Biorad (Hercules, CA). 10x Tris-Boric Acid-EDTA (TBE) buffer was purchased from USB (Cleveland, OH). 100 b.p. DNA ladder was purchased from New England Biolabs (Ipswich, MA). Glycogen was purchased from Fermentas (Glen Burnie, MD). Digital camera, camera box, transilluminator, and photo acquisition software were purchased from Eastman-Kodak (Rochester, NY).

2.5.2. RNA Isolation

RNA was isolated, as per manufacturer's instructions, using RNeasy® kits. Briefly, cells

Gene	Sense (5'-3')	Antisense (5'-3')	Product Length (bp)
AQP8	TCATTGCTACCTTGGGGAAC	GCTCCTGCTCCTGGACTATG	222
AQP9	CGCCAGGTGCCTTTGTAG	GAAGACCTCAAACCCCCATC	239
β-Actin	GAGCTATGAGCTGCCTGACG	GGATGTCAACGTCACACTTC	150

Table 2.2: Primer sequences against AQP8, 9 and β-actin. Primer sequnces were selected using IDTDNA's Primer Quest software. All primer sequences were BLAST analyzed to ensure gene specificity.

were collected directly using RLT buffer. This solution was then loaded into Shedder® spin columns and the columns centrifuged at 16,000 *x g* for 1 minute. One volume of 70% EtOH (*v/v*, in DEPC water) was added to the flow-through and transferred to an RNeasy® column. Columns were centrifuged at 16,000 *x g* for 15 seconds. Washed three times (16,000 *x g*: 15 seconds, 15 seconds, and 2 minutes) with proprietary buffers provided with the kit. A final spin (16,000 *x g*, 1 minute) was performed for drying purposes with a fresh collection tube. The last spin (16,000 *x g*) was performed with 35-50µL of nuclease free water. Typical yields were between 250-600ng RNA/µL depending on the number of cells collected (e.g. a confluent well of a 6-well plate typically yielded 250-350ng/µL in 35µL.

2.5.3. Reverse Transcription

Reverse transcription was performed, as per manufacturer's instructions using the Promega ImProm-II® RT system. Briefly, 0.5µL of random hexomers and 1µg RNA template were added to a 0.5mL PCR tube and nuclease-free water was added to adjust the final volume up 5µL. This mixture was incubated at 70°C for 5 minutes and the tubes placed on ice while a master mix was prepared. A master mix was made containing the following constituents (per reaction): 4.5µL nuclease free water, 4.0µL ImProm-II 5x reaction buffer, 4.0µL 25mM $MgCl_2$, 1.0µL dNTP mix, 0.5µL RNase inhibitor (10 units) and 1.0µL ImProm-II Reverse Transcriptase. The total master mix volume per reaction was 15µL and the total reaction volume was 20µL. After preparation of the master mix, it was aliquoted to a reaction tube. Tubes were transferred to a thermocycler and the following program was used: 95°C (2:00 minutes), 95°C (0:30 seconds), 50°C (0:30 minutes), 72°C (0:30 seconds)—28 cycles of the 30 seconds steps—72°C (5:00 minutes). Following, 2µL of 3M sodium acetate, 1µL of glycogen and 300µL of isopropyl alcohol was added to each reaction tube. Tubes were incubated at -20°C overnight. Next, tubes were

centrifuged at 16,000 *x g* (10 minutes, 4°C). The supernatant was removed and 500µL of 70% EtOH was added to each tube. The tubes were very briefly vortexed and centrifuged at 16,000 *x g* for 10 minutes at 4°C. The alcohol was removed using a pipette and the tubes were left to air dry for 10 minutes. Finally, 30µL of 1x tris-EDTA was added to each reaction tube and DNA concentration was measured using a NanoDrop.

2.5.4. Polymerase Chain Reaction

A master mix containing (per reaction): 12.5µL Green GoTaq, 1µL forward primer, 1µL reverse primer, and 8.5µL nuclease free water was prepared. A 1% agarose (*w/v*) containing 10µL/100mL of ethidium bromide (EtBr) was prepared. 5µL of DNA loading buffer was added to each tube and mixed by pipetting. 20µL from each tube was then instilled into each well of the agarose gel and the gel was resolved at 90mA until the dye front reached the end of the gel. At this point, the gel was transferred to a transilluminator and a digital image was captured.

2.6. Cell Lysate Preparation

2.6.1. Materials

β-mercaptoethanol and sodium deoxycholate were purchased from Sigma-Aldrich (St. Louis, MO). Cell scrapers were purchased from Becton Dickinson (Franklin Lakes, NJ). Laemmli buffer was purchased from Biorad (Hercules, CA). Tris buffered saline (TBS) was purchased from USB (CLEVELAND, OH). 6, 12 and 24-well plates, D100 dishes, T-75, T-175 and T-225 flasks were all purchased from Becton Dickinson (Franklin Lakes, NJ).

2.6.2. Methods

Laemmli collection buffer was prepared by adding 50µL of β-mercaptoethanol to every 950µL of Laemmli buffer. Cell collection was performed by aspirating medium, washing cells in ice cold PBS, and cell lysis using Laemmli collection buffer. The plates/dishes were then

scraped to ensure maximum protein yield. Samples were boiled for 10 minutes and stored at -80°C prior to analysis.

2.7. Western Blot

2.7.1. Materials

Methanol, glycine, ammonium persulfate (APS), N,N,N`,N`-Tetramethylethylenediamine (TEMED), Tween-20 and sodium dodecyl sulfate (SDS) were purchased from Sigma-Aldrich (St. Louis, MO). Filter paper (Fisher brand, P8 grade) Pierce ECL kit, Pierce X-ray film and a Nutator were purchased from Fisher (Waltham, MA). Nitrocellulose paper was purchased from GE-Amersham (Piscataway, NJ). Tris base, transfer sponges, 30% acrylamide/0.8% bisacrylamide solution (also referred to as "acrylamide mix"), non-fat dry milk and a Mini-Protean Western system for gel running and transfer were purchased from Biorad (Hercules, CA). See-Blue Plus 2 protein ladder standard was purchased from Invitrogen (Carlsbad, CA). A Nanodrop 1000 and a Pierce 660nm protein assay kit, with detergent compatibility reagent (Pierce catalog numbers: 22662, 22663), were purchased from Fisher (Waltham, MA).

2.7.2. Sample Protein Concentration Measurement

Sample protein concentration was measured using a 660nm protein assay purchased from Fisher (Waltham, MA). The assay was performed following the microplate procedure, except that measurements were conducted by taking 2μL from each well and measuring protein concentration using the Nanodrop. Briefly, protein concentration was measured by adding 150μL of assay reagent to each well and then adding 10μL of sample. Samples were mixed by covering the plate and placing it on a orbital shaker set to ≈ 70 RPM for 1 minute. The plate was then incubated at room temperature (25°C) for 5 minutes. Protein concentration of each well was then measured using the Nanodrop. A standard curve was generated per the manufacturer's

instructions.

2.7.3. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis Gels (SDS-PAGE)

Western blot gels were prepared using glass plates and the Mini-Protean 3 multi-casting chamber, were purchased from Biorad (Hercules, CA). 15% resolving gels were prepared as follows (per 50mL): 11.5mL ultrapure H_2O, 25.0mL 30% acrylamide mix, 12.5mL 1.5M Tris (pH 8.8), 0.5mL 10% SDS(*v/v*), 0.5mL 10%APS(*v/v*) and 0.02mL TEMED (Sambrook *et al.* 2001). 5% stacking gels were prepared as follows (per 10mL): 6.8mL ultrapure H_2O, 1.7mL 30% acrylamide mix, 1.25mL 1.0M Tris (pH 6.8), 0.1mL 10% SDS(*v/v*), 0.1mL 10%APS(*v/v*) and 0.01mL TEMED (Sambrook *et al.* 2001). Approximately 10mL of the resolving gel solution and 5mL of the stacking gel solution was used per gel.

2.7.4. Protein Resolution and Transfer

Protein samples and protein ladder were loaded onto the gel. A 50 volt (V) difference was applied until the protein sample had reached the resolving gel and then the voltage was increased to 125V until the die front reached the bottom of the gel. The gel was placed on nitrocellulose membrane between 4 sheets of filter paper (on each side) and sponges. A 300mA current was applied for 70 minutes.

2.7.5. Blocking, Antibody Probe and Developing

Following transfer, membranes were incubated in blocking buffer (5% (*w/v*) non-fat dry milk in 1x TBS), in boxes, at room temperature, on a rocking platform for 1 hour. Membranes were incubated, in bags containing antibody diluted in blocking buffer on a Nutator at 4°C overnight. Membranes were washed three times (10 minutes / wash) in 1x TBS/0.2% Tween-20 (*v/v*) and placed in bags containing a 1:5000 dilution of secondary antibody in blocking buffer, at room temperature, on a Nutator, for 1 hour. Next, the membranes were

washed three times (5 minutes / wash) in 1x TBS/0.2% Tween-20 (*v/v*), followed by three washes (3 minutes / wash) in 1x TBS. Lastly, membranes are developed using ECL following manufacturer's instructions and band intensity was measured by densitometry. Membranes were then stripped and then reprobed with an antibody specific against β-actin (house keeping protein).

2.8. Immunohistochemistry & Immunocytochemistry

2.8.1. Materials

95% and 100% ethanol were purchased from Pharmco Products (Brookfield CT). ABC staining kit and goat normal serum were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Hematoxylin(Mayer's Modified) and 10mM citrate buffer (pH 6.0) was purchased from Poly Scientific (Bay Shore, NY). Eosin was purchased from Surgipath (Richmand, IL). PBS, hydrogen peroxide, ammonium hydroxide, collagen (type I) and Accustain 10% neutral buffered formalin solution was purchased from Sigma-Aldrich (St. Louis, MO). Xylene and glass microscope slides were purchased from Cardinal Health (McGaw Park, IL). Cover glass and Permount were purchased from Fisher (Waltham, MA). A steamer was purchased from Black & Decker (Towson, MD). Glass chamber slides and plastic chamber slides were purchased from Nunc (Rochester, NY). A tissue processor (model ATP1) was purchased from Triangle Biomedical Sciences (Durham, NC).

2.8.2. Tissue Preparation

Hepatic tissues were collected and stored in 10% neutral buffered formalin for at least 24 hours (room temperature). Samples were then transferred to an appropriate tissue mold and are dehydrated by a series of graded ethanol washes and processed into paraffin blocks by a series of xylene washes followed by paraffin (these steps were automatically performed by a

tissue processor machine). The resulting embedded tissue block was cut into 4μm sections and each slice was adhered to a positively charged glass slides.

2.8.3. Immunohistochemical / Immunocytochemical Analysis

To prepare tissue samples for analysis, slides were incubated at 60°C for 20 minutes. Next slides were deparaffanized and rehydrated by three washes in xylene (3 minutes / wash), followed by two washes in 100% EtOH (3 minutes / wash), followed by two washes in 95% EtOH (3 minutes / wash), followed by one wash 70% EtOH (3 minutes), lastly, slides are placed in distilled water until the slides run clear.

A Coplin jar was filled with 10mM citrate buffer (pH 6.0) and the slides were placed in the jar. Next the Coplin jar was placed in the steamer for 40 minutes. Following, the jar was removed from the steamer and allowed to cool to room temperature for 20 minutes. Next, the slides were rinsed in two changes of deionized H_2O and one wash in 1x PBS.

Slides were incubated in 0.5% hydrogen peroxide (diluted in PBS) in a humidified chamber for 7 minutes. Then washed in 1x PBS (x2, 5 minutes / wash). Then slides processed per manufacturer's instructions provided with ABC staining kit (Cat# sc-2018. Note: all subsequent steps were carried out in a humidified histology chamber). Briefly, slides were blocked in 1.5% goat serum for 1 hour. Then slides were incubated in primary antibody, diluted in blocking serum overnight at 4°C. Next, the slides were incubated in biotinylated secondary antibody for 30 minutes, followed by 3, 5 minute washes in 1x PBS. Then, slides were incubated for 30 minutes in an a diluted enzyme solution (i.e. the AB enzyme), followed by 3, 5 minute washes in 1x PBS. Next, slides were incubated in 3 drops of peroxidase substrate until desired staining was achieved. Lastly, slides were counter stained with Gill's formulation #2 hematoxylin for 5 seconds, washed in several changes of deionized H_2O, followed by mounting of a glass coverslip.

2.8.4. Slide Scoring

3 representative shots were blind scored by at least 3 independent scorers. The mean of the three scores was used as the value for analysis.

2.9. Antibodies

Polyclonal AQP8 and AQP9 antibodies were generously provided by Dr. Francis Hughes Jr. (McConnell *et al.* 2002). Monoclonal AQP8 and AQP9 antibodies, β-actin monoclonal antibody, normal goat serum, anti-rabbit biotinylated secondary antibody and anti-goat HRP conjugated secondary antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

2.10. Densitometry & Statistics

All densitometry was performed using ImageJ (Rasband 1997-2009). All statistics were calculated and charts created using SAS JMP8.0.2 (Cary, NC). $p < 0.05$ was considered significant.

CHAPTER 3: EFFECT OF CYCLIC-AMP OR INTERLEUKIN-6 ON AQUAPORIN 8 AND 9 EXPRESSION AND LOCALIZATION IN HEPATOCELLULAR CARCINOMA *IN VITRO.*

3.1. Introduction

Aquaporins are a conserved class of proteins which play a central role in water movement across cell membranes (Gonen *et al.* 2006). Further, certain aquaporins permit the movement of neutral solutes (Gonen *et al.* 2006). In the liver, there are currently three identified aquaporins, Aquaporin 0 (AQP0), Aquaporin 8 (AQP8), and Aquaporin 9 (AQP9). AQP0 is intracellular and there have been no reports of stimuli which initiate AQP0 translocation in the liver (Masyuk *et al.* 2006). AQP 8 is the most abundant aquaporin in hepatocytes and like AQP0 is primarily intracellular (Masyuk *et al.* 2006). However in response to glucagon, in a cyclic-adenosine monophosphate (cAMP) dependent manner, AQP8 expression increases and translocation occurs from intracellular vesicles to the bile canalicular membrane (Garcia *et al.* 2001; Tani *et al.* 2001). In contrast, AQP9 is predominately found on the basolateral membrane of normal hepatocytes but currently no published study has reported AQP9 expression or localization to be glucagon/cAMP dependent (Masyuk *et al.* 2006). Work from the McKillop laboratory has reported that AQP8 and 9 expression drops to barely detectable levels in freshly isolated HCC tumors but is expressed strongly twenty-four hours after culture (Jablonski *et al.* 2007). Further, freshly isolated tumors, which were cultured and exposed to transforming growth factor β (a pro-apoptotic agent) immediatly after *in vitor* culture showed similar levels of

caspase 3 activation to serum starved cells (Jablonski *et al.* 2007). Though twenty-four hours after tumors were cultured, these cells had significantly higher caspase 3 activation in response to TGFβ (Jablonski *et al.* 2007). Our article presented no data on the mechanism by which AQP8 and 9 expression was decreased in HCC. Given the partial mechanism presented for normal hepatocytes cAMP represents a reasonable choice.

Cyclic-adenosine monophosphate is a ubiquitous, intracellular, second-messager molecule which plays a critical role in the intracellular transduction of extracellular signals following activation of membrane recptors (Pavio *et al.* 2005; Rall *et al.* 1958; Zhang *et al.* 2005). Following production by adenylate cyclase, cAMP binds to the regulatory subunits of PKA, causing these regulatory subunits to dissociate, and the activation of PKA (Cho-Chung *et al.* 2002). Activated PKA is a transcription factor which binds to the cAMP response element (CRE) thereby activating cAMP dependent gene transcription (Cho-Chung *et al.* 2002). It has been reported that HCC is characterized by reduced adenylate cyclase activity (Schmidt *et al.* 1997). Cyclic-AMP has also been reported to act as an anti-mitotic agent in HCC (Kovach *et al.* 2006; Schmidt *et al.* 1999). While a partial mechanism for the role of cAMP in AQP8 and 9 expression and localization has been demonstrated in normal hepatocytes (Garcia *et al.* 2001), it is currently unknown whether cAMP plays the same role in HCC. These studies seek to determine the role of cAMP on AQP8 and 9 expression and localization *in vitro*.

Interleukin-6 (IL-6) is a 21-28kDa cytokine which is involved in liver regeneration (Koniaris *et al.* 2003), plays a role it T-cell differentiation(Diehl *et al.* 2002), T-cell growth (Houssiau *et al.* 1992), is mitogenic in some cells (Culig *et al.* 2005) and growth inhibitory in others (Moran *et al.* 2005). Interleukin-6 signal activation is initiated by the formation of a binary complex of IL-6 and the nonsignaling IL-6α receptor (IL-6Rα) (Boulanger *et al.* 2003)

(Figure 3.1). This complex interacts with a gp130 homodimer to form a competent signaling complex (Boulanger *et al.* 2003). The complete signaling complex causes the activation of Janus kinase (JAK) and gp130. Inactive JAK constitutively binds to gp130 (Akira 1999). JAK is then activated which in turn, most commonly, activates signal transducer and activator of transcription 3(STAT3) which then forms a homodimer STAT3 (Akira 1999). This STAT3-STAT3 dimer is a competent transcription factor and translocates to the nucleus where it binds to, among other sites, CRE sites on DNA. In the liver, previous work has reported a significant role for IL-6 in liver regeneration and hepatocellular carcinoma progression (Moran *et al.* 2005; Zimmers *et al.* 2003; Fausto 2000). Previous work in our lab has reported that IL-6 inhibits H4IIE cell growth (Moran *et al.* 2005).

Along with the cAMP response element binding protein (CREB) sites on AQP8, the promoter regions of AQP8 and 9 contain many other promoter binding sites (Figure 3.2). Of particular interest are CCAAT/enhancer binding protein (C/EBP) sites, AP-1 sites on AQP9 and NF-κB sites. C/EBP both regulates and is responsive to cAMP (Wilson *et al.* 2002). Also, downstream activation of the IL-6 pathway, particularly STAT3, is known to activate genes with C/EBP sites (Alonzi *et al.* 2001; Cantwell *et al.* 1998).

Because of the role IL-6 plays in hepatocyte proliferation during liver regeneration and that IL-6 inhibits growth in this model of HCC, and because of the presence of IL-6 activated promoter regions on both AQP8 and 9 promoters, I examined whether IL-6 causes AQP8 or 9 expression or location changes in H4IIE cells *in vitro*. Also, I examined the functional role of IL-6 on water movement.

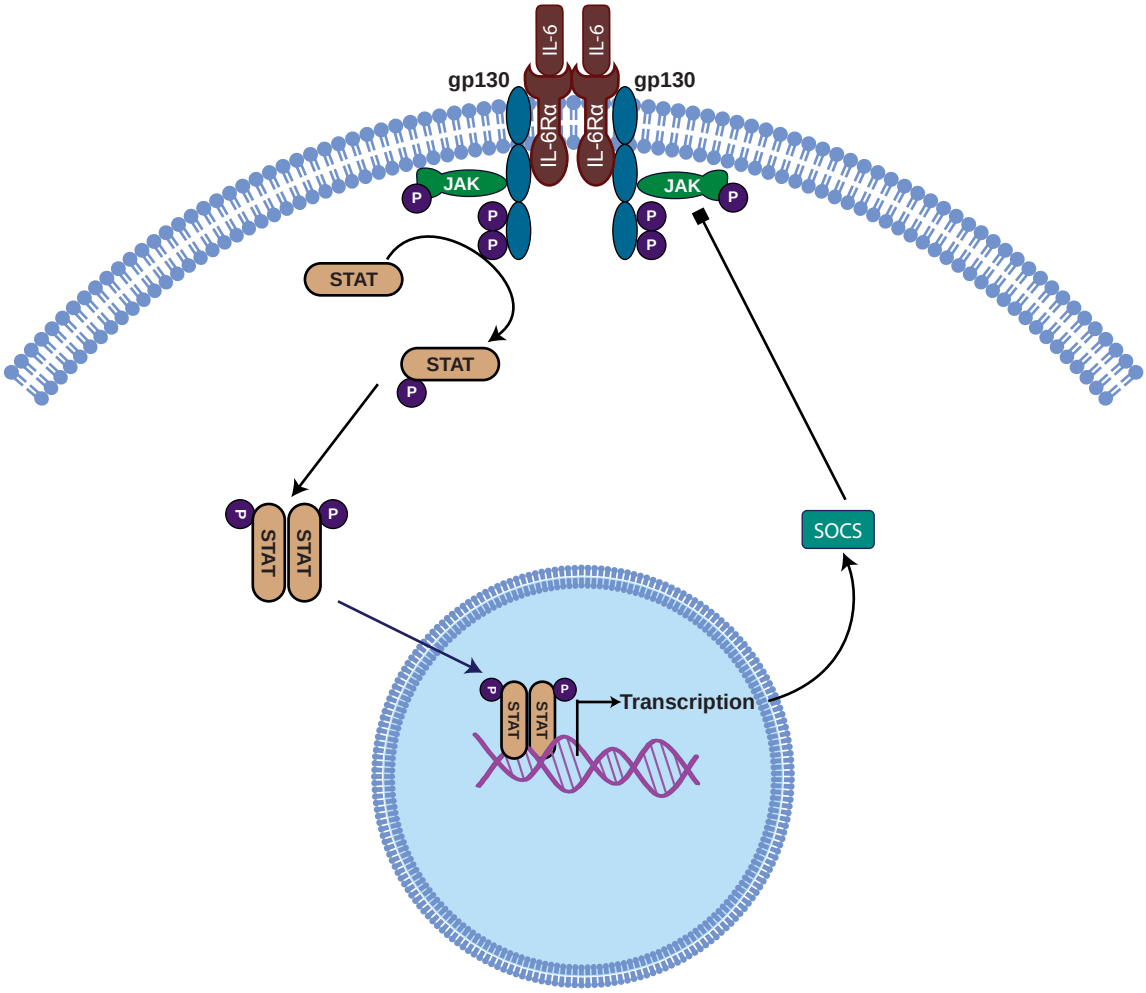


Figure 3.1: IL-6 JAK-STAT Pathway. Binding of IL-6 to IL-6Rα recuits gp130 forming a competent signaling complex. Janus kinase (JAK) is then recruited and phosphorylated which phosphorylates gp130. Activated gp130 recruits and activates STAT which then is phosphorylated (p-STAT). p-STAT forming a dimer that translocates to the nucleus. Amongst the genes stimulated by p-STAT is supressor of cytokine signaling (SOCS). SOCS inhibits IL-6 signaling by binding to JAK and the phosphorylated tyrosines on gp130 to prevent further signal propagation. (Carbia-Nagashima *et al.* 2004).

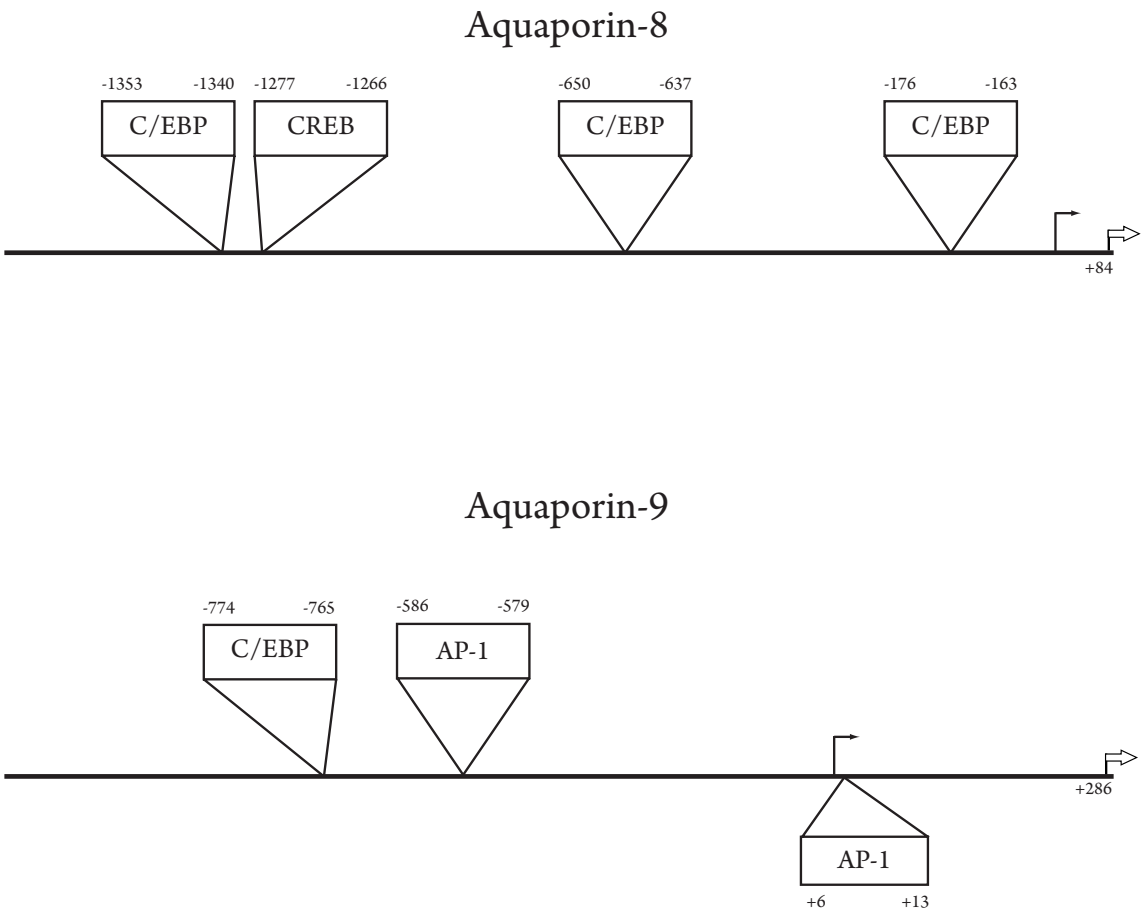


Figure 3.2: Partial promoter map for AQP8 and 9. Promoter analysis indicates a number of transcription factor binding sequences. The numbers indicate the distance (in base pairs) from the start site (black arrow). The hollow arrow indicates the direction of transcription. The promoters of interest in this study are shown.

3.2. Materials & Methods

3.2.1. Cell Line

H4IIE cells were used throughout this chapter and were stored and cultured as described in Chapter 2.2.4.

3.2.2. Protein & Pharmacological Agents

8-Bromoadenosine 3′,5′-cyclic monophosphate (8-BrcAMP) and cis-N-(2-Phenylcyclopentyl)-azacyclotridec-1-en-2-amine hydrochloride (MDL) were purchased from Sigma-Aldrich (St. Louis, MO). N-[2-((p-Bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide, 2HCl (H89) was purchased from Calbiochem-EMD (Gibbstown, NJ). Recombinant rat interleukin-6 (rrIL-6) was purchased from Invitrogen (Carlsbad, CA).

3.2.3. Sample Generation for Western blot and RT-PCR analyses

H4IIE cells were grown in 6-well plates, using 10% FBS (*v/v*) medium until ~90% confluence. Medium was then aspirated and replaced with 0.1% FBS (*v/v*) medium for 24 hours. At this point, the medium was aspirated and replaced with 0.1% FBS (*v/v*) medium alone or medium plus 10μM 8-BrcAMP, 10μM H89 or 10μM MDL for 1 hour. After the hour, 10μM 8-BrcAMP was added to the medium containing H89 or MDL.

Experiments involving IL-6 were performed similarily to cAMP experiments. H4IIE cells were grown in 10% FBS (*v/v*) medium until ~90% confluence. Medium was then aspirated and replaced with 0.1% FBS (*v/v*) medium containing IL-6 (concentration range: 0ng/mL — 100ng/mL).

For Western blot analysis, cells were collected in Laemmli buffer with 5% β-mercaptoethanol (*v/v*), boiled for 10 minutes and stored at -80°C. For RT-PCR, cells were collected in Qiagen stock RLT buffer and stored at -80°C (Chapter 2.5.2).

3.2.4. Membrane/Cytoplasm Sample Preparation

3.2.4.1. Materials

Beckman-Coulter Ultracentrifuge TL and 1.5mL ultracentrifuge tubes were purchased from Beckman Coulter (Brea, CA). Complete protease inhibitor (CPI) tablets were purchased from Roche (Basel, Switzerland). Tris(hydroxymethyl)aminomethane (Tris), Ethylenediaminetetraacetic acid (EDTA) and β-mercaptoethanol were purchased from Sigma-Aldrich (St. Louis, MO). Phosphate buffered saline (PBS) was purchased from Invitrogen (Carlsbad, CA). Laemmli buffer was purchased from Biorad (Hercules, CA). 20x Tris buffered saline (TBS) was purchased from USB (Cleveland, OH).

3.2.4.2. Method

Stock membrane buffer consisted of 50mM Tris and 1mM EDTA. One tablet of CPI was dissolved in 10mL of membrane buffer stock immediately before cell collection (referred to as "membrane buffer"). Cells were propagated and treated in D100 dishes. Medium was aspirated and cells were washed with ice cold PBS. 400μL of membrane buffer was added to each dish and cells were scraped off of the dish and collected. Samples were stored at -80°C for at least 24 hours to lyse the cells. Next, samples were thawed in ice, vortexed for at least 1 minute, and spun at 90,000 *x g* for 1 hour at 4°C. The supernatant was collected as the cytoplasmic fraction and the pellet was suspended in 100μL of Laemmli buffer (with 5% β-mercaptoethanol (*v/v*)) and collected as the membrane fraction.

3.2.5. Western Blot

Western blots were performed as described in Chapter 2.7. AQP8 and AQP9 antibodies were provided by Dr. Francis Hughes. β-actin antibodies and goat secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit secondary antibody was

purchased from Jackson ImmunoResearch (West Grove, PA). Aquaporin antibodies were diluted 1:1000 in 5% non-fat dry milk (NFDM) diluted in 1x TBS. β-actin antibodies were diluted 1:500 in 5% NFDM (diluted in 1x TBS). All secondary antibodies were diluted 1:5000 in 5% NFDM (diluted in 1x TBS).

3.2.6. Immunohistochemistry & Immunocytochemistry

Immunohistochemistry and immunocytochemistry were performed as described in Chapter 2.8.

3.2.7. Statistical Analysis

Group wide stastistical analysis was performed using a one-way, Type I ANOVA model (both single variable data and pair-match data). Pair-wise comparisons were made using Student's t-Test or Tukey-Kramer HSD analysis. All analyses were performed with SAS JMP 8.0.2 (Cary, NC). $p < 0.05$ was considered significant.

3.3. Results

3.3.1. Analysis of cAMP/PKA Signaling in Cultured H4IIE Cells.

3.3.1.1. Cyclic-AMP/PKA signaling does not significantly affect AQP8 or 9 mRNA or protein expression in vitro.

The mRNA and protein message expression was measured in H4IIE cells. Analysis by RT-PCR, using primers specific for AQP8, showed no siginificant difference in response to cAMP/PKA pathway modulation (Figure 3.3a: $p = 0.168$). Similarly, using primers specific for AQP9, no significant differences were detected (Figure 3.3a). Western blot analysis, using antibodies specific for AQP8, demonstrated no significant difference in response to cAMP/PKA pathway modulation (Figure 3.3b). Using antibodies specific for AQP9 also demonstrated no significant difference in response to cAMP/PKA pathway modulation (Figure 3.3b).

3.3.1.2. Cyclic-AMP/PKA signaling does not significantly affect AQP8 or 9 localization.

Aquaporin 8 and 9 localization was measured by isolation of total membrane and cytoplasmic fractions from cultured H4IIE cells. Western blot analysis was performed using membrane or cytoplasmic fractions. While ANOVA analysis of AQP9 membrane localization data did indicate a significant difference overall due to cAMP pathway modulation, Tukey-Kramer HSD analysis of pair-wise combination indicated no siginificant difference between any given pair of cAMP/PKA pathway modulators (Figure 3.4a). Analysis of AQP8 localization to the plasma membrane was not significant (Figure 3.4a). There was no significant difference in AQP8/9 localization to the cytoplasmic compartment in response to cAMP pathway modulation (Figure 3.4b).

3.3.2. Analysis of cAMP/PKA Signaling in Freshly Isolated Tumors.

3.3.2.1. AQP8 and 9 protein expression is unaffected by 8-Br-cAMP in ex vivo H4IIE cells.

Male, ACI rats (175-225g) were inoculated with H4IIE cells (Chapter 2.3). Tumors were then resected and cultured immediately in the presence of cAMP/PKA modulating agents for 48 hours. The medium was replaced with identically treated medium at 24 hours. Western blot analysis indicated no siginificant difference in AQP8 expression (Figure 3.5a) or in AQP9 expression (Figure 3.5b).

