ABSTRACT


Animal models of human diseases are essential to elucidate the molecular mechanisms of disease and to develop and evaluate diagnostic and therapeutic approaches. Fish are attractive model organisms due to the easy and economical maintenance, large number of embryos produced, external development of transparent embryos, low background incidence of neoplasia, well annotated genome, availability of inbred strains and established techniques to manipulate gene expression and create mutant lines. Consequently, it is not surprising that fish are increasingly used in biomedical research, high-throughput screening of chemicals, and environmental monitoring. It is known that notable mechanistic differences in disease pathogenesis often exist between humans and the relevant animal models used. These differences do not preclude using a species as a model of human disease but it is important to know and understand these differences and the limitations/special characteristics that apply.

In fact, the degree of similarity of fish models have with appropriate mouse models and humans has become a critical issue. Although it is established that certain aspects of tissue and cellular organization, reaction to injury, and neoplasms are remarkably similar histologically between humans and fish, little is known regarding the specific molecular mechanisms leading to liver pathologies in piscine models. In this dissertation, the medaka fish was used as a model for investigating liver fibrosis and regeneration following injury.

In chapter 2, we developed a dimethylnitrosamine (DMN)-induced fish model of hepatic injury in Japanese Medaka (*Oryzias latipes*) and anchored expression of key genes involved
in the pathogenesis of fibrosis with the development of hepatic fibrosis and neoplasia. We demonstrated that the main cellular and molecular events in the pathogenesis of hepatic fibrosis in mammals and medaka fish exposed to DMN are conserved. Hepatocellular injury is followed by activation of hepatic stellate cells, TGF-β pathway activation, change in the balance between matrix metalloproteinases and tissue inhibitors of metalloproteinases and increase in collagen production with the end result of excessive deposition of collagenous extracellular matrix. These data also confirm the medaka as a useful animal model of hepatic fibrosis. In chapter 3, the immunohistochemical and ultrastructural characteristics of the putative piscine oval cell/progenitor cell compartment after acute and chronic toxic hepatic injury were determined in order to characterize the cellular lineages and differentiation processes in medaka. The study demonstrated that fish oval cells/hepatic progenitor cells (HPC) shared similar morphology and immunoreactivity with rodent oval cells and human HPCs, and had similar bipotential lineage pathways. This provided additional evidence of the striking morphological similarities in the hepatic regenerative process between fish and mammals. In chapter 4, a label retention cell assay was performed to identify the location of the HPCs in the Japanese Medaka liver. Labeling was performed either during liver development in embryos and physiological growth was used to dilute the label in hepatic cells, or following acute hepatic necrosis in adult medaka and the subsequent hepatic regeneration phase was used to wash out the label. Phenotypic characteristics, location and double immunolabelling were used to confirm the identity of the BrdU retaining cells. Label retention was observed in three possible HPC niches: hepatocytes, bile preductular epithelial cells, and cholangiocytes. These data demonstrated that the process of hepatic regeneration is a complex system relying on multiple HPC niches as observed in mice.
Collectively, the results of our investigations improve comparative understanding of the liver’s response to chronic injury across taxa and demonstrate the utility of the medaka model for hepatic injury studies.
Pathogenesis of Liver Fibrosis and Regeneration in the Japanese Medaka (*Oryzias latipes*)

by

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A dissertation submitted to the Graduate Faculty of North Carolina State University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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Arnaud Van Wettere grew up in eastern Belgium in the city of Visé and later in the nearby village of Warsage. In childhood he developed a strong interest in birds, wildlife and falconry that led him to study veterinary medicine at the University of Liège. After obtaining his Doctor in Veterinary Medicine degree in 1999, he practiced small animal medicine before pursuing a residency in clinical avian medicine at The Raptor Center at the University of Minnesota. He obtained a MS degree in Veterinary Medicine from the University of Minnesota in 2003 and moved back to Europe to work as an assistant professor of avian medicine at the veterinary school of the Ludwig Maximilian University of Munich, Germany. Interaction with pathologists during his clinical residency sparked his interest in veterinary pathology and additional time working in the avian pathology section at the University in Munich led him to seek advanced training in pathology. In 2005, he returned to the United States to start a residency in veterinary anatomic pathology at North Carolina State University (NCSU) College of Veterinary Medicine. After completing the residency and becoming board certified in veterinary anatomic pathology, he stayed at NCSU to undertake a PhD in Comparative Biomedical Sciences in the laboratories of Dr. Kullman (Department of Molecular and Environmental Toxicology) and Dr. Law (Department of Population Health and Pathobiology).
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CHAPTER 1: Literature Review

Animal models of human diseases are essential to elucidate the molecular mechanisms of disease progression and develop and evaluate diagnostic and therapeutic approaches. Fish models with their easy and economical maintenance, large number of embryos produced, external development of transparent embryos, low background incidence of neoplasms as well as their well annotated genome, availability of inbred strains and established techniques to modulate gene expression and create mutant lines make them an attractive model organisms. Responsible use of laboratory animals seeks to reduce, refine and replace use of higher vertebrates by lower vertebrates, invertebrates or in vitro systems and therefore supports the use of fish as animal models (Law, 2003). Consequently, it is not surprising that fish are increasingly used in biomedical research, high-throughput screening of chemicals, and environmental monitoring (Torten et al., 1996; Okihiro and Hinton, 1999; Wakamatsu et al., 2001; Shima and Mitani, 2004; Hardman et al., 2007; Hardman et al., 2008b; Hinton et al., 2009).

It is known that notable mechanistic differences in disease pathogenesis exist between humans and the various animal model species used. These differences in molecular mechanisms do not preclude using an animal species as a model of human disease but it is important to know and understand these differences and the limitations of the model selected (Hinton et al., 2009). How similar fish models are to the relevant mouse models and humans has become a fundamental issue. Although it is well accepted that tissue and cellular organization, reaction to injury and neoplasms are typically similar histologically between
humans and fish, little is known regarding the similarity of specific molecular mechanisms leading to liver pathologies. In this thesis, we investigated the medaka liver reaction to injury, and more specifically, the pathogenesis of hepatic fibrosis and hepatic regeneration.