3.3.3. Analysis of AQP8 and 9 Responses to IL-6 Signaling in Cultured H4IIE Cells.

3.3.3.1. Recombinate Interleukin-6 has no significant affect on AQP8 or 9 mRNA or protein expression.

Cultured H4IIE cells were exposed to five concentrations of rrIL-6 (0ng/mL – 100ng/mL) for twenty-four hours and then collected. RT-PCR analysis for AQP8 demonstrated no significant difference in response to rrIL-6 (Figure 3.6a). Probing for AQP9 also indicated no significant difference in mRNA expression, in response to rrIL-6 (Figure 3.6a). Also, Western

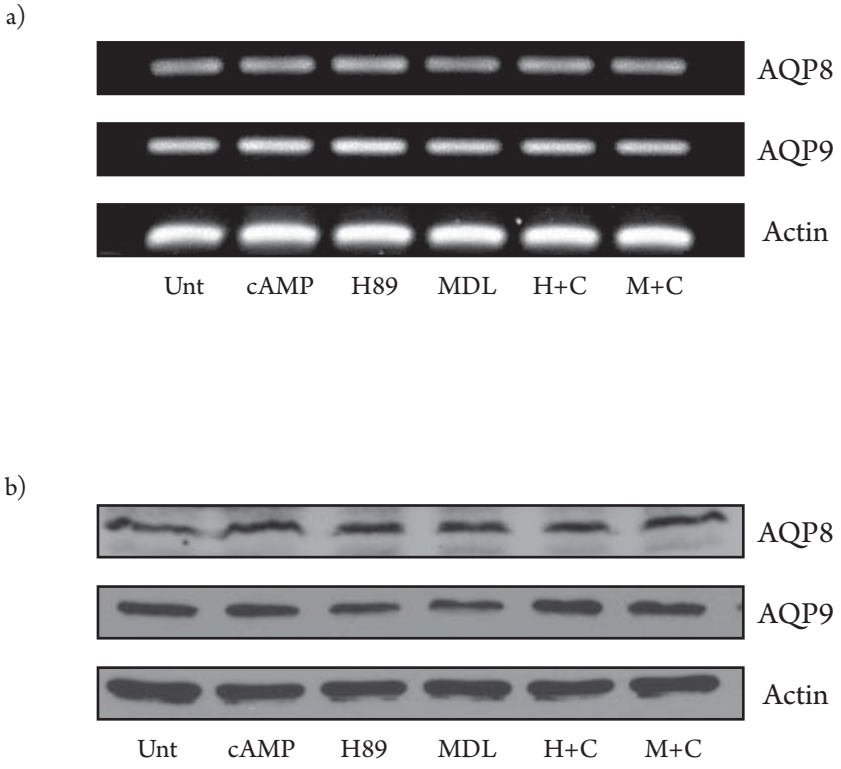


Figure 3.3: Effect of cAMP pathway modulation on AQP8/9 mRNA and protein expression. Cultured H4IIE cells were exposed to a cell permeable cAMP analogue (8-Bromo-cAMP [cAMP]; 10μM), a protein kinase-A (PKA) inhibitor (H89; 10μM) and an adenylate cyclase (AC) inhibitor (MDL; 10μM), 10μM H89 pretreatment (1hr) followed by 10μM cAMP (H+C) or 10μM MDL pretreatment (1hr) followed by 10μM cAMP (M+C). a) Representative gels from RT-PCR performed on total RNA isolated from H4IIE cells exposed to cAMP +/- pathway modulators and probed with primers against AQP8 or AQP9. b) Representative Western blot analyses preformed using whole cell lysates from H4IIE cells exposed to cAMP +/- pathway modulators and probed with antibodies against AQP8 or AQP9. β-actin expression was used as an indicator of equal RNA/protein loading and used to normalized AQP8/9 expression densitometry.

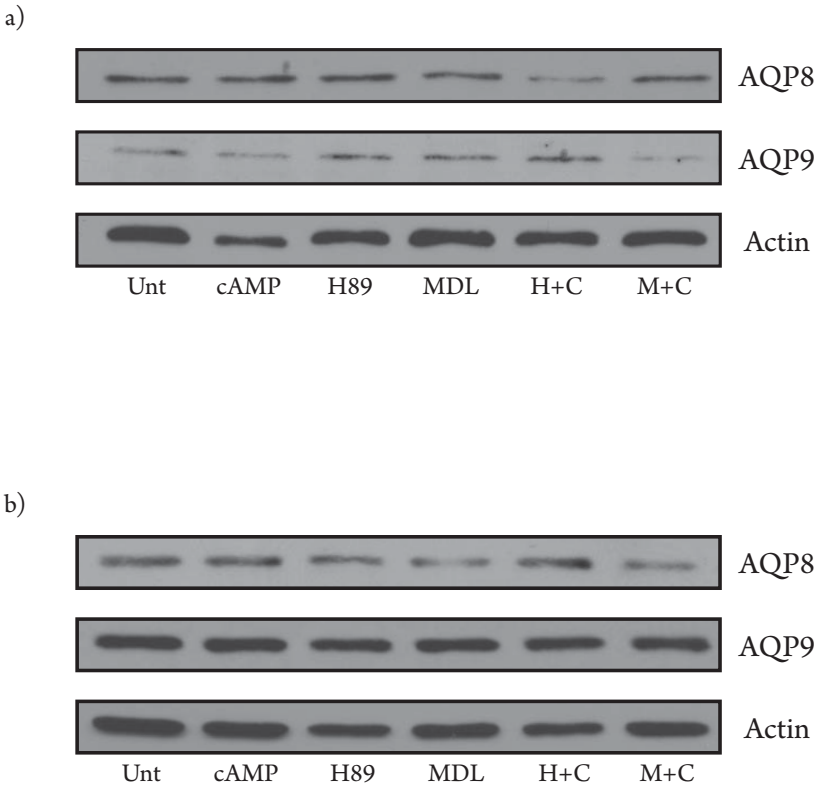


Figure 3.4: Effect of cAMP pathway modulation on AQP8/9 expression in membrane and cytoplasmic fractions. Cultured H4IIE cells were exposed to a cell permeable cAMP analogue (8-Bromo-cAMP [cAMP]; 10μM), a protein kinase-A (PKA) inhibitor (H89; 10μM) and an adenylate cyclase (AC) inhibitor (MDL; 10μM), 10μM H89 pretreatment (1hr) followed by 10μM cAMP (H+C) or 10μM MDL pretreatment (1hr) followed by 10μM cAMP (M+C). a) Representative Western blot analyses preformed using membrane fractions from H4IIE cells exposed to cAMP +/- pathway modulators and probed with antibodies against AQP8 or AQP9. Pair-wise densitometric analysis indicates no significant difference for either AQP8 or 9. b) Representative Western blot analyses preformed using cytoplasmic fractions from H4IIE cells exposed to cAMP +/- pathway modulators and probed with antibodies against AQP8 or AQP9. Pair-wise densitometric analysis indicates no significant difference for either AQP8 or 9. β-actin expression was used as an indicator of equal RNA/protein loading and used to normalized AQP8/9 expression densitometry.

blot analysis indicated no siginificant difference in AQP8 expression in response to rrIL-6 (Figure 3.6b). Lastly, there was also no significant difference in AQP9 protein expression in response to rrIL-6 (Figure 3.6b).

3.3.3.2. Recombinate Interleukin-6 significantly affects AQP8 localization to the plasma membrane.

Membrane preparations of H4IIE cells exposed to five different concentrations of rrIL-6 (0ng/mL – 100ng/mL), over twenty-four hours, resulted in significant decrease in localization of AQP8 to the plasma membrane(Figure 3.7a). Further, analysis of the data using Dunnett's method (0ng/mL rrIL-6 was used as control) showed a significant difference between 0ng/mL rrIL-6 and 10ng/mL rrIL-6 ($p = 0.038$). However, Western blot analysis demonstrated no significant difference in AQP9 membrane localization (Figure 3.7a). Also, Western blot analysis of cytoplasmic fractions from rrIL-6 treated, H4IIE cells indicated no significant difference AQP8 localization to the cytoplasm (Figure 3.7b) or in AQP9 localization to the cytoplasm (Figure 3.7b).

3.3.3.3. Functional Analysis of cultured H4IIE cells in Response to cAMP/PKA agents and rrIL-6.

3.3.3.4. cAMP pathway modulation affects cell swelling in cultured H4IIE cells.

Cultured H4IIE cells were exposed to cAMP pathway modulators for 24 hours and were then collected by trypsinization, then a cell swelling assay was performed. Analysis by paired-data ANOVA showed significant differences between cells in an isotonic environment and cells under osmotic stress (Figure 3.8: $p < 0.0001$). Further, analysis of the differences between isotonic and osmotic challenge conditions, across treatment groups, showed a significant difference within groups (Figure 3.8: $p = 0.02$). Lastly, analysis of H4IIE cells exposed to osmotic challenge showed a significant difference in cell swelling depending on the

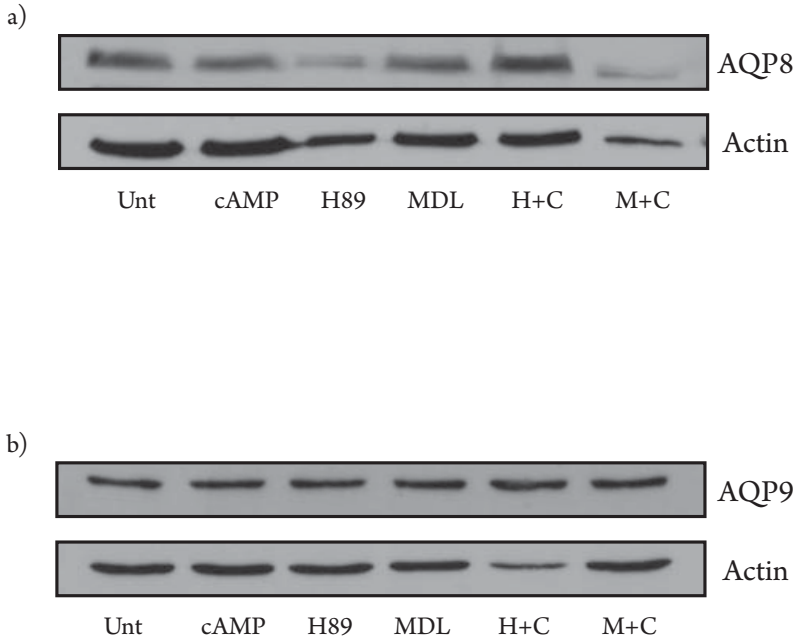


Figure 3.5: Effect of cAMP pathway modulation on AQP8 and 9 expression in freshly isolated tumor cells. Freshly isolated H4IIE tumors were exposed to a cell permeable cAMP analogue (8-Bromo-cAMP [cAMP]; 10μM), a protein kinase-A (PKA) inhibitor (H89; 10μM) and an adenylate cyclase (AC) inhibitor (MDL; 10μM), 10μM H89 pretreatment (1hr) followed by 10μM cAMP (H+C) or 10μM MDL pretreatment (1hr) followed by 10μM cAMP (M+C). Western blot analysis was performed on whole cell lysates using antibodies against, a) AQP8 or b) AQP9. β-actin expression was used as an indicator of equal RNA/protein loading and used to normalized AQP8/9 expression densitometry.

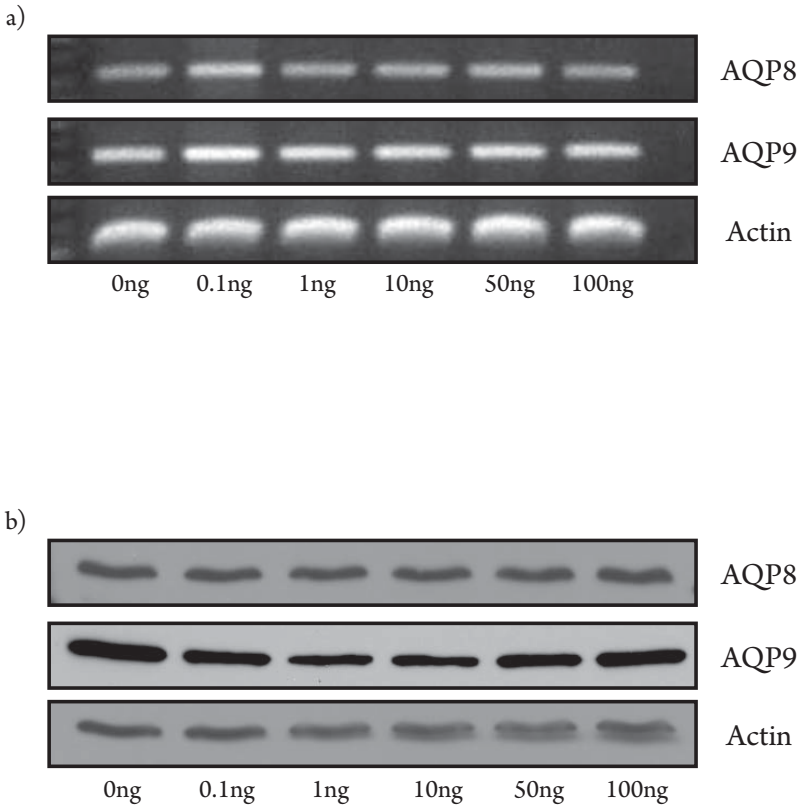


Figure 3.6: Effect of IL-6 on AQP8/9 mRNA and protein expression in H4IIE cells. Cultured H4IIE cells were exposed to recombinant rat IL-6 (rrIL-6; 0.1 -100ng/mL) for 24 hours. a) Representative gels from RT-PCR performed on total RNA isolated from H4IIE cells exposed to rrIL-6 and probed with primers against AQP8 or AQP9. b) Representative Western blot analyses preformed using whole cell lysates from H4IIE cells exposed to rrIL-6 and probed with antibodies against AQP8 or AQP9. β-actin expression was used as an indicator of equal RNA/protein loading and used to normalized AQP8/9 expression densitometry.

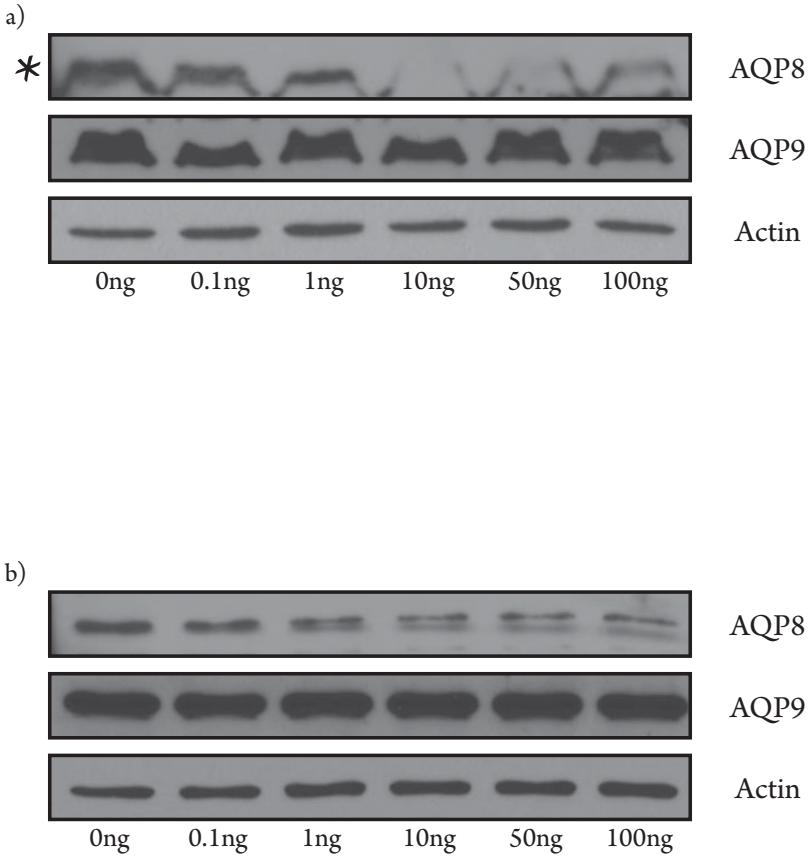


Figure 3.7: Effect of IL-6 signaling on AQP8/9 membrane and cytoplasmic localization. Cultured H4IIE cells were exposed to recombinant rat IL-6 (rrIL-6; 0.1-100ng/mL) for 24 hours. a) Representative Western blot analyses performed using membrane fractions from H4IIE cells exposed rrIL-6 and probed with antibodies against AQP8 or AQP9. Densitometric analysis indicated a significant difference in AQP8 membrane localization in response to rrIL-6. b) Representative Western blot analyses performed using cytoplasmic fractions from H4IIE cells exposed to rrIL-6 and probed with antibodies against AQP8 or AQP9. β-actin expression was used to normalize AQP8/9 expression densitometry.

pharmacological agent used (Figure 3.8: $p = 0.004$).

3.3.3.5. rrIL-6 does not affect cell swelling in cultured H4IIE cells.

H4IIE cells were exposed to rrIL-6 and a cell swelling assay was performed. Analysis by paired-data ANOVA showed significant differences between cells in an isotonic environment and cells under osmotic stress (Figure 3.9: $p < 0.0001$). However, analysis of the differences between isotonic and osmotic conditions, across treatment groups, showed no significant difference (Figure 3.9).

3.4. Discussion

These studies demonstrate that cAMP/PKA pathway modulation significantly affects water movement. However, neither cAMP nor IL-6 significantly affected membrane or cytoplasmic localization of AQP8 or 9. Taken together the data from these studies paint a picture in stark contrast to the role of cAMP, AQP8 and 9 in normal hepatocytes.

cAMP is a ubiquitous second messenger conserved across a broad array of organisms (Cooper 2003). Previous work by other investigators demonstrate that AQP8 localization is regulated by glucagon signaling and that the mechanism for this effect is partially cAMP dependent in normal rat hepatocytes (Garcia *et al.* 2001; Gradilone *et al.* 2003). Cyclic-AMP has also been reported to play a role in proliferation of human HCC cell lines (Schmidt *et al.* 1999). The aim of these studies was to examine the role of cAMP in the expression and localization of AQP8 and 9 in a rat tumorogenic cell line, H4IIE. Previous work by other investigators report that AQP8 has increased expression and translocates to the canalicular membrane in response to 8-Bromo-cAMP exposure, in normal rat hepatocytes (Huebert *et al.* 2002; Soria *et al.* 2009). In contrast, the current studies show no significant role for cAMP in the expression of AQP8/9 mRNA or protein in H4IIE cells. Previous work showed no

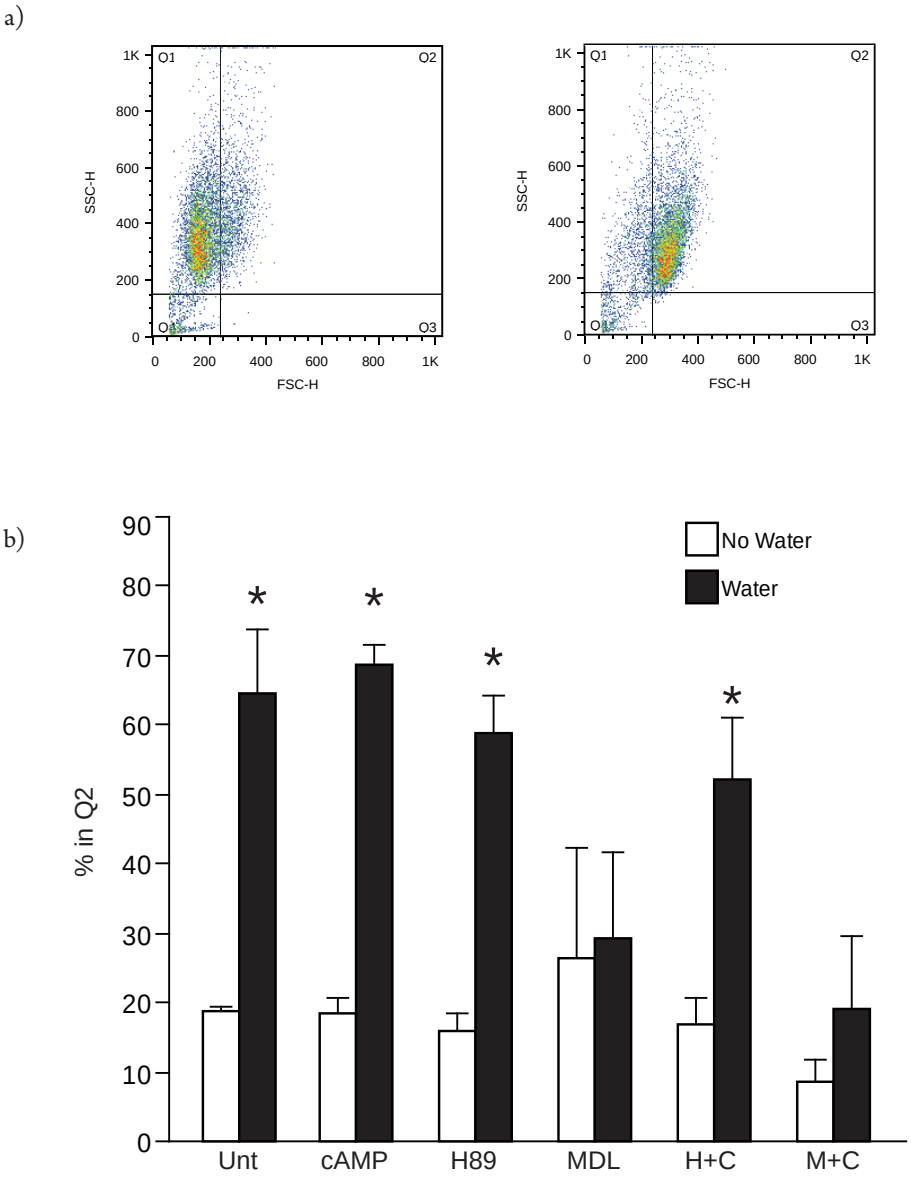


Figure 3.8: Effect of cAMP pathway modulation on cultured H4IIE cell responsiveness to osmotic challenge. Cultured H4IIE cells were exposed to a cell permeable cAMP analogue (8-Bromo-cAMP [cAMP]; 10μM), a protein kinase-A (PKA) inhibitor (H89; 10μM) and an adenylate cyclase (AC) inhibitor (MDL; 10μM), 10μM H89 pretreatment (1hr) followed by 10μM cAMP (H+C) or 10μM MDL pretreatment (1hr) followed by 10μM cAMP (M+C). Cells were processed for flow cytometry to analyze changes in cell size following osmotic challenge. a) Representative dot plots showing the gating strategy for H4IIE cells (left) and H4IIE cells under osmotic challenge (right). b) Mean percentage of total cells in quadrant 2 (Q2) in the presence (dark bars) or absence (light bars) of osmotic challenge. Bars represent 3 independent experiments. * $p < 0.05$ compared to no osmotic challenge, n = 3 independent experiments.

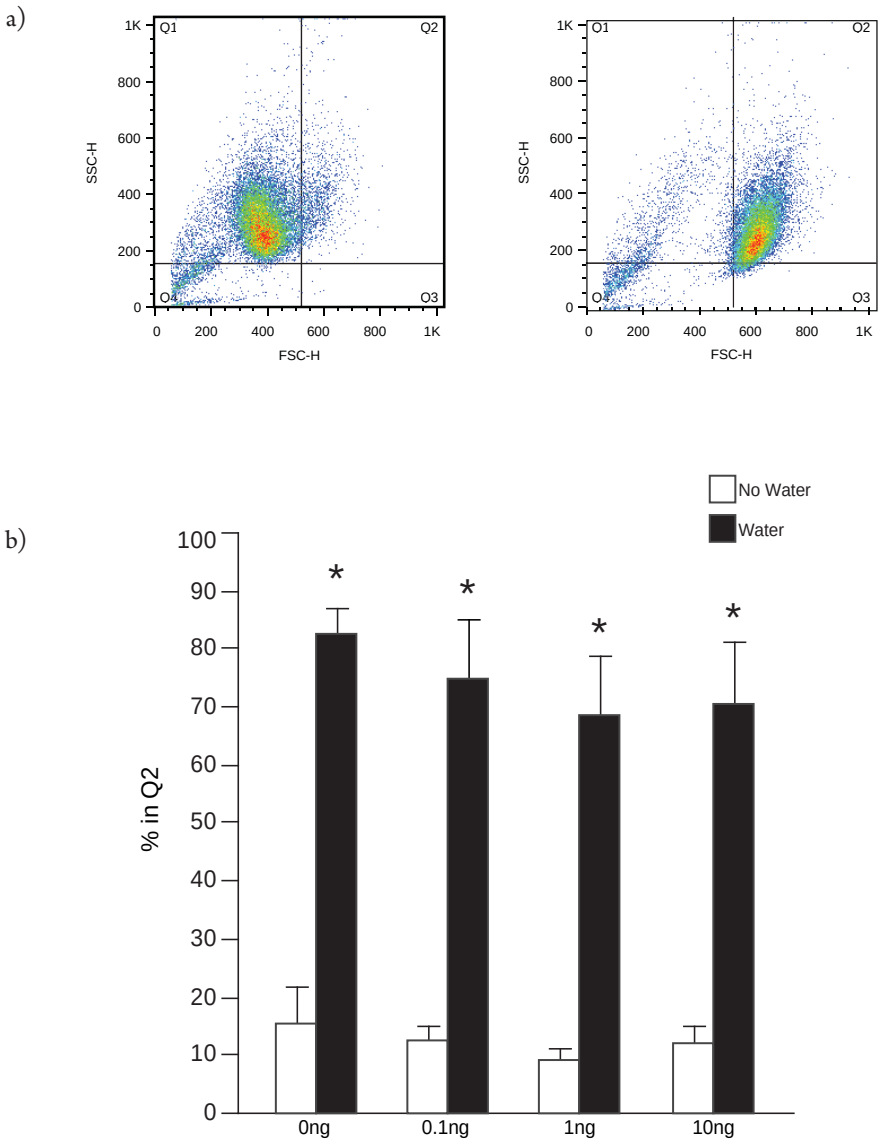


Figure 3.9: Effect of IL-6 signaling on H4IIE responsiveness to osmotic challenge. Cultured H4IIE cells were exposed to recombinant rat IL-6 (rrIL-6; 0.1-100ng/mL) for 24 hours. a) Representative dot plots showing the gating strategy for H4IIE cells (left) and H4IIE cells under osmotic challenge (right). b) Mean percentage of total cells in quadrant 2 (Q2) in the presence (dark bars) or absence (light bars) of osmotic challenge. Bars represent 3 independent experiments. * $p < 0.05$, n = 3 independent experiments.

significant effect on AQP9 protein expression or on localization (Huebert *et al.* 2002; Soria *et al.* 2009). Previous work by our lab has reported that AQP8 and 9 expression is significantly increased in resected tumor cells 24 hours post resection (Jablonski *et al.* 2007). In the current studies, cAMP pathway modulation was unable to alter this effect and by 48 hours post tumor culture AQP8 and 9 protein expression was at basal levels.

A possible explaination for these discrepancies is the role of nonspecific effects of pharmacological agents. A main criticism of small molecule protein inhibitors is that they tend to have off target effects. For example, H89 at concentrations higher than 25μM can affect myosin light chain, protein kinase C and Rho-associated coiled-coil containing protein kinase 2 (Davies *et al.* 2000). The role, if any, that these off target effects have cannot be discounted out of hand as they do pose a challenge to any case built on the usage of small molecule inhibitors but the case against them is not entirely clear. The rise of molecular gene modification techniques was, in part, a direct response to the issues that the off target effects of chemical inibitors presented. However, the advent of embryonic knockout animals, RNAi, locked nucleic acids, morpholino based technology and *Cre-Lox* conditional knockout animals presented its own set of issues. An important one comes in the form of a question: If a given a gene is so important why does the animal/cell not die when you knock it out? Part of the answer comes from the observation that a functionally (and usually structurally) related protein can compensate for a non-functional protein. In fact, the data in these studies may be presenting a case of just such an occurance. It may be that the AQP8 pathway (that is all of the proteins that go into translocating AQP8 from the cytoplasm to the membrane) is deficient at some point(s) and because of the functional, structural and promoter similarities between AQP8 and 9, AQP9 is able to compensate. Though this compensation would not appear to

be cAMP dependent. However, while protein compensation has become scientific dogma, no sufficient answer as been put forth in the literature resolving the pharmacological versus molecular question. Pharmacological agents will always have off target effects but that does not make them redundant. In fact, H89 is a very specific and potent inhibitor of PKA at low concentration. Genes can be knocked out and the cell/animal will survive but the gene is still of critical importance.

IL-6 plays a critical role in many processes, from liver regeneration (Koniaris *et al.* 2003) to promotion of Th2 T helper cells (Diehl *et al.* 2002). Interleukin-6 has also been reported to arrest HCC cell proliferation *in vitro* (Moran *et al.* 2007). The current studies examined the role that IL-6 plays in the expression and localization of AQP8 and 9. Results from these studies indicate that IL-6 has no significant affect on AQP8 or 9 mRNA or protein expression. Though in contrast to the cAMP data, where 8-BrcAMP significantly affect AQP9 localization to the plasma membrane, IL-6 significantly affected AQP8 localization to the plasma membrane. This could add another marker to further enhance diagnosis of HCC in humans. Indeed, previous studies by other groups have reported strong IL-6 cytoplasmic and membrane reactivity in 40% of HCC patients (Soresi *et al.* 2006).

Endoplasmic reticulum (ER) stress provides a potential explaination for the negative findings in these studies. First, a recent article has suggested that AQP8 may be regulated not by directly increasing its production, but by decreasing AQP8 degradation (Soria *et al.* 2009). But a known phenomena of aquaporin production is the high rate of protein misfolding, which, for example, is reported as high as 48% for AQP1 (Lu *et al.* 2000). This leads to a very interesting possibility, namely that the design of the experiment maybe backwards. It has been reported that ER stress actually drives IL-6 expression in aortic endothelial cells (Gargalovic *et al.* 2006).

If this observation is true in hepatocytes then it may be the case that instead of IL-6 driving AQP8/9 expression, stimulation of AQP8/9 expression may drive IL-6 production due to the increased ER stress.

Aquaporins play a critical role in apoptosis in that water efflux from the cell is critical for apoptotic progression (Jablonski *et al.* 2007; Jablonski *et al.* 2004). Further, previous work has reported that in normal hepatocytes cAMP exposure significantly increased water movement (Huebert *et al.* 2002). The current studies were conducted to see the functional effect of 8-Br-cAMP or IL-6 exposure on cultured H4IIE cells. H4IIE cells exposed to osmotic stress in the context of cAMP pathway modulation swelled significantly different depending on the pharmacological agent used. In contrast, IL-6 had no significant affect on cell swelling. The fact that 8-Br-cAMP affects swelling while IL-6 does not makes logical sense in that feeding animals will have increased glucagon and increased glucagon results in the increased expression of AQP8 by a cAMP associated mechanism (Soria *et al.* 2009).

The water permeability through an aquaporin is almost 100 times higher compared to diffusion (Preston *et al.* 1993). The question would be with such a high permeability is it possible to detect an increase in swelling even if cAMP or IL-6 were increasing the expression levels of AQP8/9? I would speculate that the answer to that question is no. There are physical limits at work here. First, only a certain number of water molecules can pass through an aquaporin at any one time and they must pass through single file (Wang *et al.* 2007). Also, AQP8 and 9 are open channels, there is no gating domain, therefore their activity is determined based on their location. Therefore, the difference in osmolality across the membrane is the determining factor for water flow rate, not the number of aquaporins. So even if cAMP or IL-6 is increasing AQP8/9 expression differences in flow rate would not be detected because the

flow rate would naturally increase or decrease based on the difference in water concentration. However there is undoubtedly much that is unknown about aquaporins. It has recently been reported that plant aquaporins are gated (Wang *et al.* 2007). If this is later discovered to be true in mammalian aquaporin isoforms, then total aquaporin expression, along with the gating status of those aquaporins, could play a large role it total water movement.

Future studies are required to address a number of questions. In vivo AQP8 expression and localization is partially regulated through cAMP signaling (Soria *et al.* 2009). And yet in this study AQP8 expression and localization was no affected by cAMP signaling. One possible explanation is the absence of the 3 dimensional (3D) environment in the in vitro data presented. Indeed, previous work as reported that a 3D environment has significant impact on the efficacy of anti-cancer drugs (Weigelt *et al.* 2009). With this in mind it would interesting to repeat these experiments in a 3D cell culture model to examine the effects on AQP8 and 9 expression and localization in the context of a synthetic extracellular matrix.

In summary, cAMP pathway modulation significantly affects AQP9 localization to the plasma membrane while IL-6 exposure significantly affects AQP8 localization to the plasma membrane. Though neither cAMP pathway modulation nor IL-6 exposure significantly affects AQP8 or 9 protein or mRNA expression. Cyclic-AMP pathway modulation significantly affects cell swelling while IL-6 exposure does not. Further research will be required to tease out why AQP8 is sensitive to cAMP in normal hepatocytes but not in the H4IIE rat model of HCC. It is tempting to speculate that the genetic instability that is concomitant with cancer (Hanahan *et al.* 2000) is resulting in cellular compensation for the loss of function of AQP8. Also, typically cancer cells evolve mechanisms to evade apoptosis (Hanahan *et al.* 2000) but in this case the ability to move relatively large volumes of water quickly remains intact so these cell posses the

machinery to reach the critical concentrations of potassium to activate caspases. Further, work is required to determine exactly how these cells inhibit apoptotic progression *in vivo*.

CHAPTER 4:AQUAPORIN 8 AND 9 LOCALIZATION IN A MOUSE MODEL OF HEPATOCELLULAR CARCINOMA

4.1. Introduction

In normal hepatocytes, AQP8 is expressed and translocated to the canalicular membrane in response to glucagon *via* cyclic-adenosine monophosphate (cAMP) signaling (Garcia *et al.* 2001; Gradilone *et al.* 2003). Previous work by the McKillop laboratory, demonstrated that aquaporin 8 (AQP8) and 9 (AQP9) expression are reduced to near undetectable level, in vivo, in an inoculation rat model of hepatocellular carcinoma (HCC) (Jablonski *et al.* 2007). However, 24 hours after tumors are cultured, AQP8 and 9 expression increased markedly compared to freshly isolated tumors (Jablonski *et al.* 2007). One thing these studies did not address is the time frame during which aquaporin expression changes. The rat model used in those studies is a cell inoculation model which only permits studying mid to late stage tumor progression. Human HCC is a progressive disease having multiple risk factors, the most common of which being cirrhosis (Leong *et al.* 2005). HCC has a symptom profile that remains subclinical with patients often presenting with advanced HCC (Clark *et al.* 2005). A cell inoculation model would be inappropriate to model these phenomena as early progression of the disease is missed entirely, and examination of disease progression through time is not possible. Therefore, a different model will need to be employed to study the change in aquaporin expression from foci formation to mature tumor.

The diethylnitrosamine (DEN) mouse model is a model that is suited to studying HCC

from pre-foci formation through mature late stage tumors. Unlike the cell inoculation rat model used previously, which involves the injection of MHC matched cells into a rat, the DEN mouse model most commonly involves a single of injection of DEN in young mice, with foci formation at approximately six months and mature tumor formation at approximately one year. One aim of the current study will be to compare expression and localization of AQP8 and 9 in the inoculation rat model used previously with the DEN mouse model used in this study.

DEN is a known carcinogen in mice and rats (Clapp *et al.* 1967; Laib *et al.* 1982; Vesselinovitch *et al.* 1984). A single intraperitoneal (i.p.) injection of DEN in one day old mice is sufficient to induce foci formation within 8 weeks (Moore *et al.* 1981). In animals injected at 15 days old, the first foci are detected at 10 weeks (Vesselinovitch *et al.* 1983). Further, at exposures of 0.625μg/g, a single i.p. injection of DEN is sufficient to induce HCC in treated mice in 2 years (Vesselinovitch *et al.* 1983). In mice treated with 5μg/g, all had HCC in under a year (Vesselinovitch *et al.* 1983). Our current understanding is that there are multiple mechanisms underlying DEN tumorgenecity. Previous data has shown polysomies, specifically chromosomes 11 and 19, in C3H mice (Danielsen *et al.* 1991). Additionally, previous work by others has shown mutations in the c-Ha-ras gene as early as 11 weeks following DEN injection (Bauer-Hofmann *et al.* 1992). Lastly, products from DEN metabolism result in DNA alkylation (Magee *et al.* 1964; Swann *et al.* 1971). Alkylation results in the formation of preclastogenic DNA lesions which are thought to be a primary promoter of HCC development (den Engelse *et al.* 1983; Tates *et al.* 1986). Because the DEN mouse model permits the study of HCC during the entire course of the disease, this makes it a good model for studying AQP8 and 9 expression and localization in a time dependent manner.

An interesting aspect of human HCC, which is also examined in this study, is the role of

gender in HCC. Human HCC prevalence exhibits gender differences. The ratio of men to women who develop HCC is as low as 1.3 in South America to as high as 3.6 in Western Europe (Bosch *et al.* 2005). These are region wide averages and there are localities which have much higher sex ratios. Calvados, France has a HCC incidence sex ratio of 8.8 (McGlynn *et al.* 2005). The mechanism underlying higher male predilection for HCC is poorly understood. Current hypotheses for why there is such a strong male predilection in HCC cover a broad range of aspects from higher male incidences of viral hepatitis infection and cirrhosis, to higher tobacco use in male, to androgens (Leong *et al.* 2005; McGlynn *et al.* 2005). The current study will be examining what role,if any, sex plays in AQP8 and 9 expression and localization during hepatic tumorigenesis.

In summary, previous work by the McKillop laboratory has shown that AQP8 and 9 expression is inhibited in an cell inoculation rat model of HCC compared to cultured tumors. However, this model does not permit examination of pre-foci to mature tumor development. Human males have a higher incidence rate for HCC than females. However, previous chapters of this dissertation have not modeled this phenomena of human HCC. It would logical to use a model of HCC that allows for the examination of HCC development through time and to include gender as a variable in the model. Therefore, a DEN mouse model was selected because it is a progressive mouse model of HCC which is effective in both male and female mice.

4.2. Materials & Methods

4.2.1. Assurances

All animals were housed in a vivarium approved by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) and all methods were approved by either the Carolinas Medical Center Institutional Animal Care and Use Committee (IACUC).

4.2.2. Materials

DEN and olive oil were purchased from Sigma(St. Louis, MO). C3H/HeJ (C3H) mice were purchased from Jacksons Laboratories (Bar Harbor, ME). General animal care (including food, water, housing and veterinary care) and anaesthetic were provided by the Department of Comparative Medicine, Cannon Research Center, Carolinas Medical Center (Charlotte, NC). All surgeries were carried out in the laboratory of Dr. Iain McKillop at Carolinas Medical Center (Charlotte, NC). 70 mice were used in this study. 33 were injected with DEN (initiated), 37 were not injected (control). In the control group, 20 mice were females, 17 were males. In the initiated group, 18 mice were female, 15 were males. Immunohistochemical reagents used are described in chapters 2.8 – 2.9.

4.2.3. Methods

4.2.3.1. Tumor Initiation

C3H mice, both males and females, were anaesthizied by placing mice in a sealed chamber containing gauze soaked in isofluorane. Mice were then injected with DEN (1mg/kg i.p.) and returned to their cage. From the control group, 19 animals were euthanized at 24 weeks and 18 animals were euthanized at 48 weeks. Frome the initiated group, 17 animals were euthanized at 24 weeks and 16 animals were euthanized at 48 weeks.

4.2.3.2. Euthanasia, Tissue Collection and Processing

Mice were euthanized by anesthetizing the mouse followed by exsanguination. After mice were euthanized, the liver was resected and a sample of liver was placed in 10% neutral formalin (stored at +4°C). Tissue was then processed as described in chapter 2.8.2.

4.2.3.3. Immunohistochemistry

Immunohistochemical analysis was performed as described chapter 2.8.3.

4.2.4. Statistical Analysis

Three representative fields were taken of each slide. Images were then blind scored, those values averaged and the average used in further analysis. The model used for the analyzes in this chapter was a full factorial type I ANOVA with three factors. All interactions were included in the model. Pair-wise comparisons were analyzed by Student's t-Test. $p < 0.05$ was considered significant.

4.3. Results

4.3.1. Aquaporin 8 localization is significantly affected in a DEN model of HCC.

HCC was initiated in C3H mice with a single injection of DEN i.p. (1mg/kg). Untreated mice were maintained as controls. Mice from each group were euthanized at 24 weeks and 48 weeks. Livers from these mice were resected, fixed and processed for immunohistochemical staining. Slides were stained with an antibody specific against AQP8. Representative AQP8 stainings from the 24 week control group (Figure 4.2), 24 week DEN initiated group (DEN-24wk) (Figure 4.3), 48 week control group (Figure 4.4) and 48 week DEN initiated group (DEN-48wk) (Figure 4.5) are shown.

Analysis of the scoring data (see for scoring scale) indicated significant AQP8 plasma membrane localiztion differences and these differences were dependent on exposure to DEN and the sex of the mouse or the length of DEN exposure (Figure 4.6a: $p < 0.01$). In the 24 week group mice, AQP8 localization to the plasma membrane was significantly higher in hepatocytes of female mice exposed to DEN than in hepatocytes in female control mice (Figure 4.6a: $p < 0.01$). However, in the 48 week group mice, AQP8 localization to the plasma membrane was significantly lower in female mice initiated with DEN compared to female control mice (Figure 4.6a: $p < 0.05$). Unlike the 24 week group male mice, where there was no significant difference

between male mice initiated with DEN and control, there was a significant difference in AQP8 plasma membrane localization, in male mice, between control male mice and male DEN-48wk mice (Figure 4.6a: $p < 0.01$). Female, DEN-48wk mice had significantly reduced AQP8 plasma membrane localization compared to AQP8 plasma membrane localization in female, DEN-24wk mice (Figure 4.6b: $p < 0.01$). Male, DEN-48wk mice also had significantly reduced AQP8 plasma membrane localization compared to AQP8 membrane localization in male, DEN-24wk mice (Figure 4.6c: $p < 0.01$).

Aquaporin 8 staining also indicated a significant AQP8 cytoplasmic localization differences and these differences were dependent on exposure to DEN and the sex of the mouse or the length of DEN exposure (Figure 4.7a: $p < 0.01$). AQP9 localization to the cytoplasm was significantly higher in normal hepatocytes of female mice relative to plasma membrane localization in both the 24 and 48 week group mice (Figure 4.7a: $p < 0.01$). In the 48 week group mice, AQP8 was significantly higher in the cytoplasm of female and male exposed to DEN relative to plasma membrane localization of AQP9 (Figure 4.7a: $p < 0.05$). While AQP8 localization to the cytoplasm was not significantly higher between female DEN-24wk mice and male DEN-24wk mice, there was significantly higher AQP8 cytoplasmic localization in male DEN-48wk mice compared to female DEN-48wk (Figure 4.7a: $p < 0.05$). Female DEN-48wk mice exhibited significantly increased AQP8 localization to the cytoplasm compared to female DEN-24wk mice (Figure 4.7b: $p < 0.01$). Male DEN-48wk mice exhibited significantly increased AQP8 localization to the cytoplasm compared to male DEN-24wk mice (Figure 4.7c: $p < 0.01$).

4.3.2. Aquaporin 9 localization is significantly affected in a DEN model of HCC.

HCC was initiated in C3H mice with a single injection of DEN (1mg/kg i.p.). Untreated

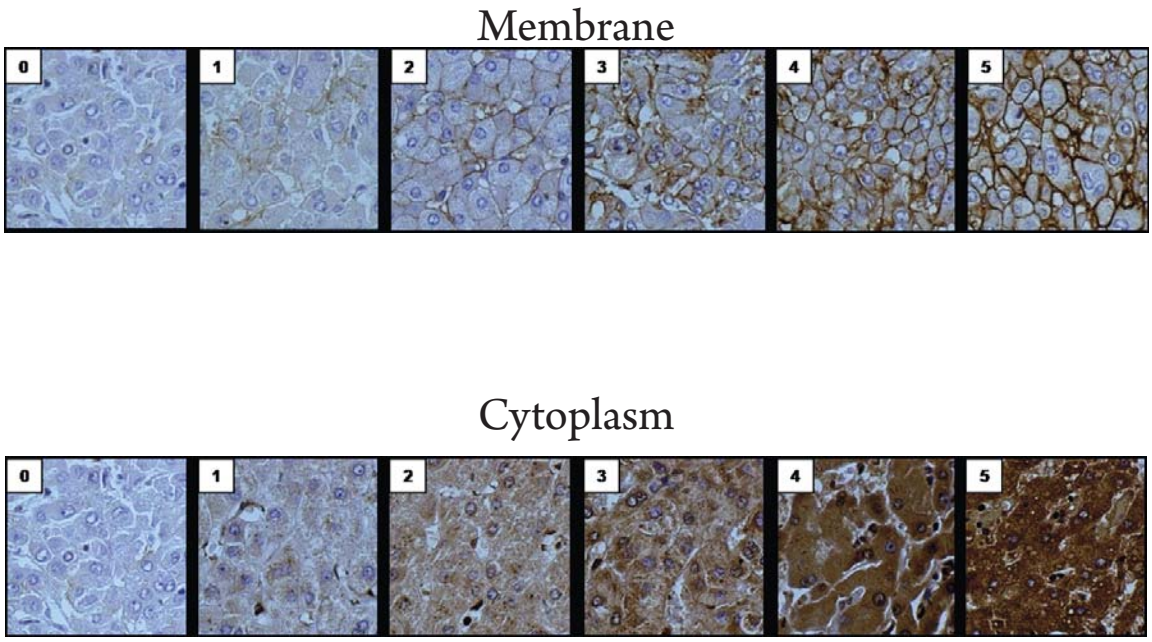


Figure 4.1: Immunohistochemical Scoring Scale. A reference scoring guide (0-5) for membrane and cytoplasmic aquaporin expression was created from human HCC tissue. This scale was used as a subsequent reference point for future immunohistochemical analysis.

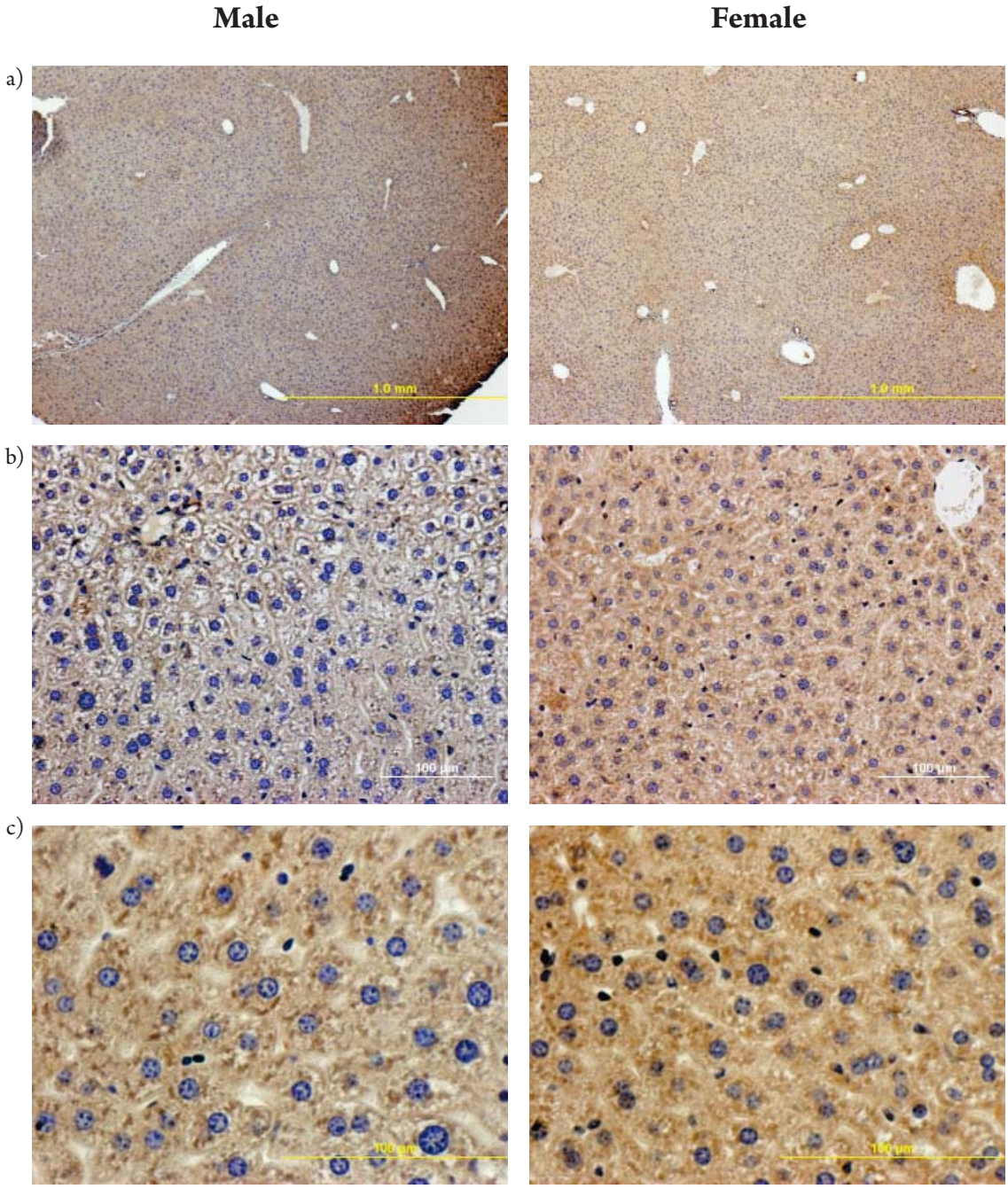


Figure 4.2: Immunohistochemical staining of liver sections for AQP8 from male and female mice in the absence of DEN. Representative immunohistochemical staining of liver sections from male and female C3H mice in the absence of DEN exposure (24 weeks). Slides were stained with antibodies against AQP8 (brown). Images taken at a) 4x, b) 20x and c) 40x magnification.

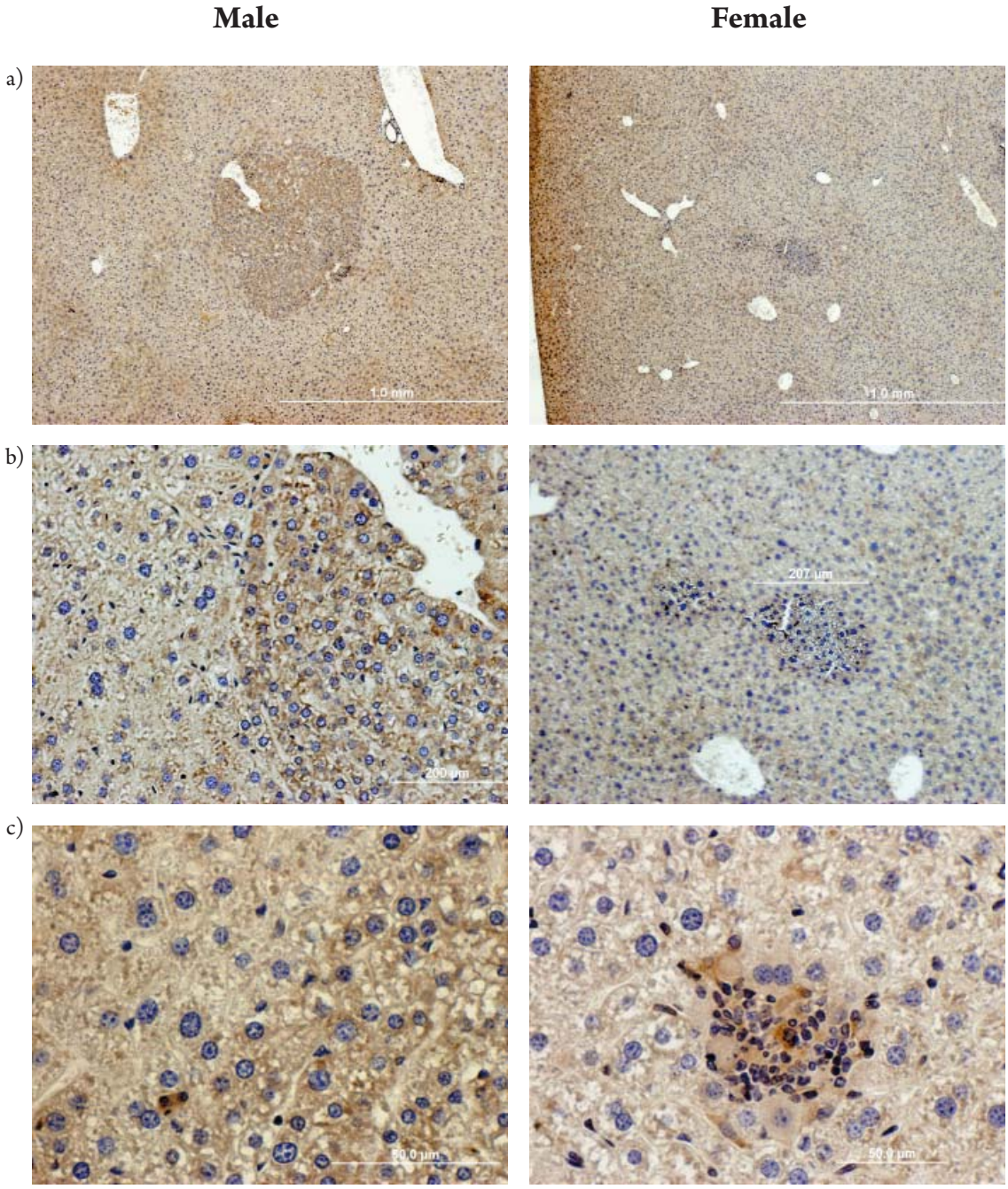


Figure 4.3: Immunohistochemical staning of liver sections for AQP8 from male and female mice exposed to DEN for 24 weeks. Representative immunohistochemical staining of liver sections from male and female mice injected with DEN (1mg/kg--ip) 24 weeks prior to euthanasia. Slides were stained with antibodies against AQP8 (brown). Images taken at a) 4x, b) 20x and c) 40x magnification.

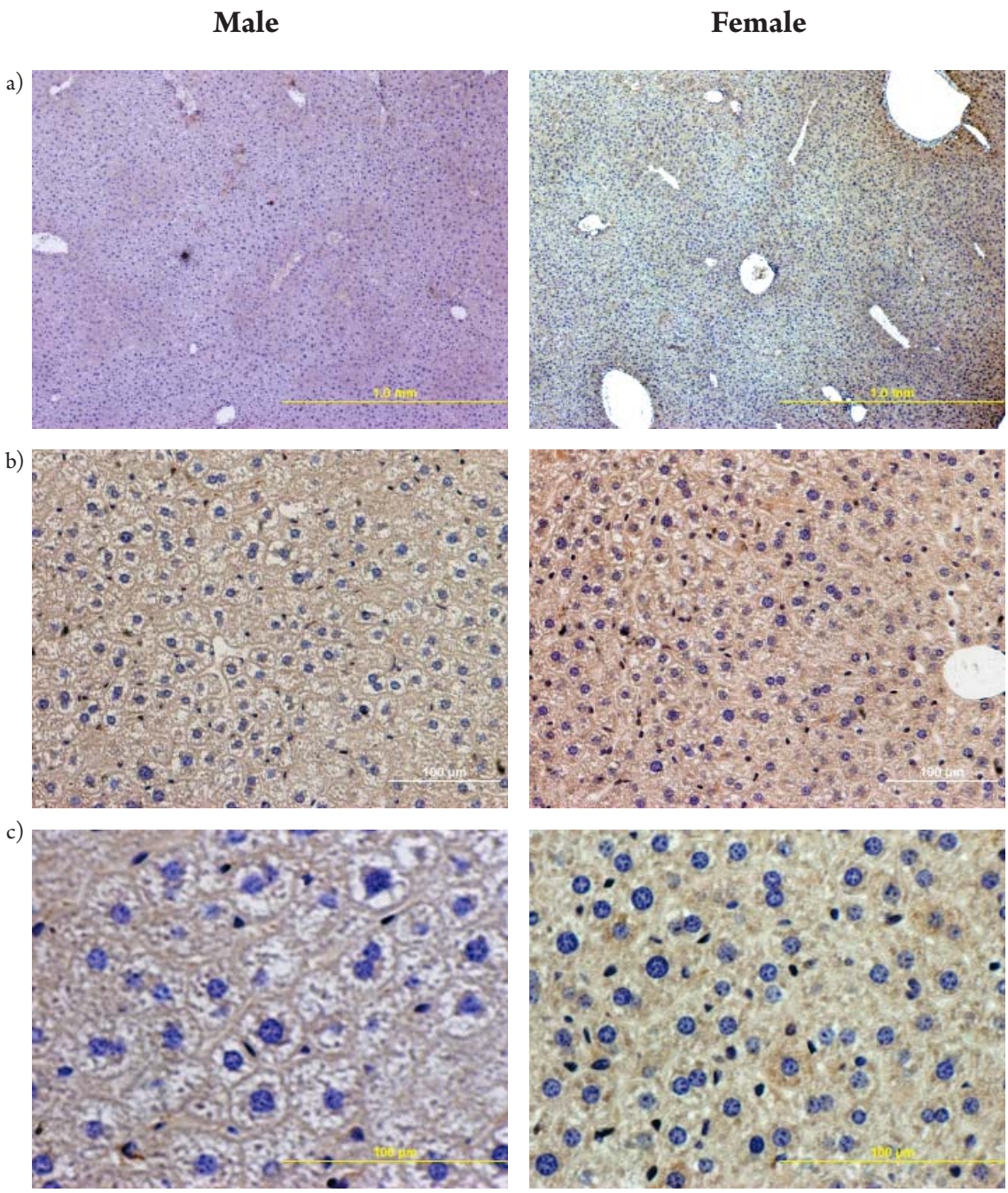


Figure 4.4: Immunohistochemical staining of liver sections for AQP8 from male and female C3H mice in the absence of DEN. Representative immunohistochemical staining of liver sections from male and female C3H mice in the absence of DEN exposure (48 weeks). Slides were stained with an antibody againist AQP8 (brown). Images taken at a) 4x, b) 20x and c) 40x magnification.

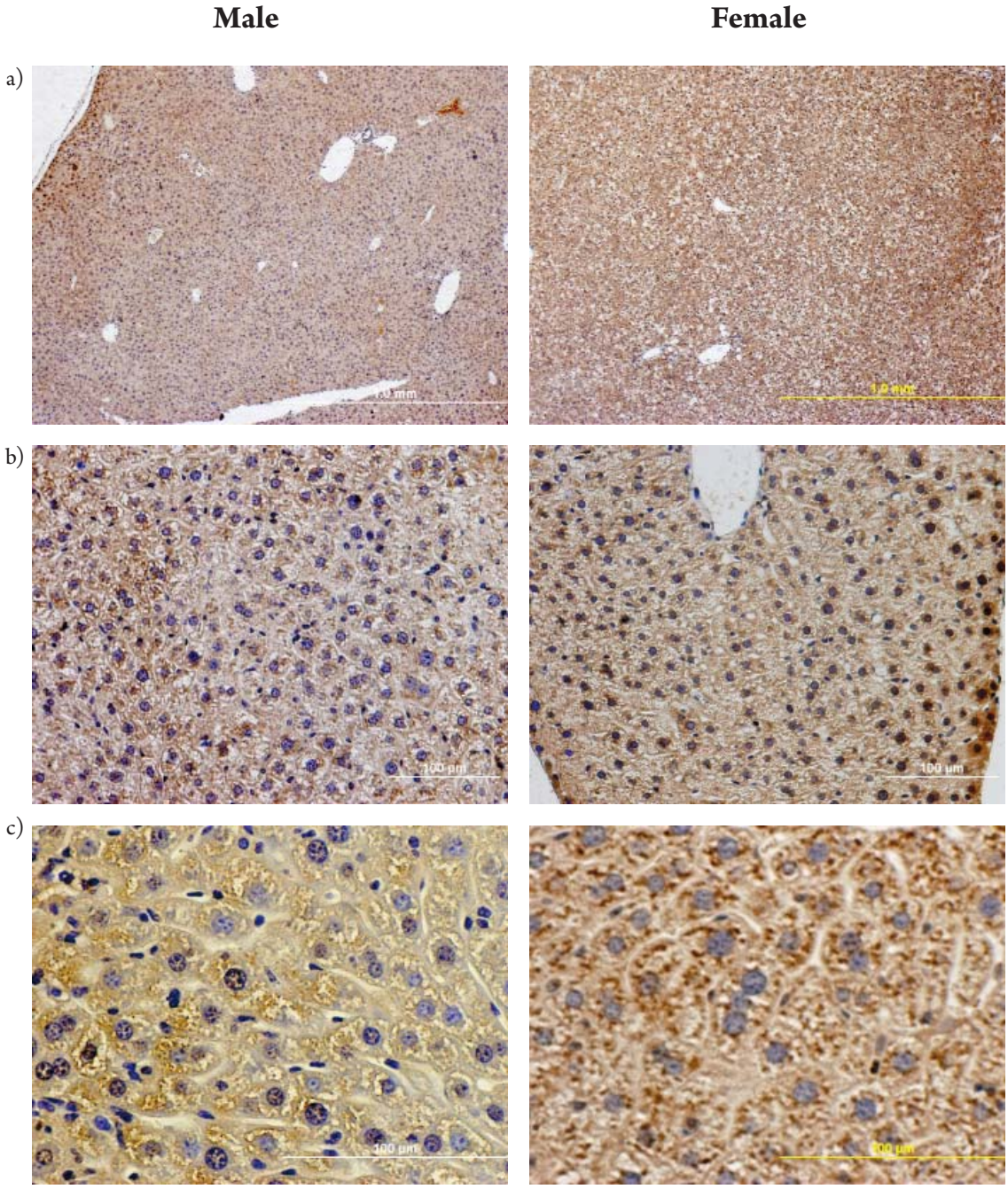


Figure 4.5: Immunohistochemical staining of liver sections for AQP8 from male and female mice exposed to DEN for 48 weeks. Representative immunohistochemical staining of liver sections from male and female mice injected with DEN (1mg/kg--ip) 48 weeks prior to euthanasia. Slides stained with an antibody against AQP8 (brown). Images taken at a) 4x, b) 20x and c) 40x magnification.