The Japanese medaka (*Oryza latipes*) is a teleost fish native to Japan, Taiwan and Southeast Asia that has been increasingly used in various biological fields including genetic regulation of development, carcinogenesis, high-throughput screening of chemicals, and environmental monitoring as well as models of human diseases (Torten et al., 1996; Okihiro and Hinton, 1999; Wakamatsu et al., 2001; Shima and Mitani, 2004; Hardman et al., 2007; Hardman et al., 2008b; Hinton et al., 2009). The medaka fish has many benefits as a model organism. Medaka are small (2.5-3.0 cm in length), hardy, prolific, tolerate a wide range of water temperatures (0-40º C) and salinity, and are highly resistant to disease (Wittbrodt et al., 2002; Furutani-Seiki and Wittbrodt, 2004; Shima and Mitani, 2004). They also have an extremely low incidence of background neoplasia (<0.5% in most studies) which is advantageous to perform low- dose chemical exposure experiments (Law et al., 1998; Williams et al., 2009). Medaka are oviparous fish. Mating, egg laying and fertilization occurs when lights are turned on under artificial lighting conditions or at dawn in natural conditions. More precisely, ovulation take place approximately 1 hour before first light, and oviposition occurs from 1 hour before to 1 hour after light onset (Iwamatsu, 2004; Shima and Mitani, 2004). In the laboratory, a daylight cycle of 14 hours light and 10 hours dark is generally recommended (Iwamatsu, 2004; Shima and Mitani, 2004). Temperatures ranging from 25 to 28ºC are
optimal for successful breeding in the laboratory. Females will spawn from 10 to 30 eggs
daily during the reproductive season. At 26°C, the eggs develop and hatch after 9 to 10 days
(Iwamatsu, 2004; Shima and Mitani, 2004). However, embryogenesis can be slowed down
significantly by reducing the ambient temperature (Shima and Mitani, 2004). The normal
stages of medaka development have been described in detail by Iwamatsu, 2004 (Iwamatsu,
2004).

The life span of medaka is sensitive to temperature. It is reported that medaka maintained
at 27°C have a life span of approximately 1 year while the life span in natural conditions at
lower temperatures outdoors in Japan can be up to five years (Shima and Mitani, 2004).
Experience in our laboratory has shown that orange red medaka tend to live at least 1.5 to 2
years at 26°C.

Multiple outbred, inbred and transgenic fish lines have been developed in medaka and
many of them are available at the National Bio Resource Project Medaka Mutant Stock
Project. Two of these strains, the STII and STIII strains, along with the zebrafish (Danio
rerio) mutant strain casper, are the only vertebrate animal models which are transparent in
the adult stage (Wakamatsu et al., 2001; Shima and Mitani, 2004; Hardman et al., 2008b;
White et al., 2008). The STII strain has no expression of leucophores and melanophores, and
minimal expression of xanthophores and iridophores while the STIII strain is recessive for all
pigment genes exhibiting no expression of chromatophores (Wakamatsu et al., 2001). These
characteristics make them an ideal animal model to study tissue response and gene
expression non-invasively in-vivo and it is possible to perform high resolution (< 5 μm)
microscopy in-vivo and generate three-dimensional (3D) reconstructions for in vivo imaging (Hardman et al., 2008b; Hasegawa et al., 2009).

Systematic mutagenesis studies in medaka have generated gene mutations that affected liver development and/or metabolism (Hata et al., 2007). Several of these mutants have been characterized and will be useful models to study liver formation, function and disease. The medaka has the smallest genome of all vertebrate genetic model systems (Wittbrodt et al., 2002). Its genome is approximately 800 Mb which is about half the size of the zebrafish genome and one-third of the mouse and human genome. They have 48 chromosomes including heterogametic XY sex chromosomes with the male sex-determining gene located on the Y chromosome (Kondo et al., 2006). Orthologue to mammalian genes involved in various biologic functions can be found in the medaka genomes and allows comparison and extrapolation between fish and mammalian systems (Broussard et al., 2009). For example, genes linked to DNA repair (e.g. AlkB, Apex1, Mgmt, Mre11a, Msh2, XPA,), carcinogenesis (e.g. Brca1, CDKN2A/B, c-Ki-ras-1, p53, RAB1, RAB2, RB1, Rev1, Rev3, XPV), and immunity (e.g. CD4, CD8, CXCR4, IgM, IL-10, IL-12, INF-γ, MHC I, MHC II, Rag1, Rag2, TCR-α, TCR-β, TLR) are found in the medaka genome (Broussard et al., 2009). Many additional orthologous genes can be found and several excellent online genomic resources are available, such as the Medaka Whole Genome Shotgun sequencing data by the National Institute of Genetics and the University of Tokyo (http://dolphin.lab.nig.ac.jp/medaka/) and the Esembl project from the European Bioinformatics Institute, European Molecular Biology Laboratory, and the Wellcome Trust Sanger Institute in Hinxton, UK (http://useast.ensembl.org/Oryzias_latipes/Info/Index).
2. **Medaka Hepatic Anatomy and Histology**