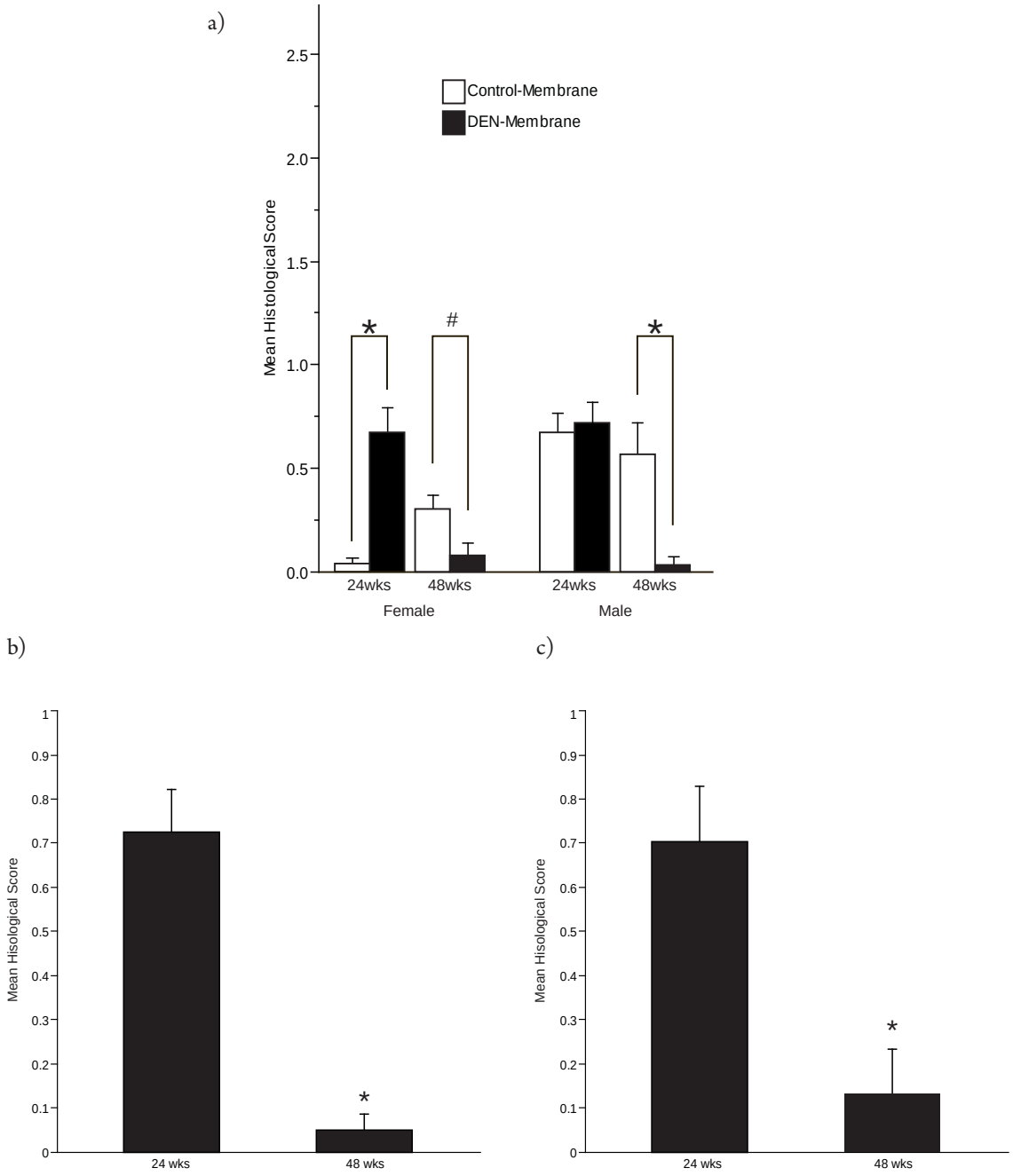


Figure 4.6: Aquaporin 8 membrane localization in the presence or absence of DEN. Female (F) and male (M) mice were initiated with DEN 24 or 48 weeks (DEN-24, DEN-48 respectively) prior to being sacrificed or were untreated as controls and also sacrificed at 24 or 48 weeks (wks). Livers from these mice were resected, processed and immunohistochemically stained with anti-AQP8 antibodies. a) Aquaporin 8 membrane localization in male and female mice, +/- DEN injection and euthanized at 24 and 48 weeks. b) Aquaporin 8 membrane localization in female DEN-24 and DEN-48 week mice. c) Aquaporin 8 membrane localization in male DEN-24 and DEN-48 week mice. * $p < 0.01$, # $p < 0.05$. For 24 week control group, n = 10(F), 9(M). For DEN-24, n = 9(F), 5(M). For 48 week control group, n = 10(F), 8(M). For DEN-48, n = 7(F), 7(M).

mice were maintained as controls. Mice from each group were euthanized at 24 weeks and 48 weeks. Livers from these mice were resected, fixed and processed for immunohistochemical staining. Slides were stained with an antibody specific against AQP9. Representative AQP9 stainings from the 24 week control group (Figure 4.8), DEN-24wk group (Figure 4.9), 48 week control group (Figure 4.10) and DEN-48wk group (Figure 4.11) are shown.

Analysis of the scoring data indicated significant AQP9 localization to the plasma membrane, which was dependent on the length of exposure to DEN (Figure 4.12a: $p < 0.01$). In the 24 week group, exposure to DEN resulted in significantly less AQP9 plasma membrane localization compared to no DEN exposure (Figure 4.12a: $p < 0.05$). In the 48 week group, exposure to DEN also resulted in significantly less AQP9 plasma membrane localization compared to no DEN exposure. DEN-48wk mice had significantly less AQP9 localization to the plasma membrane compared to DEN-24wk mice (Figure 4.12a: $p < 0.01$).

Analysis of scoring data indicated significant AQP9 localization to the cytoplasm, which was dependent on both exposure to DEN and the length of exposure to DEN (Figure 4.12b: $p < 0.01$). DEN-48wk mice had significantly higher cytoplasmic localization compared to DEN-24wk mice as well as 48 week control mice (Figure 4.12b: $p < 0.01$).

4.4. Discussion

Aquaporins play an important role in the production of bile due to the efficient movement of water across cellular membranes, which aquaporins facilitate (Masyuk *et al.* 2006). Hepatocytes express three aquaporin isoforms, AQP8, 9 and 0 with AQP8 and 9 playing a role in bile production (Masyuk *et al.* 2006). Previous reports have also suggested that AQP8 and 9 play a role in hepatocellular carcinoma (Jablonski *et al.* 2007). However that study presented no data on aquaporin localization and expression during HCC progression. In the

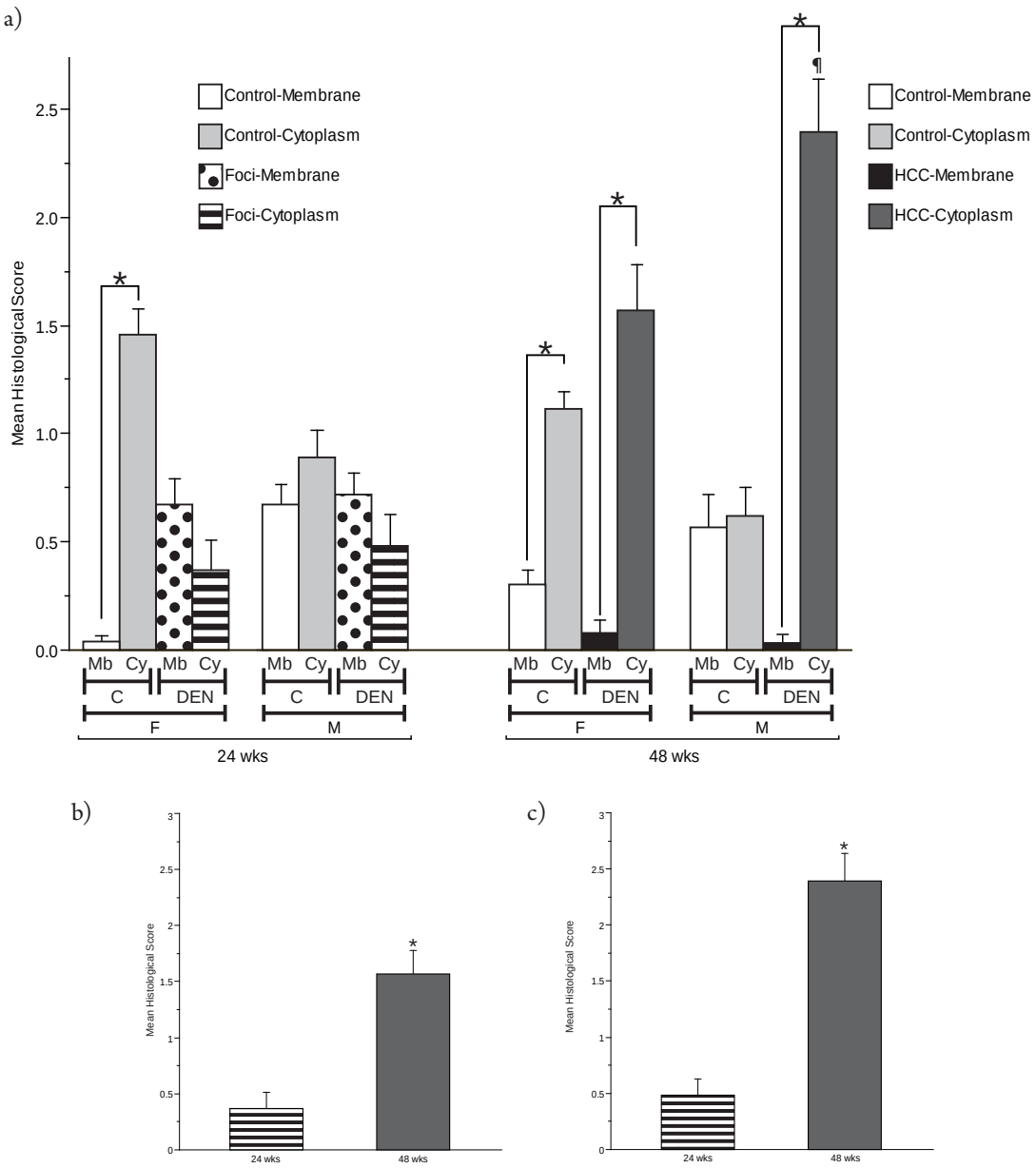


Figure 4.7: Aquaporin 8 membrane/cytoplasmic localization in response to DEN. Female and male mice were initiated with DEN 24 (foci) or 48 (HCC) weeks (wks) prior to sacrifice or were left untreated as controls. Livers from these mice were resected, processed and immunohistochemically stained with anti-AQP8 antibodies. a) Membrane and cytoplasmic localization of AQP8 in male and female C3H mice, +/- DEN injection, euthanized at 24 and 48 weeks. b) Aquaporin 8 cytoplasmic localization in female C3H mice. c) Aquaporin 8 cytoplasmic localization in male C3H mice. * $p < 0.01$, ¶ $p < 0.05$ compared to cytoplasmic staining of female mice exposed to DEN for 48 weeks. Cy = Cytoplasm, Mb = Membrane, C = Control mice, DEN = DEN initiated mice, F = Female, M = Male. For 24 week control group, n = 10(F), 9(M). For DEN-24, n = 9(F), 5(M). For 48 week control group, n = 10(F), 8(M). For DEN-48, n = 7(F), 7(M).

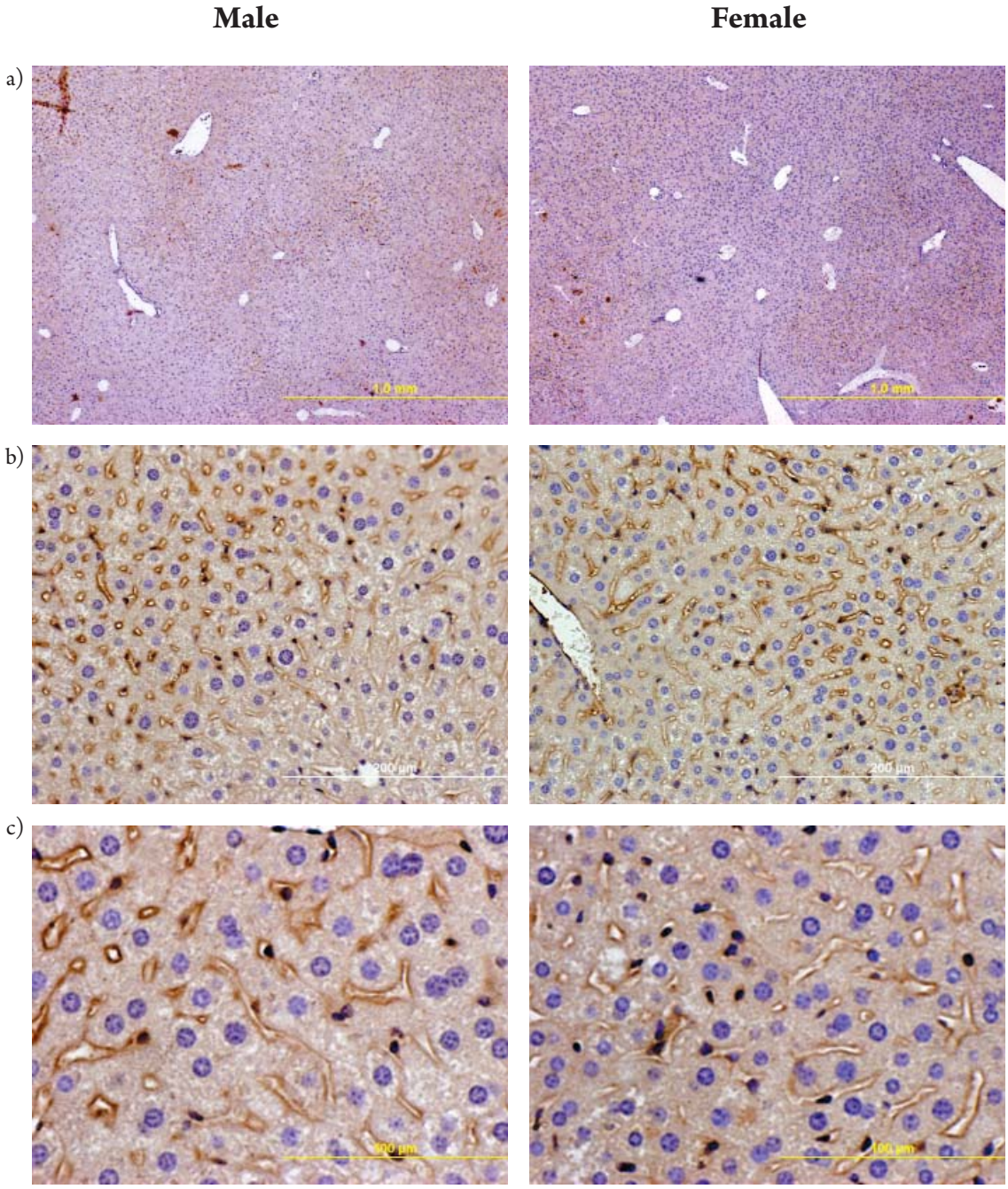


Figure 4.8: Immunohistochemical staining of liver sections for AQP9 from male and female mice in the absence of DEN. Representative immunohistochemical staining of liver sections from male and female C3H mice in the absence of DEN exposure (24 weeks). Slides were stained with antibodies against AQP9 (brown). Images taken at a) 4x, b) 20x and c) 40x magnification.

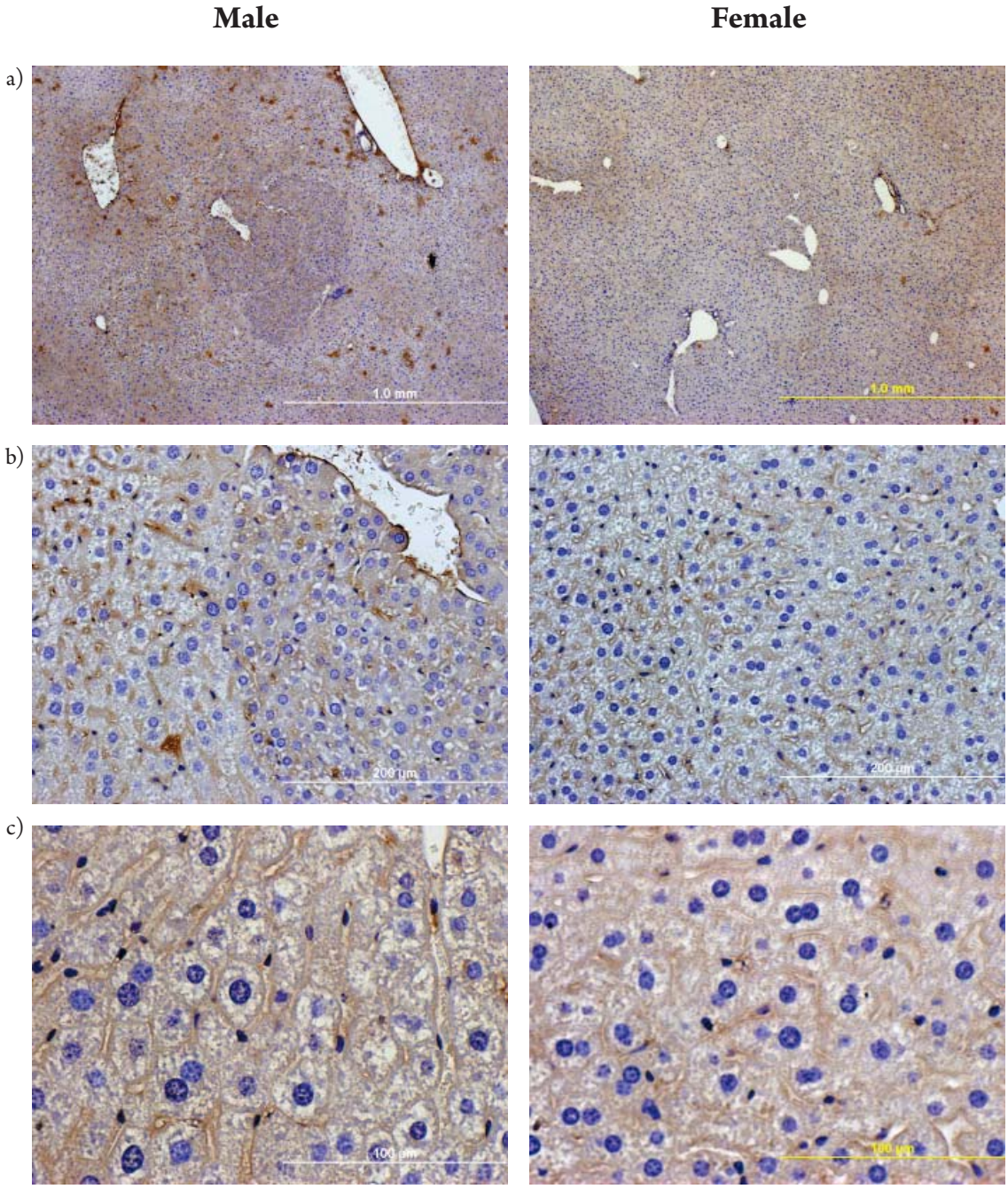


Figure 4.9: Immunohistochemical staning of liver sections for AQP9 from male and female mice exposed to DEN for 24 weeks. Representative immunohistochemical staining of liver sections from male and female mice injected with DEN (1mg/kg--ip) 24 weeks prior to euthanasia. Slides were stained with antibodies against AQP9 (brown). Images taken at a) 4x, b) 20x and c) 40x magnification.

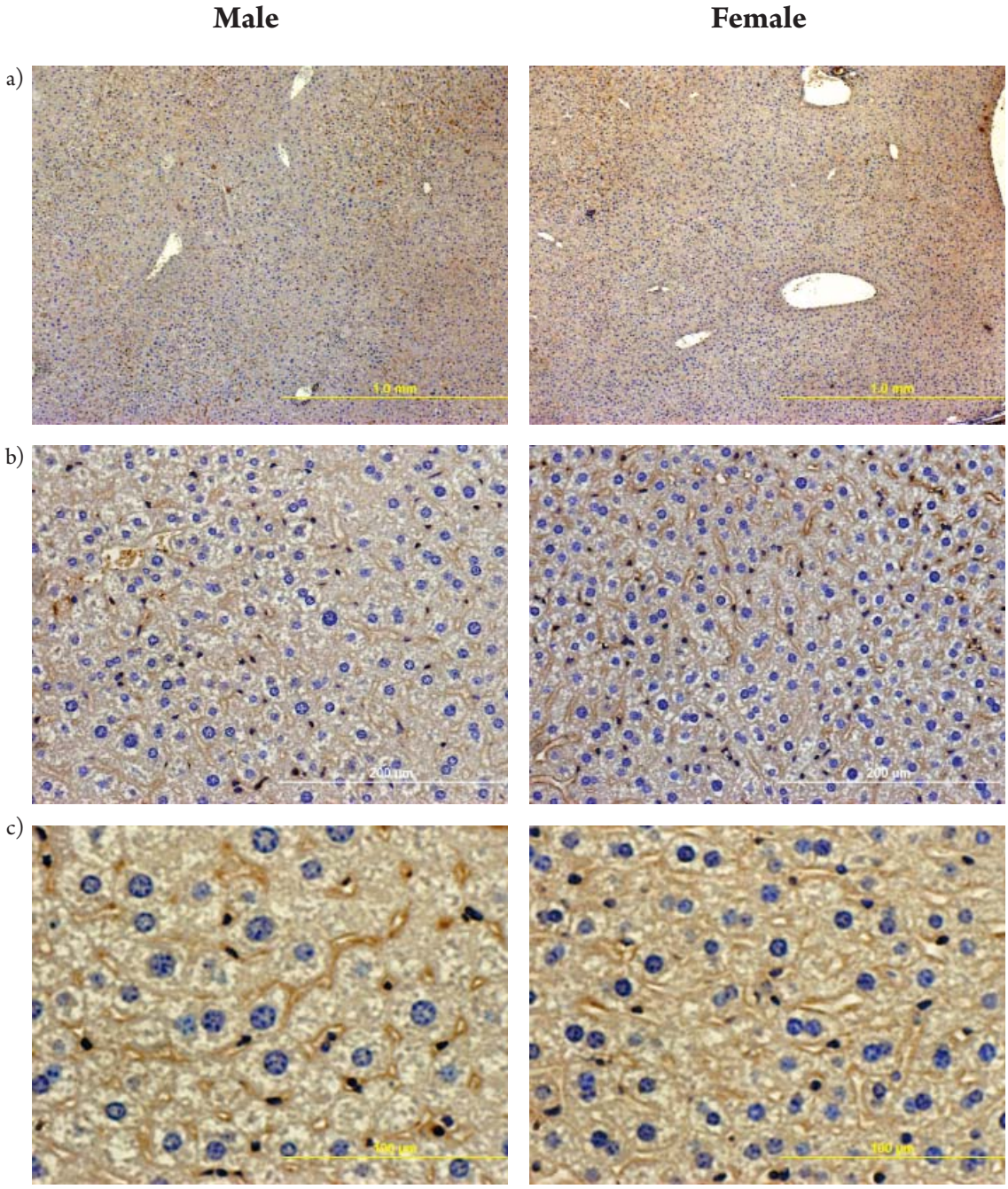


Figure 4.10: Immunohistochemical staining of liver sections for AQP9 from male and female C3H mice in the absence of DEN. Representative immunohistochemical staining of liver sections from male and female C3H mice in the absence of DEN exposure (48 weeks). Slides were stained with an antibody againist AQP9 (brown). Images taken at a) 4x, b) 20x and c) 40x magnification.

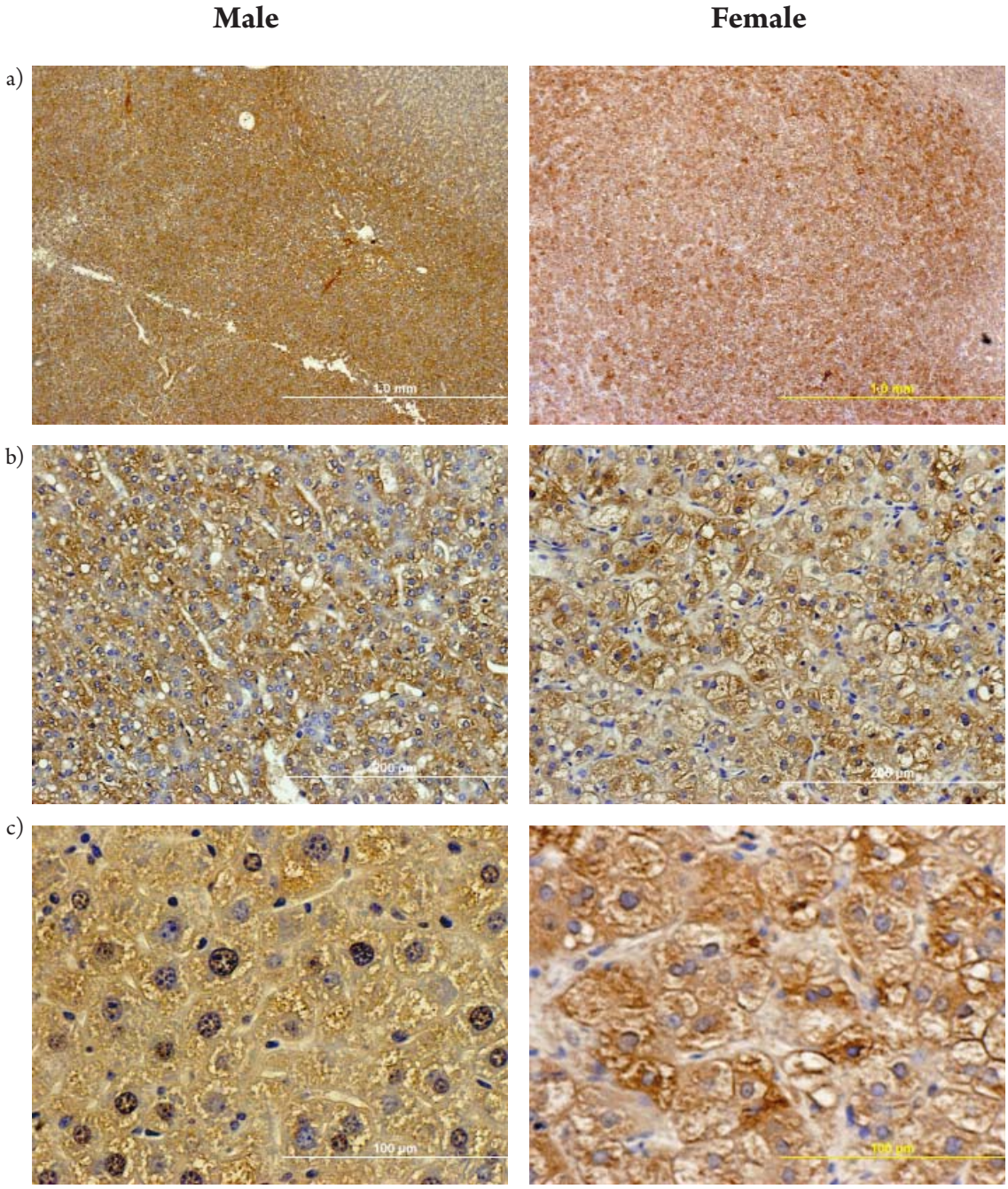


Figure 4.11: Immunohistochemical staining of liver sections for AQP9 from male and female mice exposed to DEN for 48 weeks. Representative immunohistochemical staining of liver sections from male and female mice injected with DEN (1mg/kg--ip) 48 weeks prior to euthanasia. Slides stained with an antibody against AQP9 (brown). Images taken at a) 4x, b) 20x and c) 40x magnification.

a)

b)

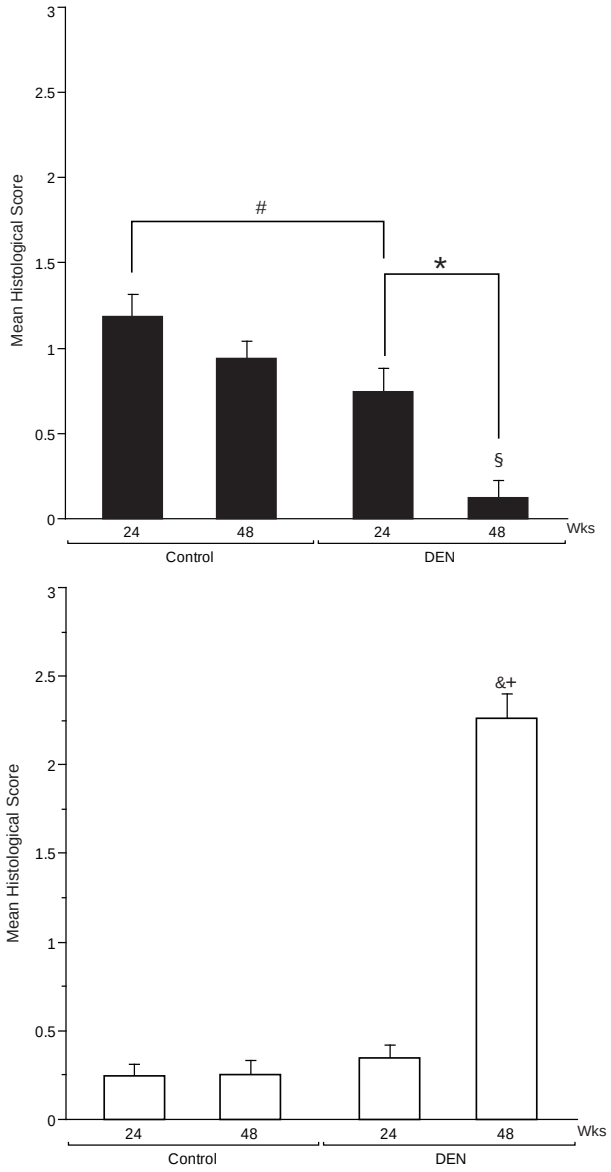


Figure 4.12: Aquaporin localization in response to DEN. Female and male mice were initiated with DEN for 24 (foci) or 48 (HCC) weeks (wks) prior to sacrifice or were left untreated as controls. Livers from these mice were resected, processed and immunohistochemical staining with anti-AQP9 antibodies indicated a) Membrane localization of AQP9 in C3H mice, +/- DEN injection, euthanized at 24 and 48 weeks. b) Aquaporin 9 cytoplasmic localization of AQP9 in C3H mice, +/- DEN injection, euthanized at 24 and 48 weeks. + $p < 0.01$ compared to mice injected with DEN 24 weeks prior to euthanasia, & $p < 0.01$ compared to 24 or 48 week control mice, # $p < 0.05$, * $p < 0.01$, § $p < 0.05$ compared to 48 week control mice. For 24 week control group, n = 19. For DEN-24, n = 14. For 48 week control group, n = 18. For DEN-48, n = 14.

current, study a progressive, DEN mouse model was used to examine AQP8 and 9 localization and progression during HCC progression. Mice injected with DEN are then said to have been "initiated". These data indicate that AQP8 localization to the plasma membrane increases early in HCC progression but then falls during disease progression. Further, as HCC progresses, AQP8 localization to the plasma membrane decreases significantly in both male and female mice. However, gender plays no significant role in AQP8 localization to the plasma membrane. There was no significant difference in AQP8 plasma membrane localization between 24 week or 48 week male and female initiated mice. However, there were significant gender differences in AQP8 localization to the cytoplasm. Male mice with later stage HCC had higher cytoplasmic AQP8 localization than female at a similar stage of HCC. Regardless of the HCC stage or gender, cytoplasmic localization of AQP8 was higher than membrane localization. In fact, AQP8 localization in the plasma membrane fell to barely detectible levels in later stage HCC. Aquaporin 9 localization was also significantly affected by HCC progression. In early stage HCC, AQP9 is primarily plasma membrane bound, but as the disease progresses transformed hepatocytes seem to withdraw AQP9 from the plasma membrane and AQP9 is then strongly expressed in the cytoplasm compared to the plasma membrane.

Gender differences play a role in a variety of different physiological and pathophysiological processes, including cancer genetics (Atwal *et al.* 2008), metastasis (Naor *et al.* 2009), T cell responses (Roden *et al.* 2004) and tumor immunology (Page *et al.* 1995). In these studies gender differences played a significant role in AQP8 localization as well. Previous reports indicate that AQP8 expression is modulated in estrogen-induced cholestasis (Carreras *et al.* 2007). Also, a previous report demonstrated that gender differences in cytochrome P450 3A4 expression is growth hormone dependent (Cheung *et al.* 2006). The current study though

is the first to show gender differences in HCC. There are reports of AQP9 regulation by androgens (Wang *et al.* 2008) and estrogen (Oliveira *et al.* 2005) so it is unexpected that gender differences was no a significant factor in AQP9 localization. Perhaps, genetic instability, which in concomitant with all cancers (Hanahan *et al.* 2000), has altered AQP9 responsiveness to gender mediated AQP9 expression and/or localization. It also might be that this phenomena is an artifact of this model of HCC.