The anatomy and histology of the medaka liver have been thoroughly described (Hardman et al., 2007). Here we will briefly review the anatomy and point out the salient differences between medaka and mammalian livers. The mammalian hepatobiliary structure/function relationship is characterized by anastomosing 1 to 2 cell thick plate of hepatocytes (muralium) forming “classic” or “portal’ lobules. In fish, the hepatocyte organization has been classically described as blind-ended, anastomosing, and branching tubules formed by two rows of hepatocytes with their apical membrane forming a biliary preductule and their basal membrane in contact with a sinusoid. However, a recent three dimensional reconstruction of the medaka fish liver challenged this view (Hardman et al., 2007). Hardman R.C. et al, 2007, have found that the medaka hepatic parenchyma is organized more in a 2 to 3 cell thick muralium than tubules. The liver parenchyma has numerous anastomosing bile preductules formed by hepatocytes and bile preductular epithelial cells (BPDEC) which are the equivalent to the canal of Hering in mammals but are distributed throughout the parenchyma rather than present only in the periportal areas. The sinusoids with their fenestrated endothelium and underlying space of Disse are organized similarly to mammals (Hardman et al., 2007; Hinton et al., 2008). Portal triads are rarely present in fish (Akiyoshi and Inoue, 2004). Small fish species like the medaka lack these vascular / biliary tract structures in their hepatic parenchyma while reminiscent anatomical structures such as venous biliary arteriolar tracts, venous arteriolar tracts, venous biliary tracts, biliary tracts, and arteriolar tracts are found in larger fish species. In medaka, an arborizing biliary tree similar to mammals is absent. Their intrahepatic biliary system consists of an interconnected
network of canaliculi and bile preductules that connect with a few branching bile ducts located at the hilus. Overall, the single lobed liver of the medaka is best regarded as the analogue to one or one part of a mammalian hepatic lobule with biliary-venous tracts (portal tract equivalent) located at the hilus and an hepatic parenchyma composed of 2 to 3 cell thick muralium rather than the mammalian 1 cell thick muralium (Hardman et al., 2007). Other microanatomical differences in medaka and most fish include the lack of lymphatic drainage system and Kupffer cells (Sailendri and Muthukkaruppan, 1975; Hardman et al., 2007; Hinton et al., 2008). Although Kupffer cells are absent, small numbers of perisinusoidal macrophages are scattered in between hepatocytes along the space of Disse (Hinton et al., 2008). Hepatic stellate cells are present in the space of Disse of medaka, although in smaller numbers than in mammals. Last, rare and small aggregates of pigmented macrophages (previously called melanomacrophage centers) are also observed in the liver parenchyma of healthy medaka (Hinton et al., 2008).

**Medaka liver embryology**

In medaka liver anlage from the ventral foregut occurs at approximately 2 days post fertilization (dpf) (50 hours post fertilization (hpf) – 18 somites – stage 25). By 58hpf, the gallbladder is formed next to left lateral longitudinal liver leaflet. A primitive hepatic tubule organization is first seen at 62hpf (28 somites – stage 28) and CYP activity is first detected. At this time only arterial blood supply is perfusing the liver. In the third dpf, (86 hpf - 35 somites – stage 30), a single prehepatic vein begins to drain the caudal liver into the left duct of Cuvier. Pigmentation of the gallbladder fluid is visible by 88dpf and suggests that metabolism of yolk by the liver is occurring. At 8dpf (stage 38), the gall bladder starts to
descend from the upper left lateral position to the ventral surface of the abdominal cavity. By 10dpf (stage 40), the liver has completed its descent in the coelomic cavity and a single hepatic vein drains into the sinus venosus directly. The liver and gall bladder rotate 90 degrees clockwise (ventral view) to a transverse position. Merger of the sinus venosus and the hepatic vein is observed at 12dpf and the hepatic portal vein is first observed at 13dpf. By 16dpf, the liver and gallbladder have achieved adult phenotype (Iwamatsu, 2004; Hinton et al., 2008).

3. The Medaka Morphologic Hepatic Response to Injury

In general, there are more similarities than differences between fish and mammalian morphological hepatic response to injury. In regards to their response to toxic injury, the main difference between fish and mammals is that fish do not display a zonal response pattern to hepatic intoxication. This difference in histological pattern of toxicity is due to the absence of metabolic zonation in fish compared to the mammalian liver (Boorman et al., 1997; Wolf and Wolfe, 2005; Hinton et al., 2008). Another important difference is that, in general, for the same exposure concentration, hepatic injury tends to be less severe in fish than in mammals (Wolf and Wolfe, 2005; Hinton et al., 2008). This relative resistance may be due to several factors. The perfusion rate of the fish liver is lower and contact between sinusoidal blood flow and hepatocyte is limited to the basal and basolateral hepatocyte membranes in fish. Also the metabolic enzyme machinery is homogenously distributed and some enzymes do not seem as readily inducible as in mammals. Interestingly, cholestasis (impairment of bile transport), a common consequence of hepatobiliary system injury in
mammals, is rarely if ever observed histologically in fish (Wolf and Wolfe, 2005; Hardman et al., 2008a; Hinton et al., 2008). However, non-invasive high resolution in vivo imaging of α-naphthylisothiocyanate (ANIT) induced hepatobiliary toxicity in STII medaka revealed an increased canalicular volume thus suggesting cholestasis occurred in vivo but was not detectable histologically (Hardman et al., 2008a). In response to toxic injury, stress, disease or decreased nutrition, fish hepatocytes frequently lose their glycogen and/or lipid content. Conversely, decrease in hepatocellular glycogen content during fasting tends to occur less rapidly in fish than in mammals (Blasco et al., 1992; Wolf and Wolfe, 2005; Ferguson, 2006). Toxic hepatocellular injury can also result in the opposite, an increase in hepatocellular glycogen and/or lipid content. If adequate controls are not available, it can be problematic to determine if the vacuolization observed is within normal limits or excessive (hepatic lipidosis) (Wolf and Wolfe, 2005; Ferguson, 2006; Hinton et al., 2008). Fish have the ability to store large amounts of lipid in their liver and hepatocellular lipid accumulation can be prominent in many fish species and is greatly influenced by the diet. (Wolf and Wolfe, 2005). Cystic degeneration, or spongiosis hepatis if the cysts are multilocular, is a frequent but non-specific degenerative change observed in fish livers (Hinton et al., 2008, Wolf and Wolfe, 2005; Ferguson, 2006; Boorman et al., 1997). Hepatocyte hyalinization is another degenerative change observed in fish and consists of enlarged hepatocytes with bright eosinophilic cytoplasm or eosinophilic hyaline intracytoplasmic 5 to 15 µm droplets (Boorman et al., 1997; Wolf and Wolfe, 2005). Single cell necrosis or apoptosis as well as coagulative or liquefactive necrosis occurs in fish like in mammals with the exception that zonal distribution does not occurs as mentioned earlier (Boorman et al., 1997; Wolf and
An increase in number and size of pigmented macrophage aggregates can be observed following exposure to toxicants but also with other causes where increased cell turnover and cell death occur such as in infectious disease (Boorman et al., 1997; Wolf and Wolfe, 2005; Ferguson, 2006; Hinton et al., 2008). Pigmented macrophage numbers also increase with age. These pigmented macrophage aggregates are not always easily distinguished from granulomas. A granuloma is a more organized inflammatory response with a center of densely packed macrophages or necrotic material surrounded by macrophages that can be epithelioid (Boorman et al., 1997; Ferguson, 2006). A lymphocytic infiltrate can be present at the periphery of the granuloma. Interestingly, unlike mammals, parenchymal hepatic fibrosis and cirrhosis is rarely observed as a consequence of chronic hepatic injury in fish (Wolf and Wolfe, 2005; Ferguson, 2006; Hobbie et al., 2011). Conversely, concentric fibrosis centered around bile duct, cholangiofibrosis, is more commonly observed and results from toxic or infectious etiologies (Wolf and Wolfe, 2005; Ferguson, 2006).

In regards to proliferative lesions, fish develop altered foci similar to rodents and they are classified as eosinophilic, basophilic, clear cell, and vacuolated foci (Boorman et al., 1997). Bile duct hyperplasia can also occur in reaction to injury to the biliary tree in fish. Primary neoplasms of the liver of fish include hepatocellular adenoma, hepatocellular carcinoma, cholangioma, cholangiocarcinoma and combined hepatocellular-cholangiocarcinoma (Boorman et al., 1997; Okihiro and Hinton, 1999; Ferguson, 2006). Proliferative neoplastic masses composed of spindle cells that sometimes whirl around blood vessels have been
observed in fish livers (Boorman et al., 1997). When the characteristic whirling pattern is present, they are called hemangiopericytoma, although the cell or origin remains unknown. The standardization of diagnostic criteria and nomenclature is essential to harmonize the reporting of hepatic lesions across studies and detailed description and classification of the proliferative and non-proliferative medaka hepatic response to injury has been published by a National Toxicology Program Pathology Working Group (Boorman et al., 1997).

4. Mechanism of Hepatic Regeneration

To date, our knowledge of the liver repair mechanisms is derived mainly from rodent studies (Michalopoulos and DeFrances, 1997; Michalopoulos, 2010). Comparatively, our understanding of the mechanisms in fish liver regeneration is much more limited (Sadler et al., 2007; Goessling et al., 2008; Kan et al., 2009). The process of liver regeneration or compensatory growth is complex and involves coordinated interactions between all liver cell types and the extracellular matrix (ECM) to produce the favorable microenvironment necessary for regeneration (Santoni-Rugiu et al., 2005; Riehle et al., 2011). Progression through the regenerative process can be artificially divided into 3 phases: the priming or initiation phase, the proliferation or growth phase and the termination or growth inhibition phase. Each phase is not an independent event but the main factors involved in each phase are different. Priming factors will prepare the hepatocytes for replication, growth factors will promote cellular replication and growth inhibitory factors will suppress cell replication. After partial hepatectomy the regenerative process is initiated by cytokines released principally by Kupffer cells, which activate hepatocyte initiation and production of growth
factors. Priming for replication and transition from $G_0$ to $G_1$ of hepatocytes is triggered by interleukin (IL) 6 and tumor necrosis factor alpha (TNF-$\alpha$) secreted by non-parenchymal cells, Kupffer cells, endothelial cells and hepatic stellate cells (Michalopoulos, 2010; Riehle et al., 2011). Subsequent to hepatocyte priming, progression through $G_1$ is dependent on hepatocyte growth factor (HGF) and transforming growth factor (TGF) alpha. The HGF produced by non-parenchymal cells trapped in the extracellular matrix is released following partial hepatectomy and binds its receptor, c-Met, on the cell membrane of hepatocytes. Several other secondary ligands/signaling factors assist with progression through the cell cycle including: epidermal growth factor (EGF), TGF$\alpha$, amphiregulin, and heparin binding EGF-like growth factor (HB-EGF) (Michalopoulos, 2010; Riehle et al., 2011). The bioavailability of growth factors is dependent on their release from the extracellular matrix (ECM) by the action of urokinase plasminogen activator, plasmin, matrix metalloproteinase (MMP) and their inhibitors (TIMP) (Haruyama et al., 2000; Mohammed et al., 2005; Riehle et al., 2011). The Wnt pathway is also activated during hepatic regeneration and its role is mainly in activation, expansion and differentiation of hepatic progenitor cells (HPC) (Goessling et al., 2008; Lade and Monga, 2011; Nejak-Bowen and Monga, 2011; Riehle et al., 2011). Proliferation of non-parenchymal cells, stellate cells, biliary epithelial cells and endothelial cells, lags 24h to 48h behind hepatocyte proliferation and is probably responding to other proliferation signals (Michalopoulos and DeFrances, 1997; Michalopoulos, 2010; Riehle et al., 2011).

In addition to the “classical” pathways mentioned, other factors influence the dynamic and complex process of hepatic regeneration. The peripheral nervous system has been
implicated in liver regeneration. However, the mechanisms by which the nervous system
influences hepatic regeneration are not clear. Direct influence of sympathetic and
parasympathetic mediators on hepatocytes and non-parenchymal cells as well as effect on
blood flow regulation are suspected (Sakaguchi and Liu, 2002; Oben and Diehl, 2004;
Michalopoulos, 2010). Platelets and platelet-derived serotonin have been shown to play an
important role in liver regeneration, however the exact mechanisms are presently uncertain
(Lesurtel et al., 2006; Nocito et al., 2007; Riehle et al., 2011).