There are a few broad categories of rodent HCC models: chemical, viral, cell inoculation (either stem cells or cancer cell lines) and xenograph (Wu *et al.* 2009). Chemical models, including DEN and carbon tetrachloride (CCl4) induced HCC by a three step process: initiation, promotion and progression (Wu *et al.* 2009). A single exposure to some of these chemicals is sufficient to initiate and promote HCC (for example, DEN). Chemicals such as CCl4 require a separate promoter, though promoter chemicals are used with chemicals such DEN to decrease time to mature HCC and to increase the aggression of a tumor (Heindryckx *et al.* 2009). The most common promoters are partial hepatectomy and phenobarbital (Wu *et al.* 2009). Promoters induce clonal expansion of initiated cells (Wu *et al.* 2009). Viral models of HCC involve the use of transgenic animals to either express viral proteins which initiate and promote HCC development through a chronic inflammation of the liver (Heindryckx *et al.* 2009). Xenograph models of HCC involve using human liver tissue which is then implanted into an immunocompromised rodent (Wu *et al.* 2009). Cell inoculation models are similar to xenograph models only that in cell inoculation models, a cancer cell line MHC matched to the species of rodent to be used are injected back into the animal to yield a tumor (Wu *et al.* 2009). All of these models are valuable tools to study HCC but they are also specialized tools which means that care must be taken when choosing between them. Cell inoculation

models produce tumors more rapidly than any other model and are suitable for studying late stage tumor progression (Heindryckx *et al.* 2009). However, these models are not particularly applicable to human HCC (Heindryckx *et al.* 2009). Viral models of HCC are suitable for studying HCC in the context of viral hepatitis infection (HBV and HCV encoded proteins are commonly used). However, this model requires the generation of transgenic mice which are controversial since there is conflicting data about the extent of liver damage caused in these models (Wu *et al.* 2009). Chemical models of HCC are the oldest and most studied being in use since 1932 (Wu *et al.* 2009). Chemical models, such as the DEN model, are preferable for studying hepatocarcinogenesis as well as tumor progression (Wu *et al.* 2009). However, the DEN model also suffers from caveats that cannot be ignored. One, DEN can be lethal to young rodents and mortality is an issue (Heindryckx *et al.* 2009). Second, this model can suffer from poor reproducibility (Heindryckx *et al.* 2009). It is very difficult to determine exactly when an animal will develop foci or mature HCC. This can be an issue if the experimental protocol requires exact time points. Third, metastasis rarely, if ever, occurs in this model (Heindryckx *et al.* 2009). With this being said, the DEN model also has a number of distinct advantages. One, tumor formation rates for this model are routinely reported to be 100% (Wu *et al.* 2009). Two, the DEN model is versatile and can model many aspects of human HCC. Instead of a single injection in young animals, long-term administration of DEN results in a close model of certain human HCC and the time to tumor formation is half that of a single injection DEN models (Heindryckx *et al.* 2009). Also, human HCC incidence is stratified by gender, with females a third as likely to contract HCC as males (Bosch *et al.* 2005). Long term administration of DEN, not only mirrors this incidence rate, but tumors in this model are aggressive and metastatic (Heindryckx *et al.* 2009). Lastly, for tumor formation approaching the same rate as cell

inoculation models, long-term DEN administration in rats, combined with partial hepatectomy, can result in tumor formation in only four weeks (Heindryckx *et al.* 2009).

Recent reports have suggested that aquaporins (AQP8 in particular) might be regulated by protein degradation (Soria *et al.* 2009). Further, a report from the McKillop laboratory indicates that AQP8 and 9 expression drops to very low levels in rodent and human HCC (Jablonski *et al.* 2007; Padma *et al.* 2009). In the current study, while there were significant differences in AQP8 and 9 localization, the expression levels in both DEN treated mice and control groups rarely scored higher than 3. However, the scoring process involves judgment on the part of the scorer so, any study based on this technique is not truly quantitative. This is not to say that it is not of value only that, one must exert caution when interpreting these types of data. While computer imaging analysis has become popular recently, I am unconvinced that it provides any more accuracy or reliability than human scoring.

In conclusion, AQP8 plasma membrane localization is significantly lower in later stage HCC compared to earlier in the disease process. AQP8 localization to the cytoplasm is significantly higher in later stage HCC compared to earlier in the disease process. Further, AQP8 localization to the cytoplasm is significantly different in female mice compared to male. AQP9 localization to the plasma membrane was also significantly lower in later stage HCC compared to earlier in the disease process. Also, AQP9 localization to the cytoplasm was significantly higher in later stage HCC compared to earlier in the disease process. These data bolster results reported by the McKillop laboratory using a rat inoculation model of HCC (Jablonski *et al.* 2007). Both models seem to be in agreement and the current study suggests that the decrease in AQP8/9 plasma membrane localization is a later occurrence in HCC.

CHAPTER 5:ANALYSIS OF AQUAPORIN 8 AND 9 IN HUMAN HEPATOCELLULAR CARCINOMA

5.1. Introduction

In previous chapters, data was presented examining a potential mechanism of aquaporin (AQP) expression and localization in a rat model of hepatocellular carcinoma (HCC). In a subsequent chapter, data was presented identifying AQP8 and 9 expression and localization in a mouse, diethylnitrosamine (DEN) model of HCC. In the present study, AQP8 and 9 expression and localization will be examined in a human tissue from HCC patients.

In 2002, HCC accounted for 5.6% of all human cancers worldwide with more than 550,000 new cases, and a similar number of deaths (World Health Organization 2002). North American men are more than twice as likely to develop HCC as women with the ratio rising to more than three and half times in western European men (Bosch *et al.* 2005). The prognosis for HCC patients was equally bleak, especially in the developing world, with HCC exhibiting a fatality ratio of approximately 1 (meaning that most HCC patients die within a year of diagnosis (Bosch *et al.* 2005)). Further, between 1995 and 2000 the 5-year survival rate of HCC patients in the United States was 8.3% (Bosch *et al.* 2005).

The most common treatments for HCC are chemical and thermal ablation, surgical resection and liver transplant. In early stage HCC (uninodular tumor, tumor size < 3 cm), ethanol ablation has produced 70% complete ablation rates (Shiina *et al.* 1993). Though the recurrence rate for ethanol ablated tumors was reported to be as high as 60%, 84% of

these recurrences were new HCC tumors distant from the original ablation site (Shiina *et al.* 1993). Despite this, retrospective studies reported 41% — 53% 5-year survival rates, simalar to survival rates for patients that underwent surgical resection (Lencioni *et al.* 2005). 5-year survival rates as high 70% were reported for patients undergoing surgical resection on single, asymptomatic, tumors <5cm (Llovet *et al.* 2000). Radiofrequency ablation was reported to be significantly more efficacious than ethanol ablation as regards recurrence free survival. In a randomized trial comparing radiofrequency ablation to ethanol ablation across 1- and 2-year survival rates, radiofrequency ablation had a 9% and 21% better survival rate, respectively (Lencioni *et al.* 2005). Liver transplant represents the radical end of the HCC treatment spectrum. Unfortunately, cadaveric liver transplantation is contraindicated for large tumors (> 5cm) or multifocal/metastatic tumors due to 5-year survival rates of only 15% (Roayaie *et al.* 2005). Further, severe shortage of donor livers results in up to 30% of patients dying while waiting for a liver transplant (Roayaie *et al.* 2005).

The poor prognosis for HCC patients has led to increased interest in HCC screening. In part this is due to the insidious nature of HCC; it remains subclinical well into disease progression. Also, the likelihood of survival increases considerably the earlier HCC can be detected (Marrero 2005). In breast cancer, the presence of *BRCA1* is correlated with young onset of cancer, low differentiation, high proliferation rates compared to *BRCA1* negative tumors and increased prevalence of grade III tumors (Klauber-DeMore 2005). Currently, human HCC lacks a marker like *BRCA1* , therefore, elucidating the underlying biology of HCC is a topic of great importance for generating efficatious clincial outcomes. To this end, identifying patient populations that have increased risk for worse clinical outcomes is a logical place to begin. An aim of the current study is to examine if AQP8 or 9 expression correlates

with other known risk factors for HCC which have known effects on clinical outcomes. This might allow for pathological determination of tumor aggressiveness and therefore allow clinicians to design better treatment plans.

Treatment of human HCC is complicated by the subclinical nature of the disease resulting in patients presenting with later stage tumors. Also, human HCC has a known resistance to chemotherapeutic agents (Befeler 2005). Part of this is due to the inhibited responsiveness to apoptosis exhibited by all cancers (Hanahan 2000). Aquaporins play a role in apopotosis such that inhibition of aquaporin function inhibits apoptotic progression (Jablonski 2007). Because there is no reported gating domain in mammalian aquaporins, proper aquaporin function requires that it be inserted into a membrane. Further, there is no published report of a cytoplasmic function for aquaporins. Therefore, if AQP8 or 9 localization correlates with other known risk factors this also would assist clinicians in treating patients. Also, if AQP8 and/or 9 has reduced localization to the plasma membrane in tumor cells, this represents plausible hypothesis as to why human HCC cells are resistant to chemothreapeutic agents. Namely, that the absence of membrane bound aquaporins inhibits apoptotic progession in tumor cells but normal hepatocytes are stimulated to undergo apoptosis. Another aim of the current study will be to examine the localization differences in human HCC compared to non-tumor liver.

The production, secretion and alteration of bile is a primary physiological function of the liver. Bile is greater than 95% water, so a relatively large volume of water must be transported from the sinusoidal space to the bile canaliculus (Calamita *et al.* 2005). Aquaporins facilitate this process by increasing membrane water permeability by up to 100 fold (Borgnia *et al.* 1999; Calamita *et al.* 2005). Hepatocytes express three aquaporin isoforms, AQP8, AQP9 and AQP0 (Huebert *et al.* 2002; Masyuk *et al.* 2006). AQP9 is constitutively expressed in

sinusoidal membranes while AQP0 is constitutively expressed on intracellular vesicles (Huebert *et al.* 2002). AQP8 is initially expressed on intracellular vesicles but in response to glucagon, *via* cyclic–adenosine monophosphate (cAMP) stimulation, AQP8 translocates to the bile canalicular membranes (Gradilone *et al.* 2003; Huebert *et al.* 2002). This mechanism for AQP8, 9 and 0 expression and localization was elucidated in mice and rats. These isoforms of aquaporins have been observed in humans but it is only hypothesized that AQP8 localization is glucagon mediated. Mice and rats act as models for human physiology and pathophysiology. As with all models, these rodent models must be validated against the human condition. Previous chapters in this dissertation have presented data using both rat and mice models, so the aim of the present study is to examine the fidelity these rodent models of HCC have with human HCC as regards AQP8 and 9 expression and localization.

5.2. Material & Methods

5.2.1. Materials

Huh7 cells were a generous gift from Dr. Herbert Bonkovsky (Carolinas Medical Center, Charlotte, NC). Apicidin, trypan blue and $HgCl_2$ were purchased from Sigma-Aldrich(St. Louis, MO). CaspACE colorimetric caspase assay was purchased from Promega (Madison, WI). Countess automated cell counter, Alexa488-conjugated goat anti-rabbit secondary antibody, Texas Red goat anti-rabbit secondary antibody, 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI) and anti-fade mounting media were purchased from Invitrogen (Carlsbad, CA).

5.2.2. Patient Information

Institutional Review Board approved the use of archived paraffin embedded surgical specimens from patients who had undergone HCC resection at Carolinas Medical Center. 23

patient samples were analyzed in total. Further, patient histories were removed of identifying information and patients were grouped according to known risk factors for HCC including: alcohol or drug use, HBV or HCV infection and cirrhosis. Patients were also grouped according to pathological factors including: serum AFP levels, the size of the tumor and cancer stage (determined either pathologically or clinically). Only patients with primary HCC were used in this study.

5.2.3. Immunohistochemistry

All immunohistochemical procedures were performed as described in chapter 2.8.4.

5.2.4. Immunofluorescent Histochemistry

Processing, slide mounting, antigen retreival and blocking were performed as described in chapter 2.8. After blocking, slides were incubated in primary antibody (1:500 dilution, 30 minutes). Detection was perfromed using either Alexa488- or Texas red-conjugated goat anti-rabbit secondary antibody (1:2000 dilution, 2 hours). Slides were counterstained with DAPI, dehydrated through graded alcohols then cover-slip mounted with anti-fade media. The sections were examined by laser scanning confocal microscopy. Each channel was recorded independently and superimposed images generated.

5.2.5. Cell Swelling Analysis

Cell Swelling analysis was preformed as described in chapter 2.4.

5.2.6. Caspase Assay

The caspase assay was performed per manufacturer's instructions. Briefly, Huh7 cells were exposed to 10μM $HgCl_2$, 2μM apicidin, 10μM $HgCl_2$ + 2μM apicidin or a caspase inhibitor (as control, Z-VAD-FMK was provided by the manufacturer). Cells alone were used as a control. Cells were collected, washed once with cold PBS, resuspended in cell lysis buffer

(provided by the manufacturer), subjected to a single freeze-thaw cycle (-80°C — +4°C) and then centrifuged at 15,000 *x g* for 20 minutes (+4°C). The supernatant fraction was collected an assayed. Quantification of caspase-3 activity was performed using a standard curve with control reagents provided by the manufacturer.

5.2.7. Statistical Analysis

Student's t-Tests were used to analyze, data with only two factors. One-way, type I ANOVA models were used to analyze multifactor data. All analyzes were performed and charts created by SAS JMP 8.0.2 (Cary, NC). $p < 0.05$ was considered significant.

5.3. Results

5.3.1. Location of AQP8 and 9 in normal, non-tumor liver.

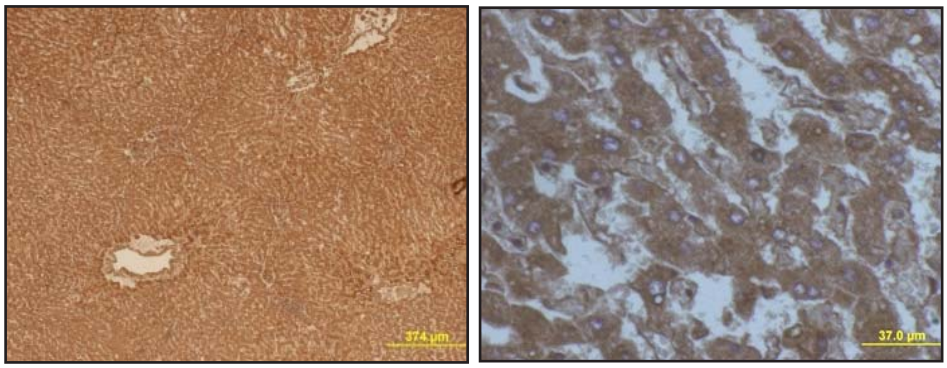
Staining of normal, non-tumor liver tissue with antibodies specific for AQP8 demonstrated predominantly cytoplasmic staining (Figure 5.1a). In contrast, staining for AQP9 indicated localization to the membrane and was zonally distributed (Figure 5.1b).

5.3.2. Localization of AQP8 and 9 in normal and HCC, in the context of underlying cirrhosis.

Staining for AQP8 in non-tumor liver of HCC patients, in the absence of underlying cirrhosis, demonstrated a significantly higher presence of AQP8 in cytoplasm compared to the membrane (Figure 5.2a-c: $p < 0.001$). In tumors, AQP8 was also significantly higher in cytoplasm compared to the membrane (Figure 5.2a-c: $p < 0.001$). Staining for AQP8 in non-tumor liver of HCC patients, in the presence of underlying cirrhosis demonstrated a significantly higher presence of AQP8 in cytoplasm compared to membrane (Figure 5.3a-b: $p < 0.001$). In tumors, AQP8 was also significantly higher in cytoplasm compared to membrane (Figure 5.3a-b: $p < 0.001$).

Next I analyzed AQP9 staining in non-tumor liver of HCC patients, in the absence of

a)



b)

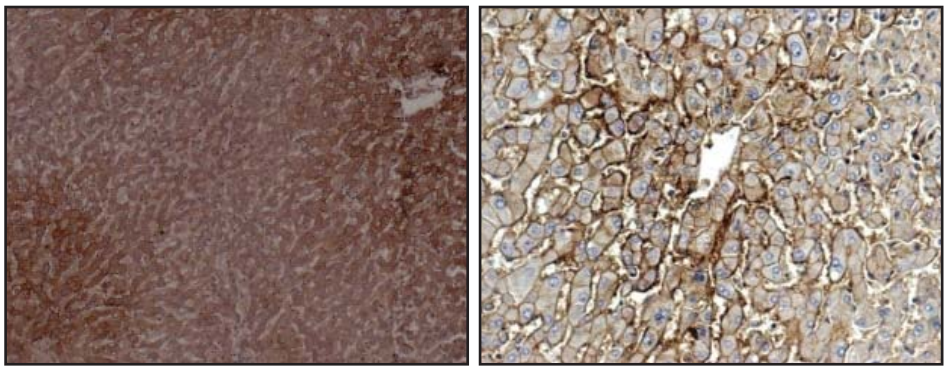


Figure 5.1: Immunohistochemical staining of normal human liver in the absence of hepatic disease. a) Representative immunohistochemical (IHC) images of normal liver sections following IHC staining using an anti-human AQP8 antibody. b) Representative immunohistochemical (IHC) images of normal liver sections following IHC staining using an anti-human AQP9 antibody.

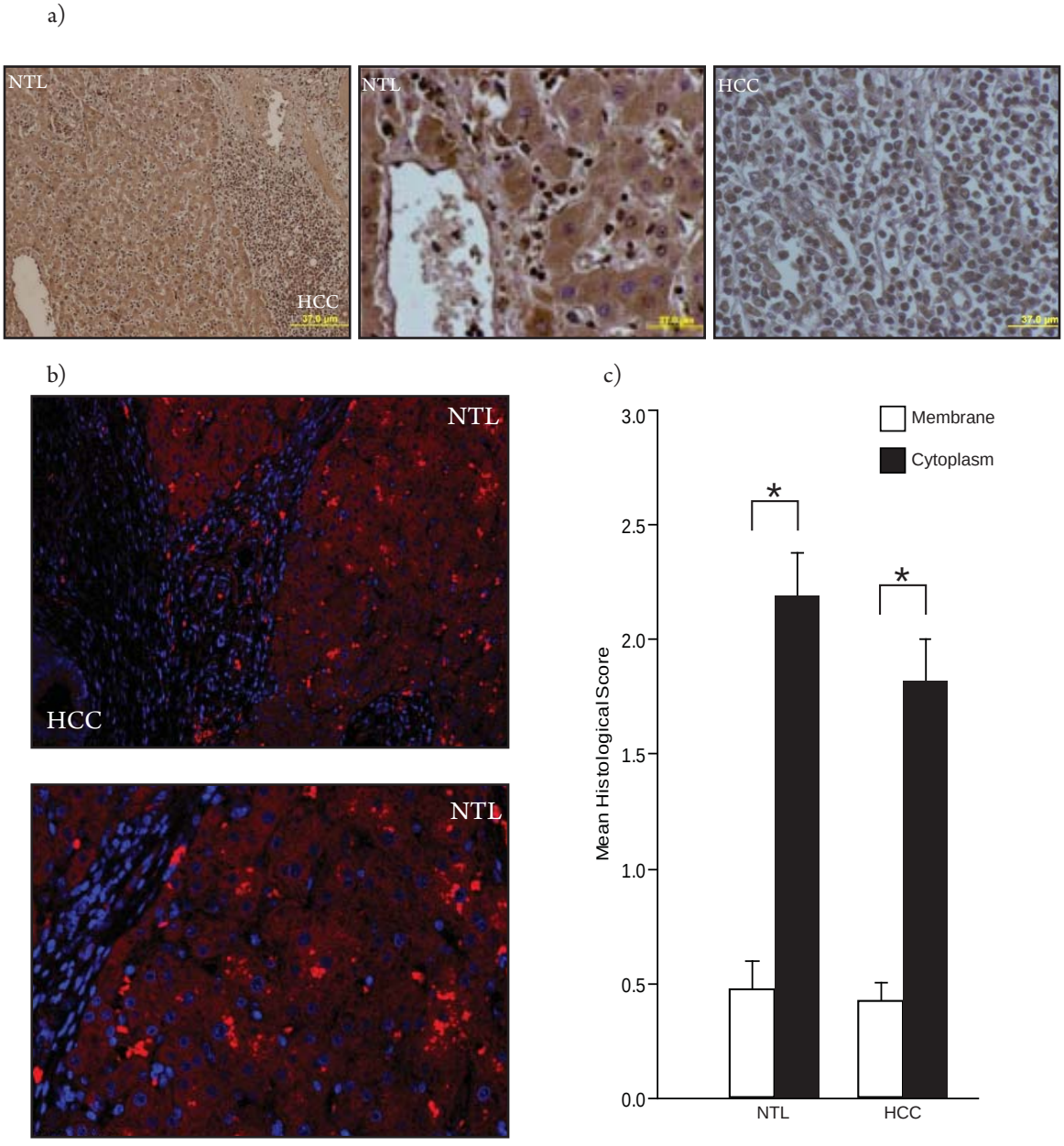


Figure 5.2: Aquaporin 8 expression/localization in human HCC in the absence of cirrhosis. a) Representative immunohistochemical (IHC) images of HCC and non-tumor liver (NTL) following IHC staining using an anti-human AQP8 antibody. b) Representative immunofluorescent histochemical (IFHC) images of HCC and NTL sections following IFHC staining using an anti-human AQP8 antibody and detection with Texas Red secondary (red). Sections were counterstained with DAPI (blue) for nuclear localization. c) Cumulative scoring analysis of membrane vs. cytoplasmic staining for AQP8 in NTL vs. HCC. Values are means +/- standard error of the mean of five separate fields scored independently. * $p < 0.01$, cytoplasmic staining compared to membrane. n = 11.

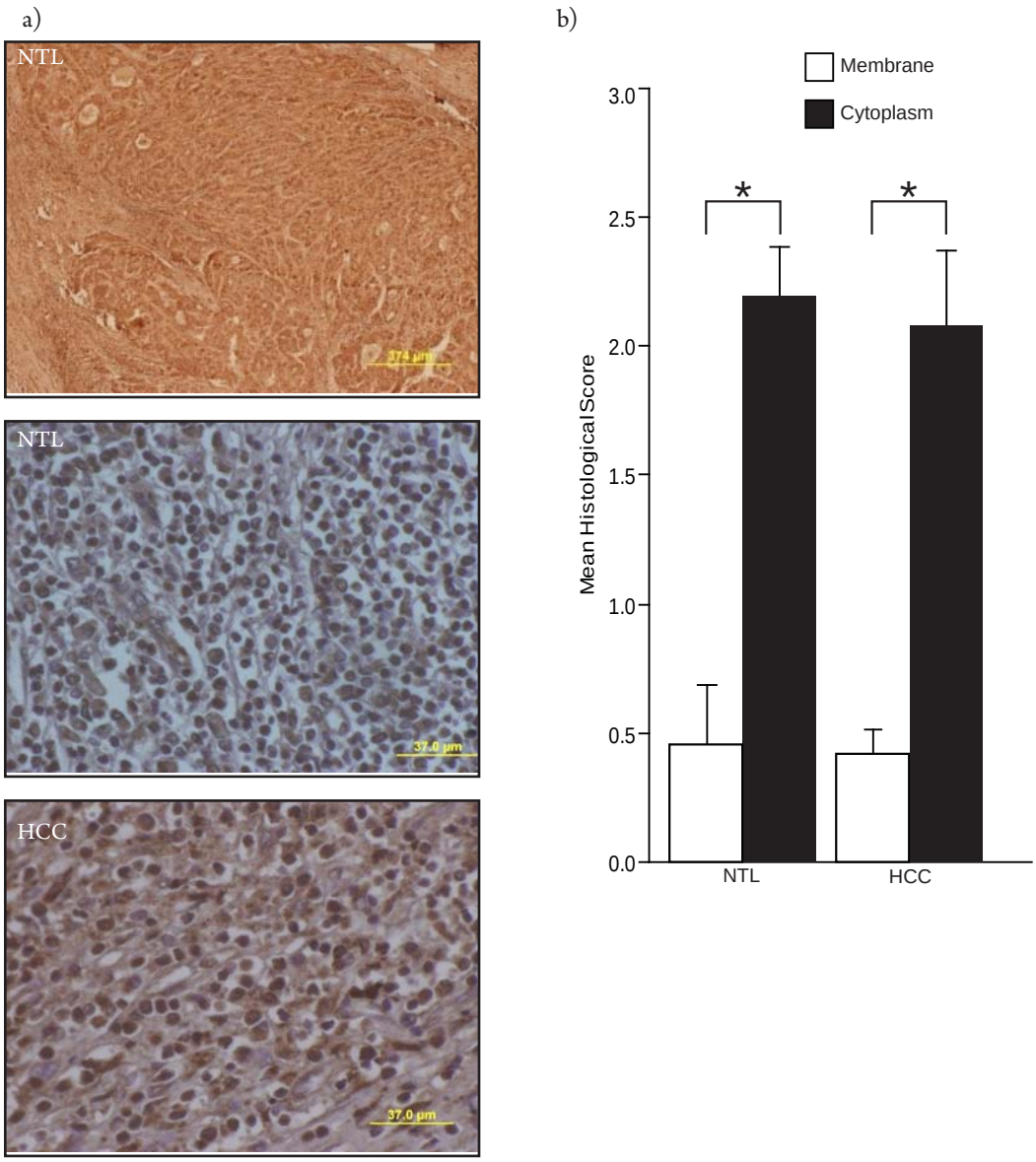


Figure 5.3: Aquaporin 8 expression/localization in human HCC in the presence of cirrhosis. a) Representative immunohistochemical (IHC) images of HCC and non-tumor liver (NTL) following IHC staining using an anti-human AQP8 antibody. b) Cumulative scoring analysis of membrane vs. cytoplasmic staining for AQP8 in NTL vs. HCC. Values are means +/- standard error of the mean of five separate fields scored independently. * $p < 0.01$, cytoplasmic staining compared to membrane. n = 12

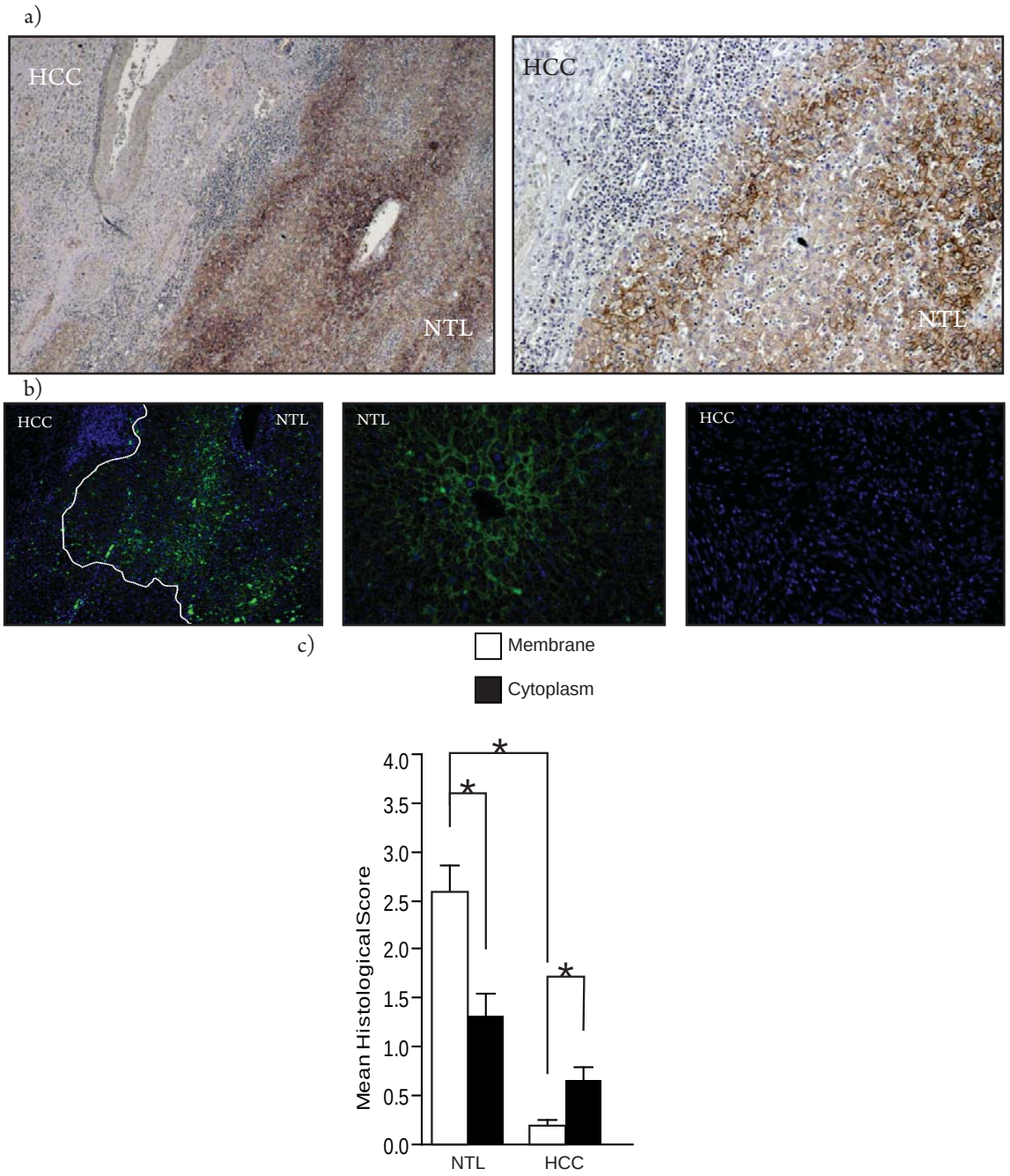


Figure 5.4: Aquaporin 9 expression/localization in human HCC in the absence of cirrhosis. a) Representative immunohistochemical (IHC) images of HCC and non-tumor liver (NTL) following IHC staining using an anti-human AQP9 antibody. b) Representative immunofluorescent histochemical (IFHC) images of HCC and NTL sections following IFHC staining using an anti-human AQP9 antibody and detection with Fluro488 labelled secondary (green). Sections were counterstained with DAPI (blue) for nuclear localization. c) Cumulative scoring analysis of membrane vs. cytoplasmic staining for AQP9 in NTL vs. HCC. Values are means +/- standard error of the mean of five separate fields scored independently. * $p < 0.01$. $n = 11$.

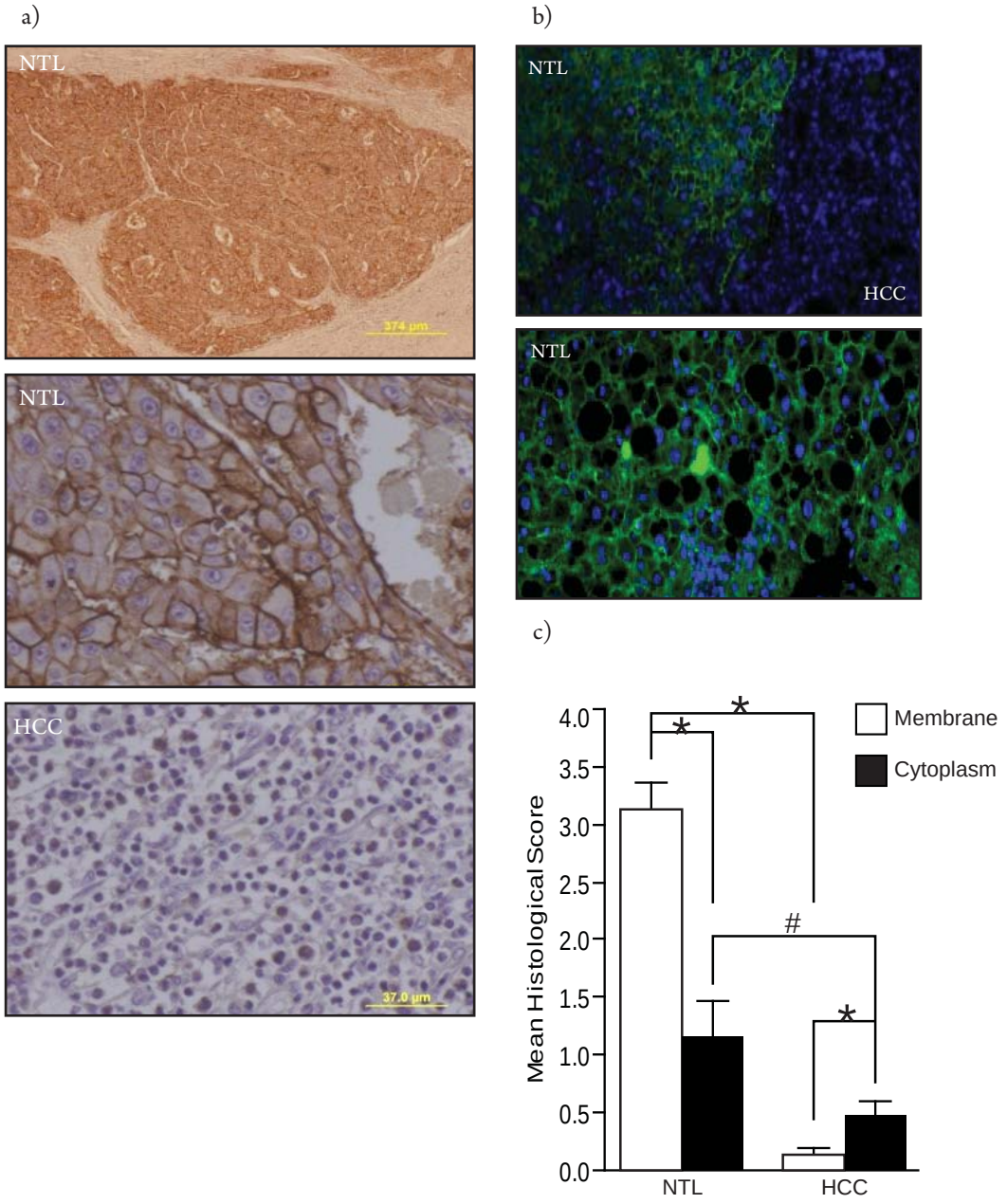


Figure 5.5: Aquaporin 9 expression/localization in human HCC in the presence of cirrhosis. a) Representative immunohistochemical (IHC) images of HCC and non-tumor liver (NTL) following IHC staining using an anti-human AQP9 antibody. b) Representative immunofluorescent histochemical (IFHC) images of HCC and NTL sections following IFHC staining using an anti-human AQP9 antibody and detection with Fluro488 labelled secondary (green). Sections were counterstained with DAPI (blue) for nuclear localization. c) Cumulative scoring analysis of membrane vs. cytoplasmic staining for AQP9 in NTL vs. HCC. Values are means +/- standard error of the mean of five separate fields scored independently. * $p < 0.01$, # $p < 0.05$. n = 12.

underlying cirrhosis. This analysis demonstrated a significantly higher presence of AQP9, where detected, in the membrane compared to cytoplasm (Figure 5.4a-c: $p < 0.01$). In tumors, AQP9 was higher in cytoplasm than in the membrane (Figure 5.4a-c: $p < 0.001$). Further, AQP9 localization in tumor membrane was significantly lower than non-tumor liver (Figure 5.4c: $p < 0.001$).