Pathways initiating and promoting the liver regeneration process have been well studied;
but, the mechanisms involved in the termination of liver regeneration are still poorly
understood. Once the hepatic mass is restored, cessation of the proliferative response is
mediated at least in part by TGF-β and activins (Michalopoulos and DeFrances, 1997;
Michalopoulos, 2010). TGF-β1 which is produced mainly by HSC and myofibroblasts is a
well-known potent inhibitor of hepatocyte proliferation (Santoni-Rugiu et al., 2005; Nguyen
et al., 2007). Its effect is mediated mainly by downregulation of c-myc and upregulation of
cyclin-dependent kinase inhibitors p15ink4b and p21Cip1 (Claassen and Hann, 2000; Pardali
et al., 2000; Seoane et al., 2001). It also suppresses HGF production and receptor activation
(Gohda et al., 1992; Mars et al., 1996). In the early phase of regeneration, hepatocytes are not
sensitive to the inhibitory effect of TGF-β because of decrease in TGF-β1 receptor
expression and the high level of TGF-α produced (Houck and Michalopoulos, 1989; Chari et
al., 1995). Then during the termination phase, TGF-β1 expression peaks, hepatocyte
replication is inhibited but production of ECM proteins is stimulated (Pepper et al., 1993;
Michalopoulos and DeFrances, 1997; Michalopoulos, 2010). In addition, TGF-β1 also likely
promotes reformation of the sinusoidal vascular network. The extracellular matrix itself also plays an important role in regulating termination phase. Integrin-linked kinases present in the ECM are known to be critical for termination of liver regeneration once the hepatic mass has been restored (Apte et al., 2009; Michalopoulos, 2010).

In mammals, liver regeneration can be separated in 2 physiological forms depending on the type of liver injury. When the injurious event targets hepatocytes or after partial hepatectomy, liver regeneration is achieved by proliferation of mature hepatocytes (Fausto and Campbell, 2003; Michalopoulos, 2010; Riehle et al., 2011). However, after severe hepatic injury or when hepatocyte division is impaired, a second system of reparative mechanisms is activated and HPCs participate in the healing process (Evarts et al., 1989; Fausto and Campbell, 2003; Riehle et al., 2011). In rodents and humans, it is well accepted that the HPC niche is located in the transition canal between bile ductules and canaliculi called the canals of Hering (Braun and Sandgren, 2000; Fausto and Campbell, 2003; Hardman et al., 2007; Zhou et al., 2007). However, this is likely an over simplification and multiple HPC niches probably exist (Kuwahara et al., 2008; Riehle et al., 2011). Work by Kuwara et al., 2008 proposed that there are maybe up to four HPC niches in the rodent liver: 1) the oval cells in the canal of Hering, 2) some epithelial cells in the intralobular bile ducts, 3) some periductal mononuclear “null” cells, and 4) some peribiliary hepatocytes (Kuwahara et al., 2008). In fish, the piscine oval cell equivalent, the BPDECs are the purported liver tissue progenitor cells (Hardman et al., 2007; Hinton et al., 2008).

An additional mechanism of hepatic regeneration is fusion or transdifferentiation of hematopoietic or bone marrow stem cells into hepatocytes, however, this process is not
considered clinically significant (Hatch et al., 2002; Menthen et al., 2004; Oh et al., 2007). Bone marrow stem cells may also contribute to repopulation of myofibroblasts and sinusoidal endothelial cells, and thus may play additional roles in liver tissue repair (Kallis et al., 2007).

5. **Mechanism of Hepatic Fibrosis**

Following an acute injury, the liver regenerative response usually results in restoration of the normal hepatic architecture and function. However, when the injurious cause persists, chronic inflammation and scar tissue deposition occurs, and progressive hepatic fibrosis develops. Chronic hepatic injury leads to cirrhosis, the end-stage of fibrosis, with profound morphological and metabolic consequences that are often lethal (Wallace et al., 2008; Hernandez-Gea and Friedman, 2010).

Hepatic fibrosis is the result of the liver wound healing response to repeated injury. When the cause of hepatic injury persists, the liver regenerative mechanisms are compromised and deposition of excessive ECM, scar tissue, occurs. The distribution of the fibrous tissue deposition in the liver depends on cause of the injury. Fibrosis first occurs around portal tracts and progresses to portal-portal and portal-central bridging fibrotic septa in chronic viral diseases and diseases affecting the biliary tree. In non-alcoholic fatty liver disease and alcohol-induced liver disease, fibrosis occurs in the pericentral and perisinusoidal regions along the space of Disse and results in a chicken-wire pattern (Wallace et al., 2008; Hernandez-Gea and Friedman, 2010). With time, portal-portal and portal-central bridging fibrosis develop as well. The excessive deposition of ECM matrix and the loss of fenestration in the basal lamina along the spaces of Disse lead to “capillarization” of the sinusoids that
result in significant impairment in metabolic exchange between sinusoidal blood flow and hepatocytes (Schaffner and Poper, 1963; Wallace et al., 2008).

Hepatic fibrosis results from complex interactions between inflammatory cells and the various hepatic cell types. Hepatocellular injury due to numerous etiologies such as viruses, bile acids, and hepatotoxicants results in the release of reactive oxygen species (ROS) and cytokines that initiate an inflammatory reaction (Bilzer et al., 2006; Hernandez-Gea and Friedman, 2010; Jaeschke, 2011). Kupffer cells in the area of hepatocyte injury detect the release of intracellular contents and apoptotic cells and secrete chemokines (e.g. MCP1, MIP2) that activate local resident leukocytes and stimulate chemotaxis of circulating leukocytes (Canbay et al., 2003; Canbay et al., 2004; Bilzer et al., 2006; Jaeschke, 2011). In addition, the Kupffer cells, but also the lymphocytes and polymorphonuclear cells, produce cytokines (e.g. TNF-α, TGF-α, TGF-β, PDGF) that activate HSCs which in turn secrete extracellular matrix, chemokines, MMPs, TIMPs, and cytokines such as TGF-β1, that modulate matrix deposition and the immunological reaction (Casini et al., 1997; Vinas et al., 2003; Winau et al., 2007; Winau et al., 2008; Atzori et al., 2009). When the injury is centered on the biliary tree like in cholestatic diseases or cholangitis, biliary epithelial cell damage triggers the inflammatory reaction and activation of the portal myofibroblast and HSCs that cause deposition of ECM predominantly around bile ducts (Kinnman and Houssset, 2002; Kinnman et al., 2003; Magness et al., 2004; Penz-Osterreicher et al., 2011). Release of cytokines like TGF-β1 present in the ECM due to injury and the inflammatory reaction also promotes HSC activation and fibrogenesis (Knittel et al., 2000; Gressner et al., 2002; Hemmann et al., 2007). With the ongoing cell death due to repeat or continuous exposure to
a hepatotoxic etiologic agent and the inflammatory reaction, a vicious cycle in which inflammatory and fibrogenic cells stimulate each other is likely to be established (Wallace et al., 2008; Winau et al., 2008; Hernandez-Gea and Friedman, 2010). HSCs are multifaceted cells that have a central role in the pathogenesis of hepatic fibrosis. Following chronic injury, HSCs transdifferentiate (activate) into myofibroblast-like cells that have contractile, proinflammatory, and fibrogenic properties. They migrate to the sites of tissue repair and secrete ECM components (e.g. collagen 1 and 3, fibronectin, proteoglycan), cytokines (e.g. IL-6, IL-10) and growth factors (e.g. PDGF, HGF, FGF, CTGF, IGF) that promote cell proliferation and ECM accumulation (Hautekeete and Geerts, 1997; Wallace et al., 2008; Atzori et al., 2009; Hernandez-Gea and Friedman, 2010). With some variation between species, they express neuroendocrine markers (e.g., reelin, nestin, neurotrophins, N-CAM, synaptophysin, and glial-fibrillary acidic protein), adipocyte markers (e.g. PPARγ, SREBP-1c, and leptin), and bear receptors for neurotransmitters (Geerts, 2001; Oben et al., 2003; Sato et al., 2003; Atzori et al., 2009). When activated, they express myogenic markers (e.g. desmin, α smooth muscle actin). Hepatic stellate cells also function as antigen presenting cells and stimulate natural killer cells, CD8 and CD4 T cells (Vinas et al., 2003; Winau et al., 2007; Winau et al., 2008).