Staining for AQP9 in non-tumor liver of HCC patients, in the context of underlying cirrhosis demonstrated a significantly higher presence of AQP9 in cytoplasm compared to membrane (Figure 5.5a-c: $p < 0.001$). In tumors, AQP9 was also higher in cytoplasm than in the membrane (Figure 5.5a-c: $p < 0.01$). Membrane localization of AQP9 in tumor cells was significantly lower compared to non-tumor liver (Figure 5.5c: $p < 0.01$). Cytoplasmic localization of AQP9 in tumor cells was significantly lower compared to non-tumor liver (Figure 5.5c: $p < 0.05$).

It should be noted here one other interesting result from these data. AQP9 is zonally distributed in absence of cirrhosis (Figure 5.4a). However, in the presence of cirrhosis zonal distribution of AQP9 is reduced (Figure 5.5a).

Patients, for which there were complete histology scoring data, were grouped according to presenece or absence of underlying cirrhosis. 5 of 8 (62.5%) patients with underlying cirrhosis had AQP8 and/or 9 membrane localization differeces of > 1 between non-tumor liver and tumor tissue(Table 5.1). For patients without underlying cirrhosis 2 of 7 (28.5%) had AQP8 and/or 9 membrane localization differences of > 1 non-tumor liver and tumor tissue. Further, in either the cirrhotic patients or non-cirrhotic patients there does not appear to be a common risk factor for the patients with largest differences between AQP8 and/or 9 membrane localization in non-tumor liver and tumor tissue (Table 5.1).

Group Summaries	No Underlying Cirrhosis							Underlying Cirrhosis							
Gender Distribution	2 Female, 5 Male							1 Female, 7 Male							
Age (Mean ± SEM, All Patients)	61.29 ± 3.87 (69.00 ± 9.00 F; 58.20 ± 3.87 M)							58.88 ± 3.67 (69.0 ± 9.00 F; 58.20 ± 3.87 M)							
Race Distribution †	3 AA, 4Ca							1AA, 7 Ca							
Individual Patient Parameters															
Gender	F	F	M	M	M	M	M	F	M	M	M	M	M	M	M
Tumor Grade +	WD	WD	PD	MD	MD	PD	MD	MD	MD	PD	PD	PD	MD	PD	MD
TNM Stage	NK	IIIB	IIIB	I	IIIA	II	I	II	II	I	II	IIIA	II	NK	I
Focality *	NK	SF	MF	SF	MF	MF	NK	MF	MF	SF	MF	MF	SF	SF	SF
Tumor Size (cm)	NK	≤5	5-10	>10	>10	≤5	≤5	5-10	≤5	5-10	≤5	5-10	≤5	≤5	≤5
R Status (clean margin)	Yes	Yes	No	Yes	No	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Vascular Invasion §	No	No	NK	No	Yes	NK	No	Yes	No	No	Yes	Yes	No	No	No
Lymph Invasion	No	No	NK	No	NK	NK	No	Yes	Yes	No	No	Yes	No	No	No
Serum AFP ¶	NK	Norm	Incr	Incr	NK	Norm	Incr	Incr	Incr	NK	Incr	Incr	Incr	Norm	Incr
Associated Disease Risk Factors #	Cryp	Cryp	HBV	Et	HBV HCV	Cryp	Cryp	EtOH HCV	EtOH HCV	EtOH HBV HCV	Cryp	DILI	HCh	HCV	EtOH HCV
Cytoplasmic AQP 8 - NTL	3.7	3.5	2.4	1.8	2.6	3.2	2.9	2	2.8	3.4	3	3.4	3.6	3	2.5
Membrane AQP 8 - NTL	0.2	0	0.4	0	0.5	0.1	0.4	0	0.2	0.1	0	0.7	0.1	0	0
Cytoplasmic AQP 8 - HCC	1.3	1.6	2	3	2	0.8	3.2	0.6	2.3	1.5	3	2.1	1.2	3	1.2
Membrane AQP 8 - HCC	0.1	0	0.5	0	0.1	0.5	0.5	0.2	0.3	1.6	0.5	0.1	0.1	0.2	0.5
Cytoplasmic AQP 9 - NTL	1.1	0.5	1.1	0	0.58	0.2	1	1.4	0.9	0.4	0.3	1.4	0.3	0.6	1
Membrane AQP 9 - NTL	1.9	3.8	3.5	2.8	1	3.4	3.7	3.6	3.9	4.6	3.4	2.3	3.8	4	1.6
Cytoplasmic AQP 9 - HCC	0.1	0.3	0.7	1.4	0.1	0.5	1	0.9	0.5	0.2	0.3	1	0	0.8	0
Membrane AQP 9 - HCC	1.8	0.9	0.6	2	1.2	0	0.6	0.8	0.1	1.4	1	1.3	0.8	0.7	0.6

Table 5.1: Summary of scoring and clinico-pathological features of a sample of the patient population. † AA = African American, Ca = Caucasian; + Tumor grade: WD = Well-differentiated, MD = Moderately-differentiated, PD = Poorly-differentiated * Focality: NK = Not known, * SF = Single focus, MF = Multiple foci; § NK = Not known; ¶ Incr = Increased, Norm = normal, NK = Not known # Et = EtOH, HCV = Hepatitis C infection, HBV = Hepatitis B infection, Cryp = Cryptogenic, DILI = Drug induced liver injury, HCh = hemachromatosis. Patients in which membrane localization of AQP8 and/or 9 were expressed by a score of ≥ 1.0 in NTL vs. HCC.

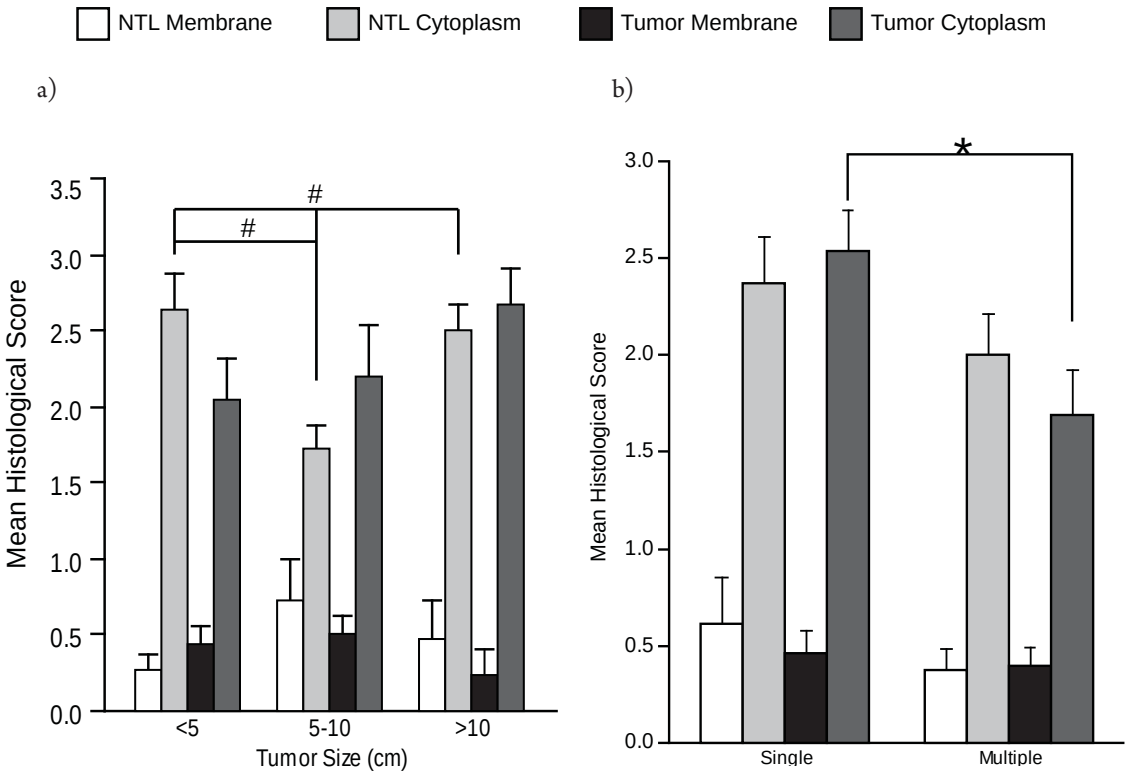


Figure 5.6: Analysis of AQP8 localization in patient subgroups. Immunohistochemical staining of sections from HCC patients which were grouped based on tumor size or whether the patient had a single hepatic tumor or multiple hepatic tumors a) Analysis indicated a significant affect of tumor size on AQP8 cytoplasmic localization in non-tumor liver (NTL). Further, Tukey-Kramer HSD analysis indicates a significant difference between patients with <5 cm tumors and patients with 5-10 cm tumors. b) Analysis of patients with either a single primary hepatic tumor or multiple primary hepatic tumors (determined clinically) indicated a significant reduction in AQP8 cytoplasmic localization in patients with multiple primary tumors. # $p < 0.05$, * $p < 0.01$. n = 23.

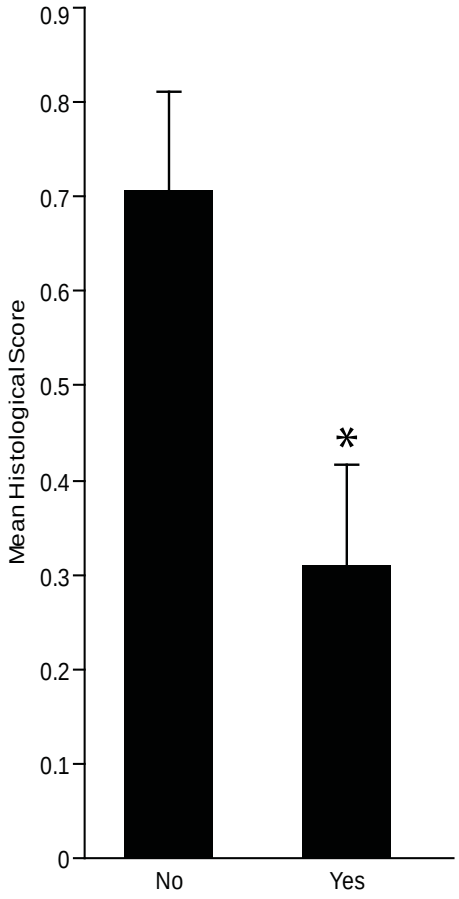


Figure 5.7: Analysis of AQP9 localization in patients with a history of chronic alcohol and/or drug abuse. Immunohistochemical staining of sections from patients with a history of alcohol or drug abuse indicated a significantly lower AQP9 localization to the cytoplasm compared to patients who did not abuse drugs and/or alcohol. * $p < 0.05$. n = 23

5.3.3. AQP8 localization is significantly affected by the tumor size, tumor origin and focality.

Analysis of normal tissue in tumor burdened livers indicated significantly different AQP8 localization in cytoplasm compared to membrane and this effect was dependent on tumor size (Figure 5.6a: $p < 0.05$). Further, Tukey-Kramer HSD analysis indicated a significant difference between tumors less than 5cm and tumors 5-10cm (Figure 5.6a: $p < 0.01$). In patients with multifocal primary HCC, AQP8 was significantly lower in the cytoplasm, compared to membrane, in tumor cells (Figure 5.6b: $p < 0.01$).

5.3.4. AQP9 localization is significantly different in patients with histories of chronic alcohol or drug abuse.

Patients with a history of chronic alcohol or drug abuse had significantly lower AQP9 localization in cytoplasm of tumor cells compared to patients without a history of alcohol or drug abuse (Figure 5.7: $p < 0.05$).

5.3.5. Role of aquaporins 8 and 9 in apoptotic progression of human HCC cells ***in vitro***.

Apicidin is a known inhibitor of histone deacetylase and is a potent pro-apoptotic compound (Kwon *et al.* 2002). Apoptosis activates a cascade of caspases which results in the cleavage activation of caspase-3, the last known regulation point for apoptosis (Lemasters 2005). Huh7 cells, a human HCC cell line, were exposed to four concentrations of apicidin (1μM – 10 μM) in the absence or presence of 10μM $HgCl_2$ (added 30 minutes prior to apicidin) for 24 hours. Cells were then collected and analyzed by flow cytometry. Cell swelling in Huh-7 cells was confirmed to be an aquaporin mediated process by conducting a cell swelling assay. Huh-7 cells were osmotically challenged in the presence or absence of 10μM $HgCl_2$. Huh-7 cells swelled in response to osmotic challenge and the presence of $HgCl_2$(Figure 5.9a-b).

Cells were gated to select live cells then the percentage of live cells under each treatment condition was measured (Figure 5.9a top left). Analysis of these data indicated a significant

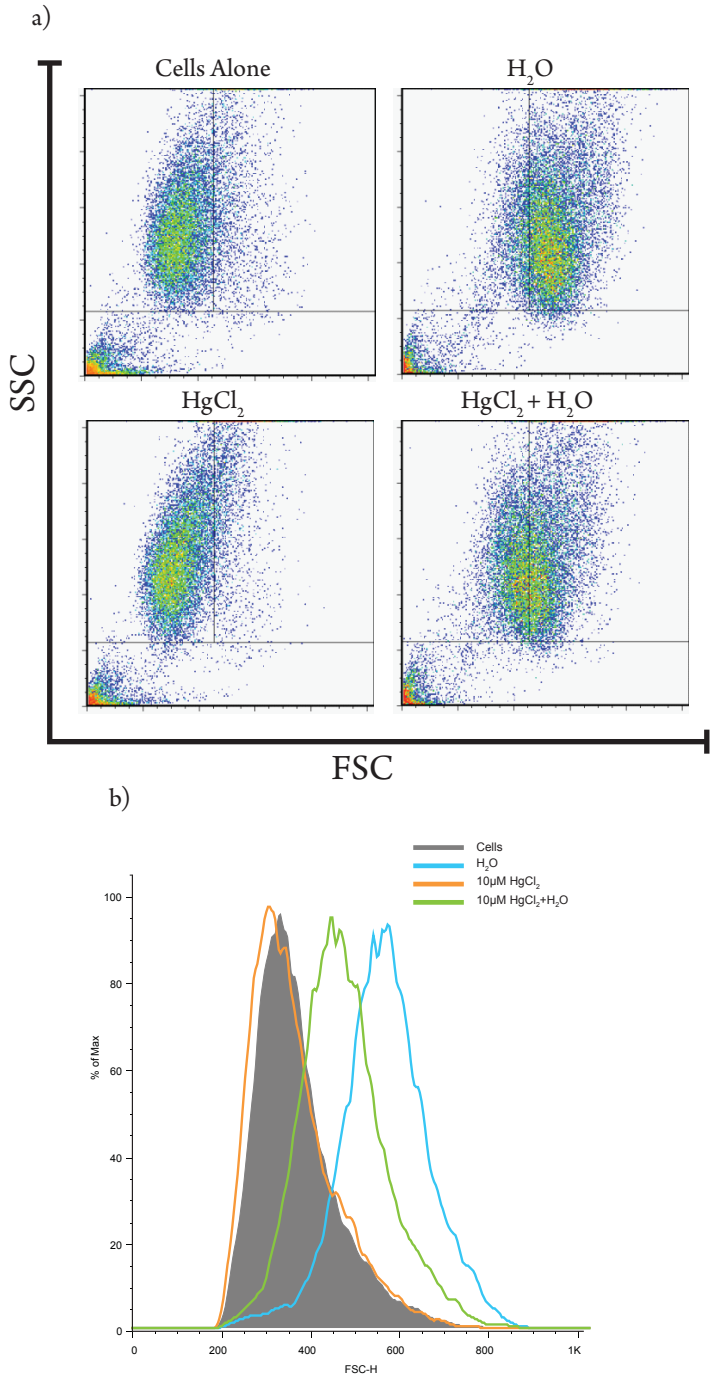


Figure 5.8: Effect of osmotic challenge on Huh-7 cells. a) Representative dot plots, showing the gating strategy used, of Huh-7 cells subjected to osmotic challenge in the presence or absence of a non-specific functional inhibitor of aquaporins ($HgCl_2$) and processed for flow cytometry to analyze changes in cell size. b) Representative histograms indicated that Huh-7 cells swells in response to osmotic challenge and swelling is reduced by $HgCl_2$ exposure.

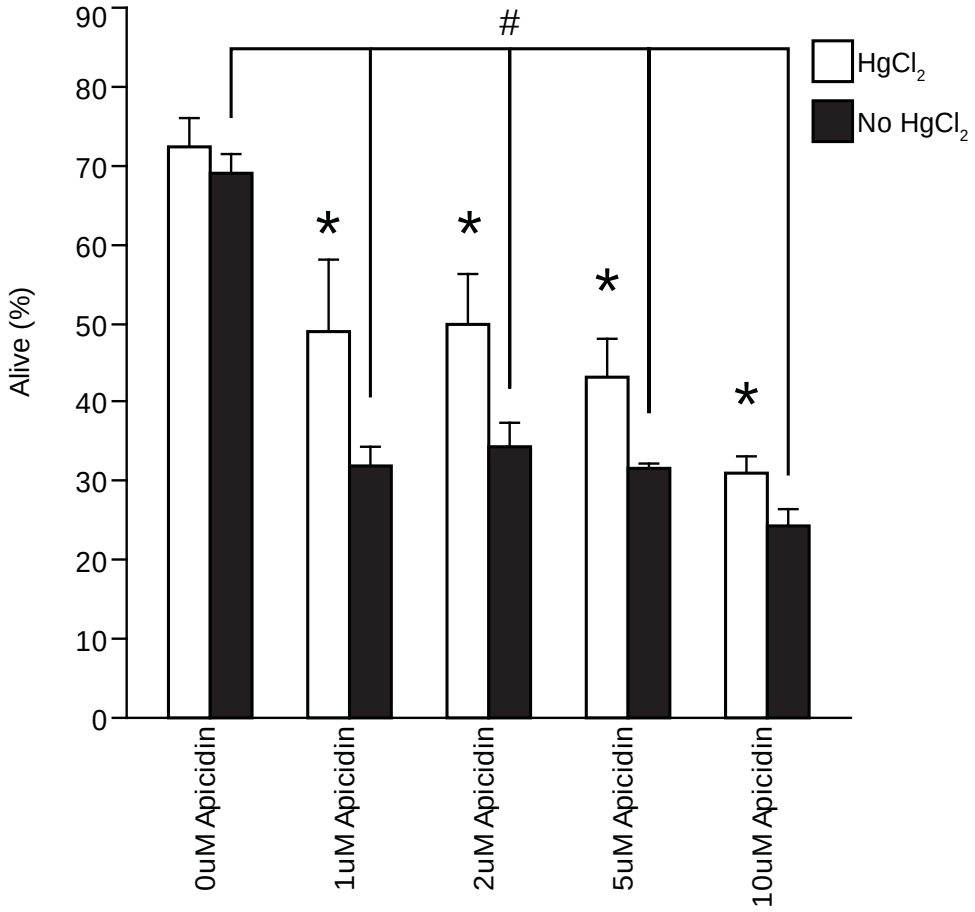


Figure 5.9: Effect of $HgCl_2$ on apicidin induced Huh-7 cell death. Huh-7 cells were exposed to four concentrations of an apoptotic stimulator (apicidin) in the presence or absence of 10μM $HgCl_2$ for 24 hours and processed for flow cytometry to analyze cell viability. # $p < 0.05$, ANOVA analysis, * $p < 0.01$, compared to no $HgCl_2$. n = 4.

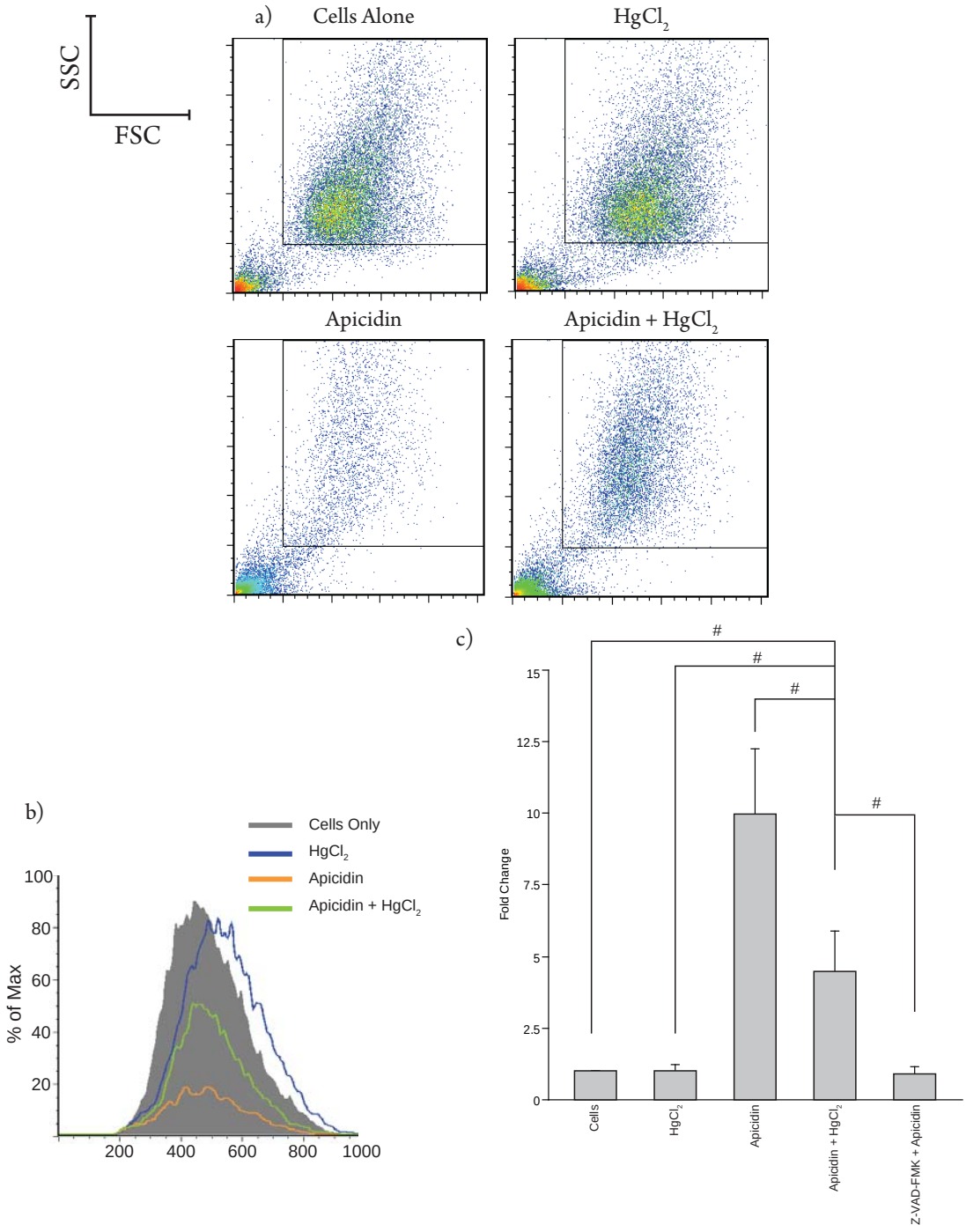


Figure 5.10: Effect of $HgCl_2$ on apicidin induced apoptosis in Huh-7 cells. Huh-7 cells were exposed to an apoptotic stimuli (2μM apicidin) in the presence or absence of aquaporin functional inhibitor (10μM $HgCl_2$) for 24 hours. a) Representative dot plots indicating gating strategy used in cells exposed to apicidin, +/- $HgCl_2$. b) Representative histograms indicating that Huh-7 cell viability is increased in Huh-7 cells exposed to apicidin and $HgCl_2$ compared to apicidin alone. c) Caspase-3 activity (bars represent fold change compared to Huh-7 cells alone) in Huh-7 cells exposed to apicidin +/- $HgCl_2$. Z-VAD-FMK is a pan-caspase inhibitor. # $p < 0.05$. n = 4.

decrease in the number of live cells in response to apicidin (Figure 5.9: $p < 0.05$). There was a significant increase in the number of live cells following apicidin exposure in the presence of $HgCl_2$ (Figure 5.9: $p < 0.01$).

To confirm that the cell death observed was due to apoptosis and was aquaporin mediated, a caspase-3 assay was performed. Huh-7 cells were exposed to 2μM apicidin for 24 hours in the presence or absence of 10μM $HgCl_2$. There was a significant difference between cells exposed to apicidin and $HgCl_2$ and Huh-7 cells or Huh-7 cells exposed to $HgCl_2$ alone (Figure 5.10c: $p < 0.05$). Also, there is a significant decrease in the level of caspase-3 activity in cells which were exposed to $HgCl_2$ and apicidin compared to apicidin alone (Figure 5.10c: $p < 0.05$). Lastly, there was a significant difference between Huh-7 cells exposed to apicidin and $HgCl_2$ and Huh-7 cells exposed to apicidin and the caspase-3 inhibitor Z-VAD-FMK (Figure 5.10c: $p < 0.05$).

5.4. Discussion

These studies demonstrated that AQP8 and 9 has altered expression and localization in human HCC. AQP8 was primarily located in the cytoplasm in both non-tumor liver and HCC. In contrast, AQP9 was localized to the membrane in non-tumor liver but in HCC AQP9 was predominantly localized to the cytoplasm. However, these differences in AQP8 and 9 localization did not appear to of clustered with any given known risk factor for HCC. The human HCC cell line, Huh-7 was used to assess the functional role of aquaporins in apoptotic progression in human HCC. Cell death was significantly inihibited as was caspase-3 activity in Huh-7 cells exposed to apicidin and $HgCl_2$ compared to apicidin alone. Indicating that apoptosis in Huh-7 cells mediated by aquaporins. This finding supports previous data reported from the McKillop laboratory indicating that apoptosis in rat HCC is also aquaporin mediated

(Jablonski *et al.* 2007).

Localization plays a critical role in aquaporin function. Only by membrane insertion can the rapid efflux of water occur (Masyuk 2006). Data from the current study indicated that AQP8 localization was low in the membrane in both non-tumor liver and HCC. More interestingly, AQP9 localization was also lower in the membrane. Therefore, these data suggest that HCC has lower total aquaporin membrane expression compared to non-tumor tissue. In fact AQP8 and 9 were barely detectable in HCC (Figure 5.2 and Figure 5.4). This presents an hypothesis. Perhaps it is this lack of membrane localized aquaporin that is responsible for chemotherapeutic resistence by HCC. Further, AQP9 is localized to the membrane in non-tumor tissue. This fact makes chemotherapy a contraindicated treatment option as the chemotherapeutic agent would be an inefficient apoptotic agent in tumor cells but a very efficient apoptotic agent in normal hepatocytes. So the question arises, is there a way to stimulate cells to express AQP8 or 9 on the plasma membrane? This question is partially addressed in this dissertation. Cyclic-AMP is known to stimulate AQP8 expression and translocation to the bile canaliculi in normal rat hepatocytes (Gradilone *et al.* 2003). So it was reasonable to examine if cAMP produces the same response to HCC cells. Data from this dissertation indicates cAMP does not stimulate AQP8 expression or translocation. However, cAMP is not the only potential candidate. There is a great deal of cross-talk in the cAMP pathway so perhaps one of the signaling molecules will stimulate AQP8 or 9 translocation to the membrane. Interleukin-6 (IL-6) is a cytokine involved in a number physiological processes, from liver regeneration (Koniaris *et al.* 2003) to T-cell differentiation (Diehl *et al.* 2002). IL-6 activates signal transducers and activators of transcription 3 (STAT3), a transcription factor that stimulates the expression of genes with cAMP response elements. Aquaporin 8

and 9 expression and localization in response to IL-6 stimulation was also examined in this dissertation. However, data indicated that IL-6 does not stimulate AQP8 or 9 expression or localization. However, data from the current *in vitro* studies suggested that perhaps apoptosis in human HCC is a partially regulated by aquaporins.

Though cAMP and IL-6 do not appear to be promising candidates for therapies for human HCC, there other possibilities for future studies. Protein kinase C (PKC) is involved in a number of physiological processes, among them the establishment of cell polarity (Rosse *et al.* 2010). Perhaps one process by which HCC arises is due to a loss of hepatocyte polarity. In normal liver, hepatocytes are polarized with distinct canilicular and sinusoidal membranes and since AQP8 and 9 are localized to these membranes (respectively) then perhaps AQP8 and 9 play some role in hepatocyte polarization. Therefore, it is tempting to speculate that since PKC plays a role in coordinating cell polarity that it might also regulate AQP8 or 9.

In conclusion, AQP8 and 9 membrane localization is significantly reduced in HCC. Further, these differences in AQP8 and 9 localization did not correlate with any given risk factor for HCC. Apoptosis of Huh-7 cells is partially regulated by aquaporin function suggesting that aquaporin function modulation might be an effective treatment for human HCC. Previous work in this dissertation reported that neither Cyclic-AMP nor IL-6 were effective at modulating AQP8 or 9 localization. There are, however, other candidiate genes which may be effective at modulating AQP8 and/or 9 localization. More work will be required to determine if a gene such as PKC modulates AQP8 and/or expression and localization.

CHAPTER 6:TETRACYCLINE CONTROLLED EXPRESSION OF AQUAPORIN 8 & 9 IN A RAT MODEL OF HEPATOCELLULAR CARCINOMA *IN VIVO*.

6.1. Introduction

Aquaporin 8 (AQP8) and 9 (AQP9) have been reported to play a role in apoptosis and expression of AQP8 and 9 are inhibited in a rat model of hepatocellular carcinoma (HCC) (Jablonski *et al.* 2007). However, no published study has reported examining tumor progression by modulating AQP8 or 9 expression *in vivo*. There are several methods available for *in vivo* genetic manipulation, popular techniques including: RNA interference (RNAi), cre-loxP knockout animals and promoter switch systems (tetracycline promoter switches being the most common) (Ryding *et al.* 2001). The strength of RNAi lies in the simplicity of constructing RNAi sequences. However, there are stability issues when using them *in vivo* (Pushparaj *et al.* 2008). Cre-loxP systems involves the generation of transgenic mice to express Cre recombinase bacteriophage topoisomerase which catalyzes site specific removal or inversion at loxP sites (Maddison *et al.* 2005). By flanking the gene cassette of interest with loxP sites, one can knockout that gene *in vivo* (Maddison *et al.* 2005). While, RNAi and cre-loxP systems are a vital tool in molecular studies both, *in vitro* and *in vivo* they are only suitable for knocking down or knocking out a gene. Aquaporin 8 and 9 expression is already inhibited in HCC *in vivo*, therefore the focus of these studies will be to overexpress AQP8 or 9 *in vivo*.

One of the oldest methods of overexpressing a gene *in vivo* is the promoter switch system (Ryding *et al.* 2001). Promoter switch systems permit controlled expression of any gene, is

relatively inexpensive, has been used extensively (Ryding *et al.* 2001) and is a validated tool, readily accepted by the scientific community. Originally developed by Gossen and Bujard, tetracycline controlled gene expression has been used in a wide variety of eukaryotic cells (Gossen *et al.* 1993a; Gossen *et al.* 1993b). The basic mechanism of the system involves the tetracycline repressor (tetR) protein which binds specifically to the tetracycline operator (tetO) DNA sequence (Gossen *et al.* 1993b). The binding of tetR to tetO renders genes with tetO in their promoters silent. An added feature of this system is that it is derived from E. coli. and eukaryotic genomes do not possess the tetO sequence, nor do they incode for tetR protein production. This serves to reduce leakage expression of the gene of interest. The original system was further enhanced by fusing the acidic domain of VP16 to tetR (this fusion being named rTA) resulting a more effective transactivator (Gossen *et al.* 1993a).

Along with being a validated and relatively inexpensive (compared to cre-loxP systems for example), tetracycline system has other advantages. First, in can provide graduated control of gene expression. Production of the tetR transactivator is dose dependent, thereby allowing for subtle manipulation of the gene of interest. Second, the system works equally well with analogues of tetracycline. Tetracycline is toxic to HeLa cells at 10μg/mL (Gossen *et al.* 1993b). Doxycycline, an analogue of tetracycline, has less toxicity than tetracycline and is more effective at inducing tetR production (Ryding *et al.* 2001). Also, doxycycline is more stable than tetracycline thereby allowing for doxycycline incorporation into rodent chow for *in vivo* activation of rTA.

The ability to use a tetracycline system *in vivo* is critically import to the study in this chapter and to the studies in this dissertation as whole. Aquaporin 8 and 9 have a document expression phenotype whereby AQP8 and 9 expression *in vivo* is barely detectable by immunofluorescent

analysis of tissue and freshly isolated tumors, *in vitro*, are relatively resistant to TGF-β induced apoptosis (Jablonski *et al.* 2007). However, culturing tumors results in significantly increased expression of AQP8 and 9 accompanied by the restoration of apoptotic sensitivity (Jablonski *et al.* 2007). One of the fundamental aims of this dissertation was to see if a small molecule, known to affect AQP8 localization in normal hepatocytes (Garcia *et al.* 2001), could prevent or delay the *in vivo* to *in vitro* expression profile change seen normally with AQP8 and 9 (Jablonski *et al.* 2007) in rat HCC. Chapter 3 looked at using cyclic-andensine monophosphate (cAMP) and interleukin-6 (IL-6) to perform this function. Unfortunately, neither cAMP or IL-6 significantly affected AQP8 or 9 expression or localization. However, this does not mean that no small molecule will work, only that it will be left to others to examine other potential candidate genes. Further, the lack of response by AQP8 and 9 to cAMP or IL-6 *in vitro* does mean that these aquaporins do not have a role in HCC initiation or progression. In fact, data from Chapter 4, examining a DEN mouse model of HCC, suggests that AQP8 and 9 play a role in HCC progression as evidenced by their being inhibited in late stage hepatic tumorigenesis. Further, data from human HCC samples suggests a role for at least AQP9 in tumor progression, since AQP9 was significantly lower in the majority of tumor plasma membrane as compared to non-tumor cells (Chapter 5). Neither of these studies, though, directly examines the role of AQP 8 and 9 in tumor progression as the model used was not an *in vivo* model of HCC and the other used humans which are typically only suitable for descriptive studies. The model of HCC used in the current study is a cell inoculation model using a clonal cell line, transfected with a tetracycline switch system (Tet-on) plasmid system. As the parent cell, H4IIE, was derived from the same rat strain into which it will be injected, the cells and the animal MHC matched. In the current study, a tetracycline switch system was used to control AQP8 or 9 expression *in*

vivo. This system placed expression of AQP8 or 9 under the control of a tetracycline promoter in H4IIE cells *in vitro*. Use of a tumorogenic cell line and customized rodent chow, thus made it possible to control aquaporin expression *in vivo*. *In vivo* manipulation of AQP8 or 9 allowed direct examination of the role these aquaporins play in tumor progression *in vivo*.

6.2. Methods

6.2.1. Assurances

All animals were housed in a vivarium approved by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) and all methods were approved by the Carolinas Medical Center Institutional Animal Care and Use Committee (IACUC)

6.2.2. Materials

Tet-On Advanced Inducible Gene Expression System (Tet-On system), HA antibody, TetR antibody, *pTRE2-pur-HA* plasmid, Doxycycline, G418 and puromycin were purchased from Clontech (Mountain View, CA). Rodent chow with 200ppm doxycycline was purchased from Research Diets (New Brunswick, NJ). Agar, LB broth, SOB, SOC and terrific broth powder were purchased from USB (Cleveland, OH). BD Falcon 5mL snap-capped tubes were purchased from Fisher Scientific (Waltham, MA). Lipofectin® reagent was purchased from Invitrogen (Carlsbad, CA). Carbenicillin was purchased from Sigma-Aldrich (St. Louis, MO). Suicide vector system (pJet1.2) was purchased from Fermentas (Glen Burnie, MD). DNA gel isolation kit and midi-plasmid isolation kit were purchased from Qiagen (Valencia, CA). Mini-plasmid isolation kit was purchased from Omega Bio-tek (Norcross, GA). *E.coli.* bacterial strain and all restriction endonucleases were purchased from New England Biolabs (Ipswich, MA). Primers were purchased from IDT (Coralville, IA).

6.2.3. Cell Culture Material and Methods

Material for cell culture were described in chapter 2.2.1. Cell culture methods were described in chapter 2.2.2-2.2.6.

6.2.4. Immunohistochemical Analysis

Materials for immunohistochemical as described in chapter 2.8.1. Immunohistochemical methods described in chapters 2.8.2 and 2.8.3.

6.2.5. Construction of ***pTRE2-AQP-HA***.

The Tet-On system works by a two plasmid system both of which must be stably expressed (Figure 6.1). The first plasmid contains a sequence encoding a modified version of the bacterial tetracycline repressor (TetR) protein. The modified TetR protein is constitutively expressed due to its promoter being fused to a CMV promoter (Figure 6.1a). The second plasmid contains a modified tetracycline response element (TRE) which permits the high affinity binding of the TetR protein only in the presence of doxycycline (a tetracycline analogue) (Figure 6.1b).

The entire CDS for AQP8 and 9 were amplified by PCR and blunt-end ligated into the pJet1.2 suicide plasmid. *E. coli.* were then transduced and plated on agar plates, containing 100μg/mL of carbenicillin. Plates were stored in a dry 37°C incubator over night. The next day, five colonies were picked and placed in 5mL tubes containing 2mL of LB broth with 50μg/mL of carbenicillin. Tubes were placed in a heated, orbital shaker (300 RPM) for ~16 hours at 37°C. Bacterial suspension was then transferred to a 2mL centrifuge tube and plasmid DNA isolated using mini-prep plasmid kit, as per the manufacturer's instructions. Primers were created to amplify the complete CDS of AQP8 or 9 along with restriction sites which were included with the primers (Table 6.1). The restriction sites were chosen because they do not

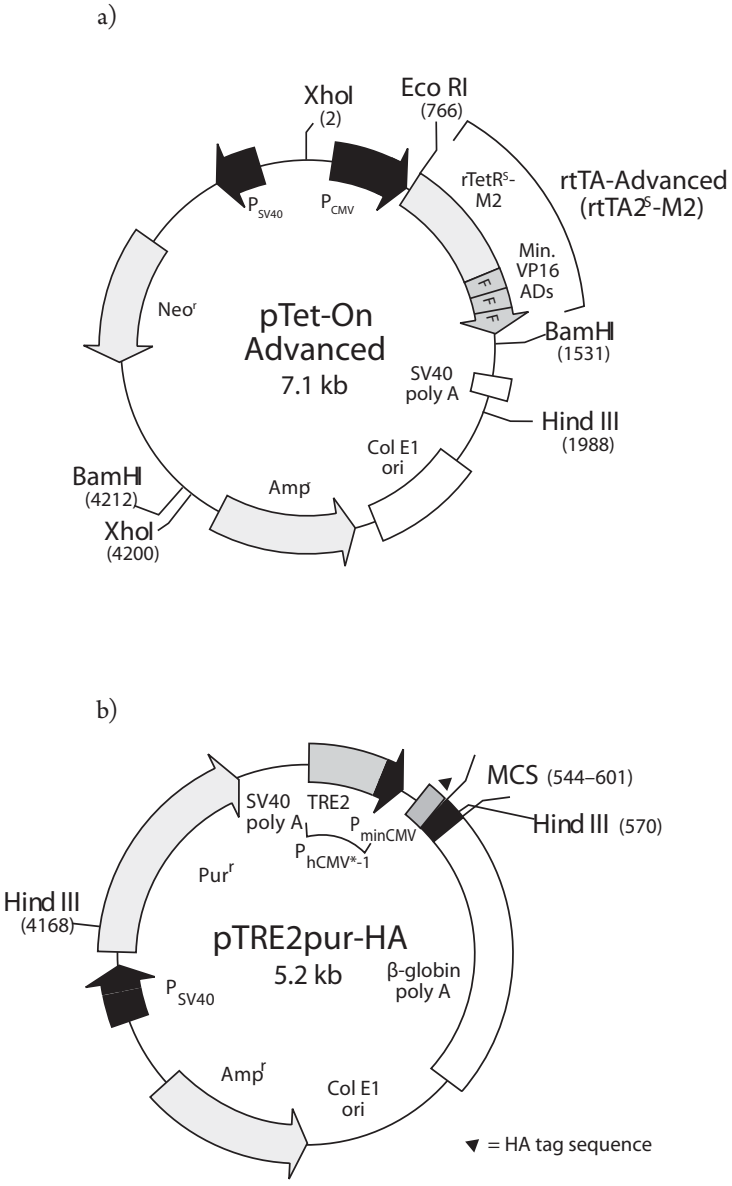


Figure 6.1: Maps of vectors used to generate pTet-AQP cells. a) The pTet-On Advanced vector expresses a transcription factor whose expression is initiated in response to tetracycline exposure. The transcription factor expressed (rtTA-Advanced) binds to the TRE2 (tetracycline response element 2). b) The pTRE2-pur-HA vector contains the gene of interest in frame with the TRE2 response element. In response to rtTA-Advanced binding, the gene of interest is expressed.

Gene	Sense (5'-3')	Antisense (5'-3')
AQP8-CDS	AGATATGTCTGGGGAGCAGA	TCACCTCGACTTTAGAATCA
AQP9-CDS	ATGCCTTCTGAGAAGGACGG	CTACATGATGACACTGAGCTCG
AQP8-Vec	ATATATCGATCCGCCATGTCT-GGGGAGCAGACGCC	ATATTCTAGATCACCTCGACTT-TAGAATCAGGCGGGTTTT
AQP9-Vec	ATATATCGATCCACCATGCCTTCT-GAGAAGGACGG	ATATTCTAGACTACATGAT-GACACTGAGCTCGTGTTTCTC

Table 6.1: Primer sequences used for gene amplification (CDS) and restriction site addition (Vec). The same restriction enzymes were used for AQP8 and 9 (ClaI and XbaI).

cleave inside either aquaporin gene and are only located on the multiple cloning site (MCS) of the *pTRE2-pur-HA* vector. The amplified AQP8 or 9 were gel purified and cut with restriction enzymes. *pTRE2-pur-HA* vector was also cut with the same restriction enzymes. AQP8 or 9 was then ligated into the ClaI, XbaI cut sites and *E. coli* were transduced with the created vector, plated on agar plates containing 100μg/mL of carbenicillin and stored in a dry incubator at 37°C overnight. The next day, five colonies were picked and placed in 5mL tubes containing 2mL of LB broth with 50μg/mL of carbenicillin. Tubes were placed in a heated, orbital shaker (300 RPM) for ~16 hours at 37°C. Following, the bacterial suspension was transferred to a 2mL centrifuge tube and plasmid DNA isolated using mini-prep plasmid isolation kit. Plasmids were then confirmed by restriction endonuclease analysis to determine the presence of AQP8 or 9. Once a clone was confirmed it was transduced into *E. coli*, plated on agar plates containing 100μg/mL carbenicillin and stored overnight in a dry incubator at 37°C. Then, a clone was picked placed into a 5mL tube containing 2mL of LB broth with 50μg/mL carbenicillin and placed in a heated, orbital shaker (300 RPM) for ~8 hours. After 8 hours, 200μL of the bacterial suspension was placed into an Erlenmeyer flask containing 50mL of LB broth with 50μg/mL of carbenicillin. The flask was incubated on an orbital shaker (100 RPM), at room temperature, overnight. The next day, plasmid DNA was isolated using a mini-prep plasmid isolation kit, following manufacturer's instructions. The plasmid yielded was used for H4IIE cell transfections.

6.2.6. Generation of double stable H4IIE cells.

Transfection of H4IIE cells was performed using Lipofectin® per manufacturer's instructions. All steps were carried out under sterile conditions. The same procedure was performed for both transfections. Briefly, 15,000 cells were plated in 6-well plates in 10%

FBS (*v/v*) medium without antibiotics. This seeding density resulted in approximately 40% confluence the following day. On that day, 20µL of Lipofectin was diluted into a total volume of 100µL of EMEM medium (medium only) and let stand at room temperature for 45 minutes. During this time, 2µg of plasmid DNA was diluted into a total volume of 100µL of EMEM medium (medium only). After the 45 minute incubation, complex formation was initiated by combining the Lipofectin solution with the DNA solution and incubated for 15 minutes at room temperature. Ten minutes after the beginning of this incubation, medium was aspirated from the 6-well plate and cells were washed once with EMEM (medium only). Next, 1.8mL of EMEM (medium only) was added to the Lipofectin-DNA complex solution, mixed gently and instilled into the 6-well plate. Plates were then incubated for 24 hours at 37°C / 5% CO_2 (*v/v*). After 24 hours, 1mL of 20% FBS medium (*v/v*) was added and the plate incubated for 48 hours at 37°C / 5% CO_2 (*v/v*). After 48 hours, each well of the 6-well plate was split into 2 D100 dishes contain selective medium. Selective medium was then changed every other day for two weeks and then every 2 days until clone colonies were visible (approximately 6 weeks in total). Clones were then picked and instilled into 12-well plates in selective medium. Clones were then expanded, tested and frozen down. For the pTet-On plasmid, clones were tested by Western blot for expression of TetR. The top three clones with the highest expression of TetR were used for transfections of *pTRE2-AQP-HA*. These transfections were also done with Lipofection and were tested by RT-PCR using forward primers internal to AQP8 or 9 and reverse a primer internal to HA (Table 6.2) (Figure 6.2).

6.2.7. Establishing tumorigenicity of transformed H4IIE cells

H4IIE cells exhibit diminished tumorigenicity *in vivo* with repeated passaging *in vitro* (Evans *et al.* 1977; Kovacs *et al.* 1977). The transfection and selection processes results in cell

of approximately P10. To ensure *in vivo* tumorgenecity, cells were inoculated subcutaneously into the flanks of male, ACI rats (175-225g). Twenty-one days later, the resultant tumors were present in approximately 15% of animals. These tumors were then harvested and cultured in selective medium, expanded. At P1 10^7 cells were inoculated into the left hepatic lobe of male, ACI rats (175-225g). Fourteen to sixteen days later tumors were resected (success rates were comparable to P0-P2 untransfected H4IIE) and cultured in selection medium. Cells were allowed to reach 80% confluency and used in the studies presented.

Prior to the experimental period, male, ACI rats (175-225g) were maintained on standard rodent chow and conditions. Seven days prior to inoculation, standard chow was removed and switched to standard rodent chow containing 200ppm doxycycline. *pTet-On/pTRE2-AQP-HA* H4IIE (*pTet-AQP* H4IIE) cells were used to initiate tumors in male, ACI rats (175-225g) as described in 2.3.

6.2.8. Necropsy

Fourteen days after *pTet-AQP* H4IIE inoculation, rats were euthanized, livers resected, and gross examination performed to detect tumor presence. Liver samples were then fixed and stored (+4°C) in 10% neutral buffered formalin until immunohistochemical analysis was performed.

6.2.9. Statistical Analysis

Tumors were considered to be cuboid in shape therefore tumor volume was calcuted by the equation: Volume = (x*y*z), where x is the width, y is hight and z the depth of the tumor. Student's t-Test was used to analyze data in this study and was performed using SAS JMP 8.0.2 (Cary, NC). $p < 0.05$ was considered significant.

Gene	Sense (5′-3′)	Antisense (5′-3′)
AQP8	TCTCATTGACGGCACCCATACACA	——
AQP9	TAAATGCCAAAGACCGTTGCAGCC	——
HA	——	ACCCATACGATGTTCCAGATTAC-GCTCTT

Table 6.2: Primer sequences for testing of pTet-AQP-HA constructs.

Figure 6.2: Test for the presence of AQP8-HA in H4IIE cells in three clones. *pTet-AQP8-HA* tumorogenicity was regained and clones were tested for the expression of HA tagged AQP8 by RT-PCR using a forward primer against AQP8 and a reverse primer against HA tag. Clone 2 was used for all experiments using *pTet-AQP8*.

6.3. Results

6.3.1. Constitutive AQP8 expression significantly affected tumor formation and tumor volume.

Male, ACI rats (175-225g) were placed on a doxycycline diet for seven days prior to surgery. After seven days, the left hepatic lobes of these rats were inoculated with 10^7 *pTet-AQP8* H4IIE cells. In parallel, 10^7 untransfected H4IIE cells were inoculated into the left hepatic lobe of different male, ACI rats (175-225g). Fourteen days later, rats were euthanized, livers harvested and a gross examination of the livers was performed. For rats with tumors, sample tissue was fixed and stored (+4°C) for further immunohistochemical analysis. Control animals (2 of 2) had tumors, and tumor volume measurements were taken (Figure 6.3), 50% (2 of 4) of doxycycline fed animals had tumors and volume measurements of the tumor were taken. Analysis of these data indicated that animals with constitutively expressed AQP8 formed significantly smaller tumors compared to regular H4IIE (Figure 6.4: $p < 0.01$).

6.3.2. pTet-AQP9 H4IIE cells fail to reestablish tumorigenicity.

Establishing tumorigenicity of *pTet-AQP* H4IIE cells requires that these cells be inoculated into ACI rats twice, once in the flank and then a second time in the liver. *pTet-AQP9* H4IIE cells formed tumors in the flanks of ACI rats with an approximate success rate of 15%. However, subsequent inoculation in livers failed to produce usable tumors. Of the liver group, one rat had an apparent cystic lesion which was later confirmed to be a tumor by histological analysis (Figure 6.5 a-b).

6.4. Discussion

Aquaporins play an important role in apoptosis by mediating the apoptotic volume decrease (AVD) (Jessica Chen *et al.* 2008). The AVD is a required event in apoptosis and

aquaporin activity affects the rate of apoptosis before AVD progression (Jablonski *et al.* 2004). Previous reports have shown that AQP9 is inhibited in human HCC (Padma *et al.* 2009). Chapter 5 of this dissertation reported decreased AQP8 and 9 expression in human HCC. Previous work from the McKillop laboratory reported decreased AQP8 and 9 expression in rat HCC (Jablonski *et al.* 2007). However, all of these studies, though important, were descriptive. The current study is the first to provide direct evidence that inhibiting AQP8 expression is sufficient to significantly inhibit tumor progression. In the current study, H4IIE cells were transfected with a vector placing AQP8 placed under the control of a tetracycline promoter. This permitted effective control of AQP8 expression. Constitutive expression of AQP8, in a rat model of HCC which was known to have inhibited AQP8 expression, significantly inhibited tumor progression. Unfortunately, technical issues prevented a similar examination of AQP9.

While these results are promising there are potential issues. First, with any stable transfection there is the possibility that the introduced DNA will be removed by the host cell. This potential was reduced by strigent antibiotic selection the transfected cells were placed under during *in vitro* propagation. Second, there is the potential that transfection reagents and/or high concentrations of antibiotics modified these cells to decrease their tumorgenecity. I submit that this potentiality is reduced by the multiple times these cells were passaged through a rat to restore the tumorgenecity of these cells. The greatest technical issue in this study was the failure to reestablish tumorigenicity in pTet-AQP9 H4IIE cells. There are many possible explanations but the one that I submit is most likely is a well known phenomena to molecular biologists but I have yet to see published in a peer reviewed journal. The recurrent issue that molecular biologists face has to do with cells, somehow, ejecting the gene of interest but retaining the resistance gene. That is, a cell loses the gene of interest but cannot

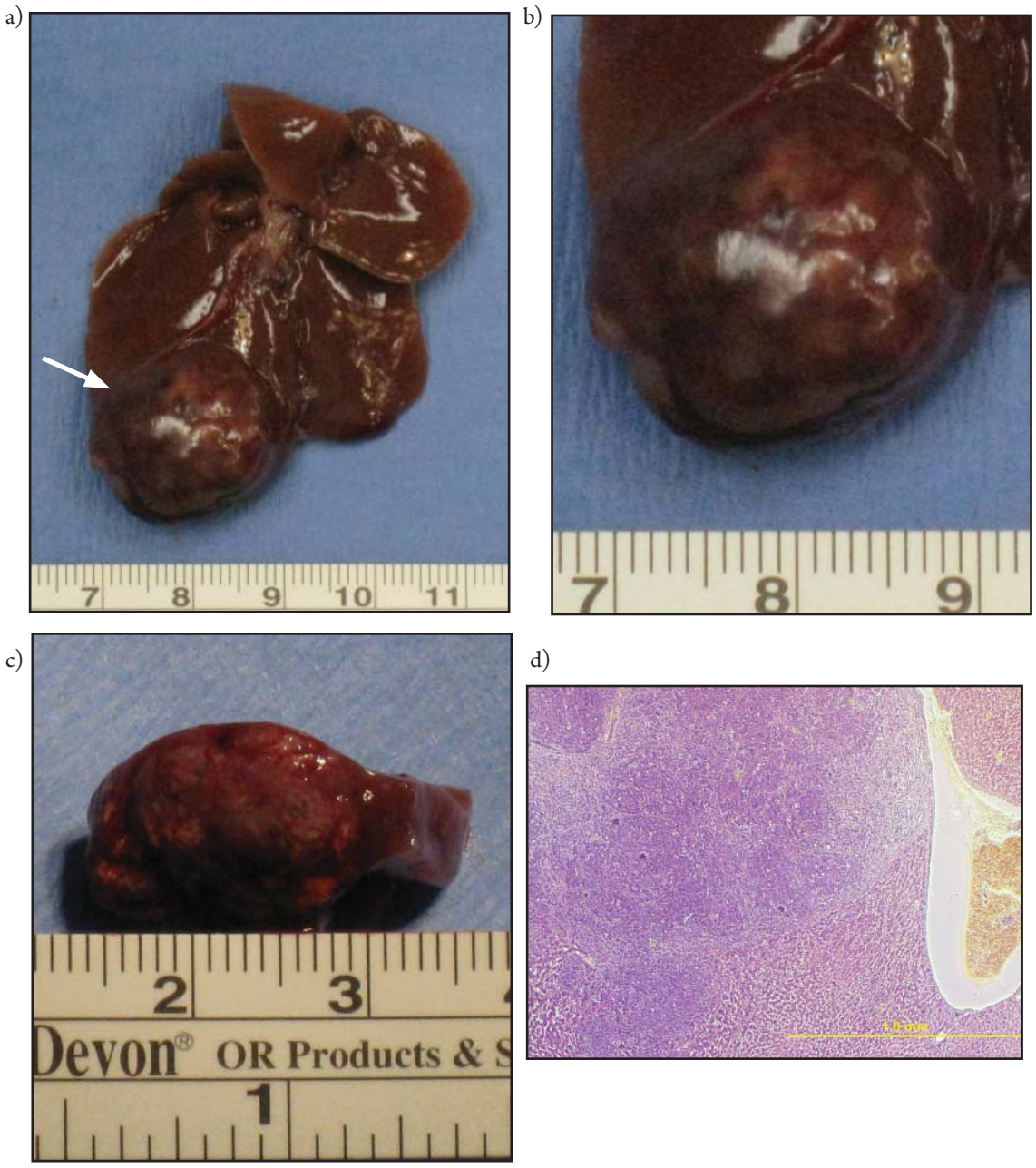


Figure 6.3: *pTet-AQP8* H4IIE cells form tumors in the absence of doxycycline. *pTet-AQP8* cells were injected into the left hepatic lobe of male, ACI rats. Approximately 14 days later livers were resected and a-c) tumors were evident upon gross examination. d) Tumor presence was confirmed histologically.

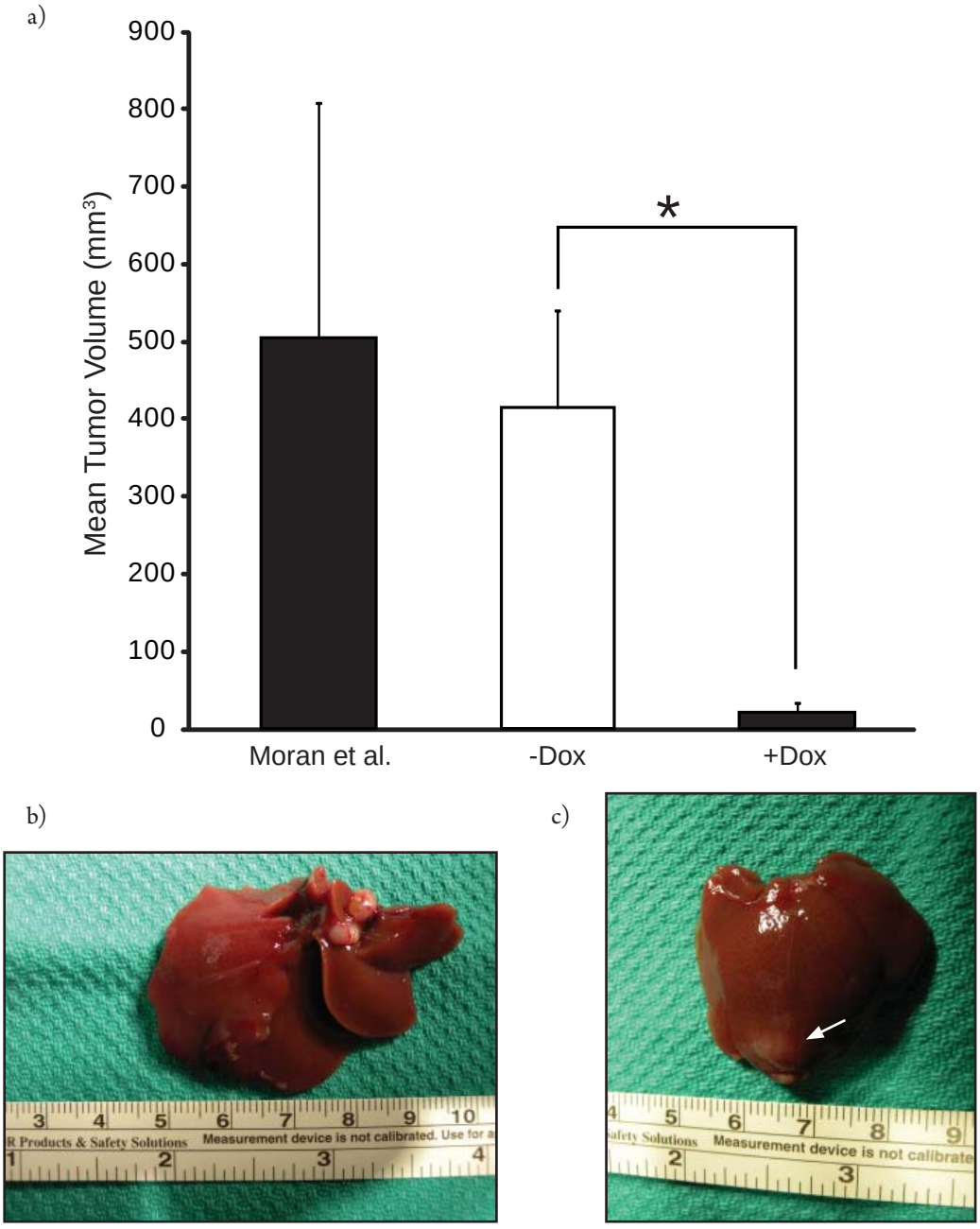


Figure 6.4: Effect of doxycycline on *pTet-AQP8* cells. *pTet-AQP8* cells were injected into the left hepatic lobe of male ACI rats in the presence (200ppm, 7 days prior to operation) or absence of doxycycline (Dox). a) Approximately 14 days later livers were resected at tumor volume measurements calculated. Of the rats fed dox, b) half of the animals did not have tumors, c) while the other half did have tumors. * $p < 0.01$. n = 4.

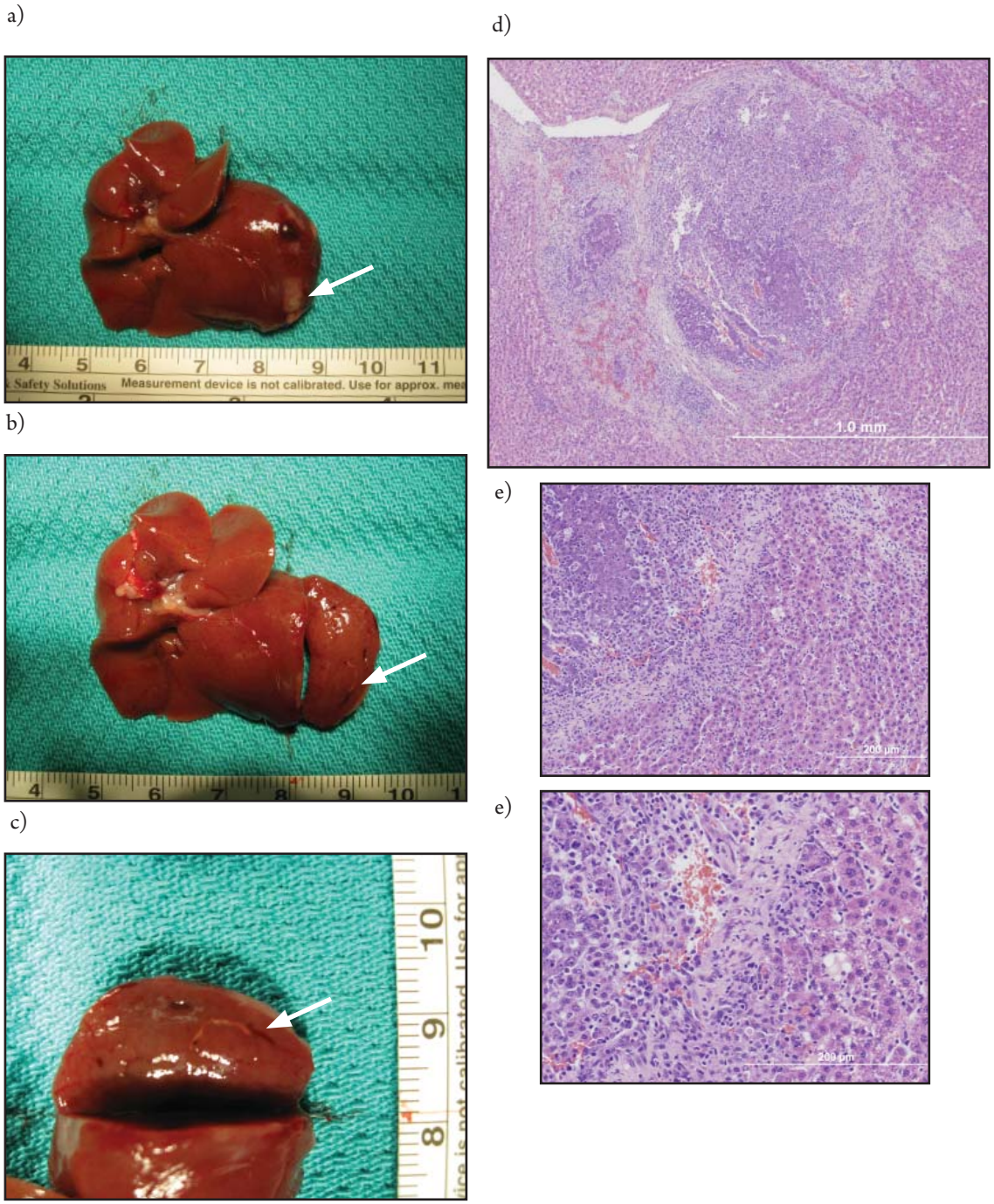


Figure 6.5: *pTet-AQP9* H4IIE cells did not regain tumorigenicity. *pTet-AQP9* H4IIE cells were injected into the left hepatic lobe of male, ACI rats in the absence of doxycycline (dox). Approximately 14 days later the livers were resected and examined for the presence of tumors. a-c) All animals had no clearly evident tumors upon gross examination, though an injection cyst was evident on one rat (white arrow). One animal had an apparent injection cyst but upon histological analysis it was confirmed to be a tumor by examination at d) 4x, e) 20x and f) 40x magnifications.

be killed by the selection antibiotic because it still retains the enzyme which neutralizes the antibiotic. Unpublished work by the McKillop laboratory suggest that the incidence rate of this phenomena can be as high as 90%. This puts a premium on conducting tests to confirm the presence of your construct. However, an issue that arose in the course of acquiring data for this dissertation is the difficulty transfecting H4IIE cells. Preliminary experiments involving siRNAs against AQP8 and 9 had to be abandoned because of the inability to get effective knockdown. Later, a collaboration between Dr. McKillop and Dr. Qi Lu (Carolinas Medical Center), using morpholino antisense oligonucleotides also yielded unsatisfactory results. However, using fluorescently labeled morpholinos yielded close to 30% transfection efficiency but still poor knockdown. Examination of the cells showed what appeared to be the morpholinos becoming localized within vesicles which were then being "ejected" from the cell. A potential side project is to examine this phenomena in greater detail. A first step might be to inhibit vesicular trafficking to examine if these morpholinos are indeed trapped in a vesicle.