HSCs are the main fibrogenic cell when injury occurs in the pericentral areas while portal myofibroblasts predominate when liver injury is centered on portal tracts (Magness et al., 2004). Yet, the bone marrow is also an important source of fibrogenic cells in the injured liver. Bone marrow derived HSCs and myofibroblasts make significant contributions to the myofibroblastic cell populations in fibrotic livers (Russo et al., 2006; Miyata et al., 2008).
(Scholten et al., 2011). However, these bone marrow derived cells may not be the main producer of ECM (Higashiyama et al., 2009). The relative contribution to fibrosis of liver or bone marrow derived HSCs and myofibroblasts likely depends on the type of liver injury (Kisseleva et al., 2006; Russo et al., 2006; Miyata et al., 2008). In bile duct ligation-induced liver injury in mice, HSCs were not recruited but collagen-producing fibrocytes were recruited from the bone marrow (Kisseleva et al., 2006). Presently, the precise contribution of bone marrow cells versus the liver derived collagen producing cells to liver fibrogenesis following various types of injuries is unclear and additional studies are necessary.

It has been suggested that epithelial-mesenchymal transition of hepatocytes and cholangiocytes to myofibroblasts contribute to liver fibrogenesis (Kaimori et al., 2007; Zeisberg et al., 2007; Rygiel et al., 2008; Harada et al., 2009). However, results of other studies did not support the occurrence of epithelial – mesenchymal transition in liver fibrosis (Scholten et al., 2010; Taura et al., 2010). The concept of epithelial-mesenchymal transition itself is still debated and, even in the kidney, where epithelial mesenchymal transition was first described and seems most accepted, this concept has been challenged (Humphreys et al., 2010). To date, in light of the evidence for epithelial–mesenchymal transition in chronic liver disease, it appears that hepatocytes and cholangiocytes can acquire mesenchymal markers associated with cell survival and migration ability but are not involved in active collagen/ECM secretion (Pinzani, 2011). Therefore they are not true pro-fibrogenic cells. Further, Pinzani, 2011 suggests the possibility that the acquisition of mesenchymal markers and migration of these epithelial cells away from the injury site is a self-protective mechanism that could also be aimed at limiting fibrogenesis (Pinzani, 2011).
The process of hepatic regeneration and fibrosis are intimately linked and cross-talk between HPCs and HSCs has been documented (Paku et al., 2001; Santoni-Rugiu et al., 2005; Roskams, 2006). HSCs are a significant source of growth factors that act on hepatocytes and HPCs such as TGF-α, TGF-β, HGF and FGF. Inflammatory cells, like Kupffer cells, are also a source of factors that activate hepatocytes and HPCs but also HSCs. In a mouse model of hepatic fibrosis, activation of HSCs and ECM deposition preceded expansion of the HPC population (Van Hul et al., 2009).

In normal livers, the ECM synthesis and degradation is a dynamic process that is strictly regulated. In liver fibrosis, the balance between synthesis and degradation is altered and the accumulation of ECM results from both increased synthesis and decreased degradation (Kossakowska et al., 1998; Hemmann et al., 2007; Wallace et al., 2008; Hernandez-Gea and Friedman, 2010). The decrease in ECM degradation is essentially due to an increase in expression of TIMPs by HSCs and myofibroblasts (Herbst et al., 1997; Kossakowska et al., 1998; Knittel et al., 2000; Hemmann et al., 2007). While the ECM deposited during liver fibrosis is excessive, it also differs in quality with increased cross-linking compared to the ECM present in normal livers (Issa et al., 2004; Wallace et al., 2008; Hernandez-Gea and Friedman, 2010). The ECM deposited during fibrosis includes collagens (I, III, and IV), fibronectin, laminin, elastin, hyaluronan, and proteoglycans (Hemmann et al., 2007). The ECM plays a critical role in cell polarization, adhesion, migration, proliferation, and survival, and the interaction between ECM and adjacent cells is bidirectional. Therefore, after injury, change in the ECM influence the activation and proliferation of HSC, regeneration, angiogenesis as well as the availability of growth factors (e.g. TGF-β, TNF-α, PDGF, HGF).
and MMPs (Olaso et al., 2001; Schuppan et al., 2001; Hernandez-Gea and Friedman, 2010). For example, collagen 1 has been shown to stimulate HSCs activation and proliferation via their discoidin domain receptor 2 and integrins (Olaso et al., 2001).

a. **TGF-β Signaling Pathways in Liver Fibrosis**

TGF-β is a multifunctional cytokine with key roles in fibrosis. It activates HSCs, stimulates production of ECM, potentially suppresses the proliferation of hepatocytes and can mediate apoptosis (Dooley et al., 2001; Gressner et al., 2002; Leask and Abraham, 2004). In mammals, TGF-β has three major isoforms (TGF-β1, TGF-β2, and TGF-β3). An additional isoform, TGF-β6, is present in fish, including medaka (Funkenstein et al., 2010). TGF-β1 is the primary isoform involved in liver fibrosis and is produced predominantly by monocytes, macrophages and HSCs (De Bleser et al., 1997; Gressner et al., 2002; Leask and Abraham, 2004). TGF-β1 is stored in the ECM in an inactive form and released from the ECM by the action of MMPs and inflammatory reaction (Haruyama et al., 2000; Mohammed et al., 2005; Riehle et al., 2011). Once activated, TGF-β signals through a serine/threonine kinase membrane receptor consisting of type I (TGFbRI) and type II (TGFbRII) components. Binding of TGF-β to TGFbR-II triggers heteromerization with and activation of TGFbR-I. The signal is propagated through phosphorylation of receptor-regulated Smads (Smad2 and 3; R-Smads), which combine with the common mediator Smad4 (co-Smad). The phosphorylated Smads 2 or 3 with Smad4 complex translocate to the nucleus, where it affects transcription of target genes (Leask and Abraham, 2004). Although TGF-β signaling occurs through both canonical (Smad) and noncanonical pathways, Smad3 appears to be the primary
transducing protein mediating the pro-fibrotic effect of TGF-β (Flanders, 2004; Roberts et al., 2006).

In fibrosis, TGF-β plays a major role in HSC and myofibroblast activation and is considered necessary for the development of fibrosis (Qi et al., 1999; Nakamura et al., 2000; Gressner et al., 2002; Arias et al., 2003; de Gouville et al., 2005; Liu et al., 2006; Roberts et al., 2006; Patsenker et al., 2008; Patsenker et al., 2008; Fu et al., 2011). However, TGF-β1 alone is not sufficient for HSC activation and 3 conditions are required for HSC activation to occur: high levels of TGF-β1, presence of fibronectin splice variant EDA and increase local mechanical tension (Hinz et al., 2007; Serini et al., 1998). TGF-β1 also acts on sinusoidal endothelial cells to up-regulate the production of fibronectin EDA. Transcription of several genes involved in fibrosis are up-regulated by TGF-β1. Upregulation of collagen (COL1A1, COL3A1, COL5A2, COL6A1, COL6A3), CTGF, plasminogen activator inhibitor-type 1 (SERPINE1), and TIMP1 genes has been directly linked to TGF-β signaling activation (Grande et al., 1997; Dennler et al., 1998; Holmes et al., 2001; Verrecchia et al., 2001).

TGF-β signaling plays a key role not only in hepatic fibrosis but also in carcinogenesis. The role of TGF-β in the latter is complex and intricate (Wakefield and Roberts, 2002; Mikula et al., 2006; Massague, 2008; Mishra et al., 2009). TGF-β acts as a tumor suppressor regulating cell proliferation but also as a tumor promoter exacerbating neoplastic cell motility and invasion. This dual role is well documented but the mechanisms of how neoplastic cells escape the suppressor effect of TGF-β while remaining responsive to the promoter effect are still unclear (Wakefield and Roberts, 2002; Massague, 2008). TGF-β signaling tumor suppressor activity is derived, in part, from its inhibitory growth effect on epithelial and
lymphoid cells which is mediated mainly by downregulation of c-myc and upregulation of cyclin-dependent kinase inhibitors p15ink4b and p21Cip1 (Claassen and Hann, 2000; Pardali et al., 2000; Seoane et al., 2001; Wakefield and Roberts, 2002). TGF-β also promotes cell differentiation and apoptosis. Conversely, TGF-β has direct pro-oncogenic effects on the tumor cells themselves and indirect promoting effects on the stromal environment and immune cells. Mechanisms of tumor promotion by TGF-β include deregulation of cyclin-dependent kinase inhibitors, microenvironment modification, evasion of immune surveillance and increased angiogenesis (Wakefield and Roberts, 2002; Massague, 2008). In general, decreased or altered TGF-β signal responsiveness and increased expression of the TGF-β ligand is observed during tumor progression to metastatic disease (Wakefield and Roberts, 2002; Massague, 2008). In hepatic neoplasia, malignant cells are frequently associated with intra- and peritumoral accumulation of activated HSCs, myofibroblasts and increased ECM. These activated HSCs and myofibroblasts are involved in a TGF-β dependent manner in carcinogenesis by promoting autocrine TGF-β signaling and nuclear β-catenin accumulation in neoplastic hepatocytes (Mikula et al., 2006).

6. Dimethylnitrosamine Induced Hepatic Cirrhosis Model

a. Dimethylnitrosamine (DMN)

Dimethylnitrosamine (DMN), of the nitrosamine family, is a potent hepatotoxin and genotoxic carcinogen with both initiating and promoting capabilities (Swenberg et al., 1991; Hasegawa et al., 1998). This model compound has been used to study fibrosis in rodents and carcinogenesis in the rat, mouse, hamster, and Japanese medaka (Jenkins et al., 1985;
Neoplasms induced by carcinogenic nitrosamines have been reported in over 40 animal species (Swenberg et al., 1991; Hasegawa et al., 1998). DMN induced neoplasms have been reported in various tissues but liver and to a lesser extent kidney, esophagus, lung, and nasopharynx are the predominant tissue types (Couch and Courtney, 1987; Petto et al., 1991). Among liver tumors, hepatocellular neoplasms predominate in rodents (Peto et al., 1991). In medaka, hepatocellular carcinoma predominates as well but the proportion of biliary neoplasms and mixed neoplasms is higher than in rodents (see chapter 2) (Hobbie et al., 2011). Low level natural DMN exposure in humans is common, and studies have shown that daily exposures to DMN results in an increased risks of gastric, esophageal, oropharyngeal, and lung cancer (Liteploo and Meek, 2001). Human DMN exposure occurs through tobacco smoke, automobile exhaust, and diet (nitrate- and nitrite treated food and certain beverages) as well as endogenous formation in the gastrointestinal tract (Kyrtopoulos, 1998; Liteploo and Meek, 2001; Souliotis et al., 2002). Given the results from studies of animal models and the fact that DMN metabolism is qualitatively comparable in humans and rats, exposures to DMN is a significant health hazard in humans (Kyrtopoulos, 1998; Liteploo and Meek, 2001). DMN has initiation and promotion abilities. The initiation mechanism of DMN results from DNA alkylation but its promotion mechanism is less well understood (Souliotis et al., 2002). Promotion is thought to result from release of mitogenic stimuli from damaged cells inducing cellular proliferation (Souliotis et al., 2002).