Another solution to the technical issues encountered in this study would be to switch models entirely. The rat model used has several advantages. It is a well characterized model, is reproducible, uses a readily available strain of rats and a commercially available cell line. However, the difficulty transfecting H4IIE cells is enough to warrant consideration of other models. A similar model to the cell inoculation model used in this study is a mouse inoculation model. Hepa1-6 cells are a mouse hepatoma cell line derived from C57L mice. However, Hepa1-6 are MHC matched to C57BL/6 mice. In fact, a recent article demonstrated that Hepa1-6 cells form tumors in C57BL/6 mice following inoculation into the portal vein, resulting in widespread tumor nodules two weeks post inoculation (Chen *et al.* 2007). There are two advantages in switching to a mouse model. One, from a practical standpoint, standard

strains of mice are less expensive to purchase than rats and mice are less expensive to house. Two, from a scientific standpoint, there is a wide array of tools that are available when using a mouse background that just are not available on a rat background.

Findings such as the ones in this study always present the possibility that they might be translated into clinical therapies . However, the structure of AQP8 and 9 seem to preclude this. Despite best efforts, every proposed small molecule aquaporin inhibitor has failed (Verkman 2009). There is a known genetic loss-of-function mutation of AQP2 which result in diabetes (Fujiwara *et al.* 2005). However, this mutation is rare with less than one in 20 million births resulting in this mutation (Verkman 2009). The rarity of this event strong limits its therapeutic potential. There are no published hereditary conditions resulting from AQP8 or 9 loss-of-function mutation. In Chapter 3 of this dissertation, cAMP and IL-6 both failed to modulating AQP8 or 9 expression or localization. This is in contrast to published studies, in normal hepatocytes, that cAMP increases AQP8 expression and causes its translocation to the canalicular membranes (Garcia *et al.* 2001; Gradilone *et al.* 2003). However, these studies suggest that AQP8 plays a decisive role in HCC progression. Therefore, the answer may not lie in small molecule design, instead it might lie in effect non-viral construct delivery *in vivo*. Despite the lack of success, there is a new technology for introducing non-viral vectors *in vivo* and in situ. Electroporation has typically been used to introduce foreign DNA to cells *in vitro*. Recent advances have produced a technique involving the electroporation of tissue *in vivo* to introduce a non-viral construct to cells around the electroporation probe (Wells 2010). This technique suggests a future experiment whereby a rodent liver is inoculated with a tumorogenic cell line and then the introduced cells are transfected in situ with a construct containing AQP8 or 9.

CHAPTER 7:DISCUSSION

Hepatocellular carcinoma (HCC) accounts for 5.6% of all human cancers worldwide (Bosch *et al.* 2005). There are several risk factors for HCC. Aflatoxin, Hepatitis B and C virus infection, steatohepatitis and ethanol are known risk factors for HCC development (McGlynn *et al.* 2005; McKillop *et al.* 2006a; McKillop *et al.* 2005). However, the largest risk factor for HCC is cirrhosis, with 80-90% of HCC patients having underlying cirrhosis (Leong *et al.* 2005). Of the top fifteen most lethal cancers in the United States between 1997 and 2006, only HCC has an annual average percent increase of at least 2% in men and greater than 1% in women (Edwards *et al.* 2010). In fact, no other cancer has an average year-over-year percent change greater that 0.5% over the same period (Edwards *et al.* 2010). With a yearly fatality ratio of approximately one (meaning most patients die within a year of diagnosis) and a worldwide 5-year survival rate of just 6.5%, HCC is a highly lethal cancer compared to other types of cancer (Bosch *et al.* 2005). The clinical outcomes for HCC are in part due to the fact that no cancer is genetically stable (Hanahan *et al.* 2000). Therefore, no two HCC tumors are the same. Though some cancers do have known genetic aberrations which strongly contribute to their tumorigenicity (*BRCA1* in breast cancer being a heavily studied example) which present as therapeutic and/or diagnostic targets (Klauber-DeMore 2005). Unfortunately, there is no genetic marker for HCC (Leong *et al.* 2005).

Diagnosis of HCC typically starts with a blood test and an ultrasound examination of the liver. Measuring the levels of α-fetoprotein (AFP) in blood is a common test administered to

aid in the diagnosis of HCC (Ferenci *et al.* 2010). However, AFP is a poor diagnostic tool. In patients with HCC, 40% of these patients produce no AFP (Ferenci *et al.* 2010). Further, black African patients have normal AFP levels above the diagnostic level of 500ng/mL (Ferenci *et al.* 2010). Currently, ultrasound, computer-aided topography (CT) and magnetic resonance imaging (MRI) scans are the most common non-invasive means of diagnosis for HCC (Sherman 2007). Ultrasound is the first diagnostic tool used and with contrast-enhanced ultrasound an overall accuracy in differentiating malignant tumors from benign of 85% is achieved (Liu *et al.* 2010). However, ultrasounds are not practical for screening purposes and most people only present in clinic at the onset of symptoms by which point patients are usually presenting with advanced HCC (Clark *et al.* 2005).

The low 5-year survival time, the increasing death rate, the subclinical nature and the absence of accurate screening places a premium on effective treatment for HCC. Unfortunately, treatment options for HCC are limited for intermediate to advanced HCC and typically HCC is not detected early. However, with early detection the best clinical outcomes are possible since the most effective treatments are usually available (Llovet *et al.* 2000). For early stage HCC in the absence portal hypertension, surgical resection provides the best clinical outcome, with 5-year survival rates of greater than 75% (Llovet *et al.* 2000). Unfortunately, few patients present this early (Clark *et al.* 2005). For intermediate to advanced HCC, typical treatment options included surgical resection (in special cases), liver transplantation, ablation and embolization. For small tumors surgical resection is an option in certain cases, namely, a single tumor less than 5cm (or no more than 3 tumors less than 3cm) and absence of both portal hypertension and vascular invasion (Truty *et al.* 2010). Ablation therapies fall into two broad groups, chemical and thermal. Chemical ablation therapies involve ethanol, acetic

acid and hot saline (Lencioni *et al.* 2005). Thermal ablation therapies involve using radio waves, microwaves and lasers to destroy the tumor (Lencioni *et al.* 2005). In early stage HCC, ethanol ablation has comparable 3- and 5-year survival rates to surgical resection (Yamamoto *et al.* 2001a). Radiofrequency (RF) ablation also has survival rates comparable to surgical resection but RF has better 3- and 5-year survival rates than ethanol ablation (Lencioni *et al.* 2005). Embolization involves injecting microspheres which block blood flow to the tumor and typically, chemotherapeutic agents are included (Befeler 2005). Chemoembolization however does not significantly extent 1-year survival times and are not curative therapies (Befeler 2005). It should be noted that systemic chemotherapy is not effective in HCC because typically patients present with compromised liver function so higher dosages of chemotherapeutic agents are required (Tanaka *et al.* 2010; Zhu 2010). These higher dosages result in unacceptable toxicity effects for patients, including death (Tanaka *et al.* 2010). All of these therapy options suffer from the same weakness, 60% of patients die within five years and 80% of patients die within ten years (Yamamoto *et al.* 2001a).

Orthotopic liver transplantation (OLT) is the most effective treatment for HCC, providing a worldwide 5-year survival rate of 71% (Dutkowski *et al.* 2010). However widespread OLT for HCC treatment is compromised by the severe shortage of donor organs (Mazzaferro *et al.* 2008). The shortage of livers has resulted in the adoption of living donor liver transplantation (LDLT) (Dutkowski *et al.* 2010). However, OLT and LDLT has generated a number of ethical issues. Is transplanting a high risk patient (e.g. a recovering alcoholic or HCV positive patient) a justified usage of a scarce resource such as a cadaveric liver? The viability of cadaveric livers decreases with time where transplant within 24 hours is necessary to maintain optimum liver function (Guarrera *et al.* 2008). Therefore, if there are two potential patients for a liver but

one more than 24 hours away, is it ethical to transplant a recovering alcoholic because he is closer? Lastly, is it always permissible for a parent to donate a liver graft to their child? It would first appear that the answer would be yes but the eleven deaths that were reported in 2006 has slowed the rush to fill the shortage of cadaveric livers with live donors (Fan 2006). Regardless of the answers to these questions, given the rising mortality from HCC one thing is clear; standard treatments have not been sufficient in reducing deaths from HCC.

A central function of hepatocytes is to produce bile. Bile is comprised of more than 95% and is a major function of normal hepatocytes. In order to make bile, hepatocytes must transport water from sinusoidal space into the bile canaliculi. Previous work has demonstrated a role for aquaporins in bile formation by facilitating this water movement (Huebert *et al.* 2002). All cancers share six functional abilities: 1) self-sufficiency in growth signals, 2) insensitive to anti-growth signals, 3) they invade adjacent tissue and metastasis to distance sites, 4) they have limitless replicative potential, 5) sustained angiogenesis and 6) they evade apoptosis (Hanahan *et al.* 2000). For HCC, one other can be added, moderately to well differentiated HCCs do not typically produce bile (Dominguez-Malagon *et al.* 2001). This lack of bile production however carries other potential consequences; it may also assist an HCC cell in evading apoptosis. Previous work has demonstrated that aquaporins play an important role in apoptotic progression (Jablonski *et al.* 2007; Jablonski *et al.* 2004; Seitz *et al.* 2007). Previous work has also demonstrated that AQP8 expression and membrane localization is increased in response to cAMP (Garcia *et al.* 2001; Gradilone *et al.* 2003). Work in this dissertation has demonstrated that AQP8 and 9 localization to the membrane is lower compared to non-tumor tissue (Chapter 5). Proper function of aquaporins is dependent on membrane insertion as aquaporins are passive membrane channels (Frigeri *et al.* 2007). Aquaporins play a role in apoptosis and AQP8

and 9 membrane localization is reduced in HCC. Thus if HCC cells could be forced to express AQP8 and/or 9, then this would sensitize these cells to apoptosis. This hypothesis was an initial motivation for the work presented in this dissertation.

A previous report from the McKillop laboratory demonstrated that AQP8 and 9 expression is decreased in a rat, H4IIE inoculation model of HCC, *in vivo* (Jablonski *et al.* 2007). So I used this model to examine the possibility of maintaining the *in vivo* state *in vitro*. Previous work had demonstrated that in rat hepatocytes, AQP8 expression and localization to the canalicular membrane was stimulated by glucagon and that glucagon was signaling *via* a cAMP dependent pathway (Garcia *et al.* 2001; Gradilone *et al.* 2003). Therefore, cAMP may also regulate AQP8 (and potentially AQP9) expression and localization in HCC. The Jablonski paper also indicated that AQP8 and 9 was strongly expressed (compared to tumors) 24-36 hours after tumors were isolated from the liver and placed in cultured (Jablonski *et al.* 2007). So I examined whether I could preserve the *in vivo* state by culturing tumors in the presence of cAMP pathway modulators. These studies demonstrated, modulation of the cAMP pathway does not affect AQP8 or 9 expression in freshly isolated tumors immediately exposed to cAMP pathway modulators. So a logical next step would be to examine AQP8 and 9 in long term cultured H4IIE cells. Unfortunately, cAMP does not alter AQP8/9 mRNA or protein expression, nor does cAMP affect AQP8/9 localization (membrane or cytoplasm). In normal rat hepatocytes, AQP9 expression and localization are not affected by cAMP signaling (Soria *et al.* 2009).

IL-6 is a pleiotropic cytokine involved in liver regeneration (Koniaris *et al.* 2003), is mitogenic in some cells (Culig *et al.* 2005) and growth inhibitory in others (Moran *et al.* 2005). Further, as described in Chapter 3, the promoter regions of AQP8 and 9 contain response elements some of whose transcription factors are activated by IL-6 signaling. Also, because of

the results from the cAMP pathway modulation experiments, it would be interesting to examine if another signaling pathway would affect AQP8 and 9 localization the same way as cAMP pathway modulation did. As with the cAMP pathway modulation experiments, recombinant rat IL-6 (rrIL-6) did not affect AQP8 or 9 mRNA or protein expression. However, AQP8 membrane localization was affected by rrIL-6 exposure.

To examine the function affect of cAMP pathway modulation and rrIL-6 exposure, I performed a cell swelling assay following exposure to cAMP pathway modulators and rrIL-6. Interestingly, while cAMP pathway modulation significantly affected H4IIE cell swelling in response to osmotic challenge, rrIL-6 did not.

An unresolved question in HCC is, by what mechanism is AQP8 and 9 expression inhibited *in vivo*? Perhaps a better way to state the question is not, "by what mechanism" but "by what mechanisms". There is substantial cross-talk between IL-6 and cAMP (Irvin *et al.* 2001) and it is reasonable to examine whether they stimulate the same response from AQP8 and 9. In this case however, they stimulated AQP8 or 9 expression in a counter intuitive way. The AQP8 promoter region contains a cAMP response element and yet cAMP pathway modulation did not affect AQP8 expression or localization. The AQP9 promoter region does not contain a cAMP response element and yet cAMP pathway modulation significantly affected AQP9 localization to the membrane. Conversely, the AQP9 promoter region contains AP-1, NF-κB and C/EBP response elements (all known downstream target of IL-6 pathway activation (Fausto 2000)) and yet activation of IL-6 signaling did not significantly affect AQP9 expression or localization. A possible explanation of these data is that AQP8 and 9 signaling occurs through entirely different signaling pathways and because the amount of cross-talk between cAMP, IL-6 and the rest of the signaling pathways for which cAMP and/or IL-6

are constituents. Table 7.1 shows a complete list of promoter binding sites for AQP8 and 9. Perhaps AQP8 and 9 expression and localization are stimulated through a pathway that is not *a priori* apparent. For example, perhaps instead of 8-Br-cAMP, expose H4IIE cells to glucagon. Perhaps due to genetic instability, signaling through glucagon receptors activates a G-protein other than G_s, or maybe glucagon activates a signaling pathway not associated with glucagon in normal hepatocytes. Future study will be required to further elucidate how cAMP and IL-6 signaling regulates AQP8 or 9 expression and localization.

One fundamental question about the inoculation rat model of HCC is: to what extent does this model accurately represent human HCC? A rationale way to approach such a question is by switching the model and observing if the observations from the inoculation rat model hold. A potential criticism of the H4IIE inoculation model is that it is essentially using rats as "an *in vivo* cell culture" model. ACI rats are immunocompetent but the H4IIE cell line was derived from ACI rats and is MHC matched. Therefore, the probability of host rejection of H4IIE cells is very low. Also, the usual immune response to tumors would also be absent. The immune system of ACI rats would, most likely, not be stimulated in a way that a foreign tumor would. In brief, what is missing from the H4IIE inoculation model is tumor initiation.

In 1971, Alfred Knudson demonstrated that retinoblastoma arises through biallelic disruption (Knudson 1971). Biallelic disruption is the core of the two-hit hypothesis. It states that cancer requires that both alleles of a tumor suppressor gene be mutated. Further, the mutation of both alleles is sufficient for cancer to arise. The two-hit hypothesis was controversial in Knudson's day since data presented at the time indicated that multiple gene mutations were also observed in patients, suggesting that biallelic disruption alone was insufficient for cancer to arise (Ashley 1969). Indeed, the two-hit hypothesis is still a

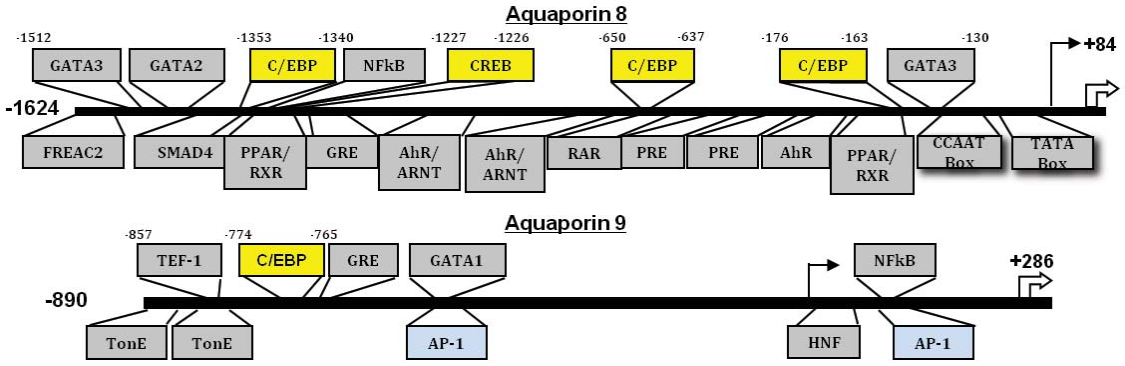


Table 7.1: Complete promoter map for AQP8 and 9. Downstream targets for cAMP (yellow) and IL-6 (blue) are highlighted.

controversial topic (Paige 2003). Though, in the thirty-nine years since Knudson published his hypothesis many tumor suppressor genes have been identified that support his "two-hit hypothesis" (Cook *et al.* 2000; MacPherson *et al.* 2007). However, there have also been several genes identified that are tumor suppressors but a single mutated allele is sufficient to abrogate the function of these genes, thereby resulting in cancer (Paige 2003). One area of agreement is that whether a given cancer requires two mutations or many, cancer does arise through a two stage process (Ashley 1969; Knudson 1971). The two stage hypothesis of cancer states that for a tumor to arise an "initiation" stage must occur whereby cells experience a mutational event which confers neoplastic potential and a "promotion" stage whereby any number of agents confers limitless replicative potential to the cell (Berenblum *et al.* 1949; Hanahan *et al.* 2000). Later, a "progression" stage was added to described the complex events that lead to, and promote, metastasis. Therefore, the answer to the question regarding to what extent does the H4IIE inoculation model accurately represent human HCC suggests that this model is one best suited to study HCC progression but is silent in studies where initiation and promotion are of interest. Thus, this would make the inoculation model useful for studying the progression stage of human HCC. Human cancers however do follow the two stage model (Ashley 1969). An additional point to be highlighted is that the incidence of human HCC is not gender neutral (Bosch *et al.* 2005). Males are more than three times more likely to develop HCC than women (Bosch *et al.* 2005). Therefore, to gain a more complete picture of the role of AQP8 and 9 in cancer requires the usage of an animal model that includes an initiation step and both genders.

To examine AQP8 and 9 expression and localization in a model of cancer involving initiation, a diethylnitrosamine (DEN) mouse model, with males and females, was used. Fifteen day old C3H mice were injected with DEN (1mg/kg). Mice separated into groups where one

group of mice exposed to DEN was euthanized at 24 weeks (DEN-24wk) and another group of mice were euthanized at 48 weeks (DEN48wk). Untreated mice were euthanized in parallel as control. For both AQP8 and 9, DEN48wk mice had significantly lower membrane localization compared to DEN-24wk mice. Further, DEN-48wk mice had significantly lower AQP8 and 9 membrane localization compared to either normal 24 week mice or normal 48 week mice. DEN-48wk mice had significantly higher AQP8 and 9 cytoplasmic localization compared DEN-24wk mice. Lastly, male, DEN-48wk mice had significantly higher AQP8 cytoplasmic localization compared to female, DEN-48wk mice.

In the H4IIE inoculation model, expression of AQP8 and 9 was inhibited not localization. However, in the DEN mouse model, AQP8 and 9 expression does not appear to change, rather AQP8 and 9 localized differently depending on exposure to DEN. Interestingly AQP8 was localized differently depending on the sex of the mouse, while AQP9 was not. Human HCC has gender differences as well and perhaps human AQP8 plays a role in the higher incidence rates in males compared to females. However, these differences must be viewed cautiously. First, at no point did I detect AQP8 or 9 membrane staining greater than 2.2 or cytoplasmic staining greater than 3.1 in the mouse model samples. I would not classify this scoring as indicating heavy expression. This leads to the second point, immunohistochemical scoring has a subjective component. It is semi-quantitative and for this reason the detected differences, while important, must be approached carefully. In the end, the fact that H4IIE inoculation model and the DEN mouse do not match exactly is not surprising. These models start from different stages of cancer and the differences detected may just be a reflection of this fact. Diethylnitrosamine is carcinogenic in rats as well and in similar dosages, so perhaps to truly compare these models we either need to use a DEN rat model or a cell inoculation mouse model; one does exist. Hepa1-

6 cells are a mouse hepatoma cell line derived from C57L mice. C57L mice MHC matched to C57BL/6 mice and there is a published study demonstrating the tumorigenicity of Hepa1-6 cells in C57BL/6 mice (Chen *et al.* 2007).

What these differences between the mouse and rat models actually indicate is that AQP8 and 9 expression and localization need to be examined in human HCC samples. At bottom, these models are designed to mimic the human disease and it is by examining human disease that an honest evaluation of these models can take place. Therefore, human HCC samples were acquired and immunohistochemical analysis was performed. Analysis of these samples demonstrated that AQP8 is predominantly localized to the cytoplasm in both non-tumor liver and HCC. Further, AQP8 membrane localization is unchanged between non-tumor liver and HCC. This was true whether patients had underlying cirrhosis or not. Aquaporin 9 however, did exhibit a dramatic reduction in membrane localization in HCC compared to non-tumor liver. Though there was no difference in AQP9 membrane localization in HCC, in cirrhotic versus non-cirrhotic patients. An original hope for this study was that AQP8 and/or 9 expression and/or localization would correlate with a risk factor for HCC. There are not any effective screens for human HCC (Marrero 2005). Further, there have been no confirmed inheritable genetic factors that contribute to HCC (Leong *et al.* 2005). So finding a protein marker that correlates with a subset of patients whom could then be studied to determine if that patient population has worse clinical outcomes is of clear clinical value. Unfortunately, the greatest AQP8 or 9 localization changes in membranes between nontumor liver and HCC did not reveal any common risk factor. Though one must use care when interpreting studies in humans. First, the sample size in this study was small (23 patients). Carolinas Medical Center specializes in providing thermal ablation therapies. The number of surgical resections done

is smaller compared to other hepatopancreobilliary surgical centers. Therefore, the issue may be that there is a risk factor for HCC that correlates with AQP8 and/or 9 expression and/or localization but the sample size we have available is too small to detect it.

As part of the human study, I also examined the functional effect of aquaporins on cell swelling and apoptosis in a human cancer cell line (Huh-7). Exposing Huh-7 to osmotic stress resulted in cell swelling and cell swelling was inhibited by blocking aquaporin function. Further, exposing Huh-7 cells to an apoptotic agent in the presence or absence of $HgCl_2$ (a non-specific aquaporin inhibitor) demonstrated that inhibiting aquaporin function inhibited caspase-3 activity. These data support similar experiments performed in H4IIE cells (Jablonski *et al.* 2007).

So far I have provided data that suggests that AQP8 and 9 can be stimulated to localize to the membrane by activating either the IL-6 pathway or the cAMP pathway (Chapter 3). I have also shown that inhibiting aquaporin function, inhibits caspase-3 activity (Chapter 5). What ties these two sets of experiments together is an effort to alter the balance between pro- and anti-apoptotic factors in favor pro-apoptotic factors. We know that cancer cells, in general, evade apoptosis (Hanahan *et al.* 2000). So inhibiting that ability to evade apoptosis would most certainly be a valuable clinical tool in treating HCC. However, systemic IL-6 injections would probably have extremely unsatisfactory side effects and I do not believe that systemic injection of a cell permeable analogue to cAMP would fare any better. So if stimulating an HCC cell to express AQP8 and/or 9 on the plasma membrane is not feasible, then perhaps inserting AQP8 and/or 9 into the plasma membrane might be a more efficacious course of action. Though even if one can get AQP8 or 9 into a cell, would introduction of a either of these be sufficient to inhibit tumor growth? To find out I transfected H4IIE cells with a Tet-on construct which

allows for the controlled expression of AQP8 or 9 by the introduction of tetracycline (or analogues of tetracycline such as doxycycline). The Tet-on system is a double plasmid system that requires that a cell stably express both plasmids. But once this is achieved, the gene of interest can be expressed by exposing the cell to doxycycline. H4IIE cells bearing this Tet-on system, engineered to express AQP8 in response to doxycycline, were injected into ACI rats pretreated with standard rodent chow containing doxycycline. The rats injected with the transfected H4IIE cells, which also received doxycycline formed significantly smaller tumors than untransfected H4IIE cells. This finding is significant because it is direct evidence which suggests that modulating aquaporin expression *in vivo* can inhibit tumor progression. There was however an interesting issue which arose. The tumorigenicity of H4IIE is inversely proportional to the passage number (Evans *et al.* 1977). The transfection protocol results in H4IIE cells that are approximately passage 10 by the time they stably express both plasmids. Unfortunately, these cells are not tumorigenic in the liver of ACI rats. But tumorigenicity can be restored by inoculating the cells into flanks of male, ACI rats, culturing and expanding the resultant tumors, then inoculating these cells into the liver, culturing and expanding the resultant tumors and at the end of this H4IIE cells are tumorigenic in the liver. For H4IIE cells transfected with the AQP8 construct this is exactly what we observed, for AQP9, liver tumors never developed. The least likely possibility as to why the H4IIE cells with the AQP9 construct did not work as expected is that the construct was not there to begin with or degraded by the cell at some point. These cells were almost always under very hard selection and always the week before any inoculation into rats. To give an indication of the selection pressure these cells were under consider the following: the plasmids provided resistance to G418 and puromycin. The recommended dosage (from Sigma) of G418 is 400μg/mL and the recommended dosage of

puromycin is 1-10μg/mL. I used 1mg/mL of G418 and 40μg/mL of puromycin. One potential possibility why *pTet-AQP9* H4IIE did not regain tumorigenicity is that AQP9 is a potent inhibitor of tumor progression and the leaking expression of AQP9 was sufficient to halt tumor formation. It is rare that tetracycline switch systems are completely binary, there is always some leaking expression of the gene of interest. In this case though this is not a confounding variable for the actual experiment. There is little to no AQP8 or 9 expression with *in vivo* tumors (Jablonski *et al.* 2007) so what leaking expression there was would not affect the results as I continuously treated the rats with doxycycline. But if AQP9 is in fact a potent inhibitor, much more so than AQP8, then the leaking expression would result in no tumor formation in the preliminary stages as the tumorigenicity of these cells is already reduced. The tumors resulting from inoculation with the AQP9 constructs did exhibit some interesting phenotypes. First, fewer flank tumors resulted from inoculation with the AQP9 constructs compared to the AQP8 constructions. Second, while this was not quantified, the flank tumors from the AQP9 constructs appeared to have a size limit. For untransfected H4IIE, inoculation into the flank can result in extremely large tumors if the tumor is not resected within approximately 14-20 days. But in the case of the AQP9 construct, the tumors tended to appear in the small range (4-6mm) in the few instances (≈ 15%) that tumors formed at all. Now that data suggests that expressing AQP8, in a context where it would not normally be expressed, inhibits tumor progression, the question of what inhibits AQP8 and 9 expression in HCC becomes very important.

Future studies on this project should focus on the question of by what mechanism is AQP8 and 9 down regulated in HCC. Other small molecules can certainly be examined. Protein kinase C (PKC) has been shown to regulate aquaporins in the brain (Yamamoto *et al.* 2001b). Further, it also plays a role in cell polarity (Rosse *et al.* 2010). In normal hepatocytes,

AQP8 and 9 are inserted into lipid rafts, into different membranes of the cell (Mazzone *et al.* 2006), perhaps they too play a role in cell polarity. Another possibility is ionic calcium (Ca^{2+}). Aquaporin 2 trafficking is regulated by intracellular Ca^{2+} mobilization (Balasubramanian *et al.* 2008; Szaszak *et al.* 2008). Further, the HBV antigen, HBx, has been reported to activate Ca^{2+} signaling (Bouchard *et al.* 2001). This is not an exhaustive list of candidates and while they should be studied, I submit that efficacious treatments for HCC lie elsewhere; specifically, they lie with gene therapy.

Work has already begun on brining gene therapy to the bedside and there are several clinical trials ongoing (Hernandez-Alcoceba *et al.* 2006; Hwang 2006). All but one of the trials in progress uses viral vectors as the delivery system (Hwang 2006). I submit that a better way to proceed would be non-viral plasmids introduced *in situ*. My objection to the usage of viruses in this instance is that the benefits do not out weight the costs. When introducing a virus into a patient you run the risk of initiating autoimmune disease (Chistiakov 2010). Also, the viral vector can only be used once as the host will develop an immune memory response to the virus. Further, there is no need to use viral vectors, there are other technologies which not only permit the introduction of foreign DNA into the cell but that can also be performed *in situ*.

Electroporation has been used for the introduction of foreign DNA for almost 30 years (Neumann *et al.* 1982). However, for most of that 30 years it was of limited use for *in vivo* studies due to having to put the sample in a special sample chamber (Neumann *et al.* 1982). Recently, however electroporation has been successfully used in various tissues, including the liver (Wells 2010). Further, the recent introduction of commercial ultrasound and microbubble-aided transfection, has also been successfully used in liver and adds another tool for *in vivo* gene transfer (Wells 2010).

The place to start examining how AQP8 and 9 are inhibited in HCC is by having detailed data on when this inhibition occurs. Data presented in this dissertation suggests that inhibition occurs between 24 and 48 weeks. In order to perform a detailed analysis, a tighter time frame will be needed. Bioluminescence imaging would be an ideal technology for doing detailed time courses of AQP8 and 9 inhibition. A relatively simple method to examine this would be to use Hepa1-6 cells which would be transfected with AQP-firefly luciferase fusion protein. These cells would then be inoculated into the liver and bioluminescence measures would be taken daily. With these data, a detailed examination of the genes involved as AQP8 and/or 9 expression is inhibited could be performed using micro arrays to select potential targets.

Another experiment would be to breed condition *cre-loxP-AQP* mice which will allow for a liver specific AQP8 or 9 knockout. These mice can then be used in a DEN model to examine what role AQP8 and/or 9 play in tumor initiation and early progression. Lastly, a Tet-on conditional *cre-loxP* mouse could be generated. By floxing the DNA sequence encoding the Tet-on transactivator protein, it would be possible to specifically, potently inhibit AQP8 or 9 expression at an investigator chosen time point. Further, AQP 8 and 9 knockout mice are available, both of which are on a C57BL/6 background (Rojek *et al.* 2007; Yang *et al.* 2005). This is important because *cre-loxP* animals have to be breed from crossing *cre* mice with mice which have the gene of floxed (i.e. flanked by *loxP* sequences). Using a common mouse strain such as C57BL/6 means a *cre* animal can be purchased instead of breeding. Therefore, using AQP8 or 9 knockout mice for the *loxP* mice in the system, generates mice which have a liver specific, controllable expression of AQP8 or 9.

In conclusion, AQP8 and 9 membrane localization, in an *in vitro* model of HCC, are affected by IL-6 and cAMP, respectively. Further, in a DEN mouse model of HCC, AQP 8

and 9 membrane localization is lower the longer HCC progresses. Though during early HCC, in the DEN model, AQP8 membrane localization is lower in female mice compared to males. In human HCC, AQP9 membrane localization is lower in HCC compared to non-tumor but there is no difference in AQP8 membrane localization between non-tumor liver and HCC. This suggests that while neither the rat nor mouse models of HCC are a perfect fit, each is suitable for modeling specific stages of cancer. Also, there appears to be no common human HCC risk factor which correlates with the largest differences in membrane localization between non-tumor liver and HCC. Lastly, constitutive AQP8 expression results in inhibited tumor progression in a rat model of HCC. The role of AQP9 expression in this model remains unclear.

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