DMN-induced cellular damage results from 2 predominant pathways: 1) formation of DNA and protein adducts and 2) oxidative stress. In rodents, DMN is rapidly absorbed by
ingestion or inhalation, has a wide tissue distribution, is metabolized in the liver and other tissues, and is excreted in urine (Liteplo and Meek, 2001). Metabolic activation of DMN in the liver of rodents is dependent on cytochrome P450 2E1 (CYP 2E1) via enzymatic denitrosation and/or alpha hydroxylation (Kyrtopoulos, 1998; Liteplo and Meek, 2001; Souliotis et al., 2004). In alpha hydroxylation, the unstable intermediate metabolite, hydroxymethyl-methyl-nitrosamine (HOCH2CH3N-N=O) is formed and quickly transformed into formaldehyde and methylidyazonium ion [CH3-N=N]+ (Frei et al., 1999). Cellular damage results from formation of nucleic acid and protein adducts caused by methylidyazonium ion alkylation of DNA, RNA, and proteins. Methylidyazonium ions also react with water to form methanol and nitrogen gas (N2) (Frei et al., 2001; Liteplo and Meek, 2001). DNA adducts caused by methylidyazonium ion’s interaction with nucleophilic sites on DNA include N7-methylguanine (N7-MeG), O6-methylguanine (O6-MeG), N3-methyladenine (N3-MeA), O4-methylthymine (O4-MeT), as well as other minor adducts (Dobo et al., 1998). Although not the main DNA adducts formed, O6-MeG and O4-MeT are the most premutagenic. O6-MeG results in GC-AT transitions by direct mispairing of guanine with thymine and O4-MeT giving rise to AT-GC transitions through mispairing of adenine with cytosine during DNA replication (Dobo et al., 1998). DMN-induced mutations include transversions (GC-TA, GC-CG, AT-TA, and AT-CG) as well as frameshifts, deletions, and insertions (Shane et al., 2000). Sister chromatid exchanges, chromosome aberrations, and recombination have also been documented (Lin et al., 1999; Margison et al., 2002). Cytotoxicity also results from generation of reactive oxygen species that lead to
oxidative stress, glutathione (GSH) depletion, lipid peroxidation, and 8-OHdG adduct formation (Lin et al., 1998; Lin and Hollenberg, 2001).

b. DMN Induced Rat Model of Hepatic Fibrosis

In addition to its carcinogenic properties, DMN is a potent hepatotoxin that is used in rodents to model human hepatic fibrosis and cirrhosis (Jezequel et al., 1987; Shiba et al., 1998; George and Chandrakasan, 2000; Nakamura et al., 2000; Tada et al., 2001; de Gouville et al., 2005; Hyon et al., 2011). Anecdotally, unintended acute and chronic exposure to DMN in humans has resulted in hepatic cirrhosis and subsequent death further supporting the validity of this model (Liteplo and Meek, 2001). Among the animal models of hepatic fibrosis/cirrhosis, the DMN induced rodent models are attractive and commonly used models due to similar aspects of the resultant pathology induced to the human disease counterpart (Nakamura et al., 2000; Tada et al., 2001; de Gouville et al., 2005; Wallace et al., 2008; Hyon et al., 2011). Hepatic fibrosis develops within 3 weeks after exposure, and the morphological changes induced such as fibrous septa formation, nodular regeneration, portal hypertension, and development of ascites resemble the human disease (Jenkins et al., 1985; Ala-Kokko et al., 1987; George and Chandrakasan, 2000; George et al., 2001; Tada et al., 2001; de Gouville et al., 2005). Protocols for the induction of hepatic fibrosis in rats are variable but all involve multiple intraperitoneal (IP) injections of DMN over several days to a few weeks (Jenkins et al., 1985; Ala-Kokko et al., 1987; George and Chandrakasan, 1996; Shiba et al., 1998; George et al., 2001; de Gouville et al., 2005; Ohara et al., 2007). DMN administration results in centrilobular hemorrhagic necrosis followed by neutrophilic
inflammation and progressive collagen deposition over a 3 week period. By day 21 after the first dose of DMN, bridging necrosis and fibrosis surrounding the central veins, multifocal fatty degeneration, inflammation, ductular reaction and nodular regeneration are present (Jenkins et al., 1985; Ala-Kokko et al., 1987; George et al., 2001; George et al., 2004; George, 2006). Metabolic abnormalities documented in rats include hypoproteinemia, hypocholesterolemia, hypophosphatemia, impaired glucose tolerance with insulin resistance, elevated serum and urinary hydroxyproline and uric acid levels (George and Chandrakasan, 2000). Elevated malondialdehyde levels in liver and serum were also observed indicating increased lipid peroxidation and oxidative damage (George and Chandrakasan, 2000). Activation of HSCs, which is a critical event in the pathogenesis of hepatic fibrosis, was documented in rats as well. Stellate cell activation was demonstrated by an increase in smooth muscle actin positive cells using immunohistochemistry, in \( \alpha \)-smooth muscle actin mRNA and in KLF5 (also known as BTEB2) a regulator of the phenotypic modulation of mesenchymal cells (George et al., 2004; Ohara et al., 2007). Increased deposition of collagen fibrils, fibrosis, was associated with an increase in type I, III and IV collagen and MMP1 mRNA (Ala-Kokko et al., 1987; Shiba et al., 1998). The collagen deposited in fibrotic liver had increased cross-linkage compared to control liver (George and Chandrakasan, 1996). The TGF-\( \beta \) pathway, an essential pathway in the pathogenesis of fibrosis, was also shown to be of critical importance in this rat model. Inhibition of TGF-\( \beta \) signaling by either TGF-\( \beta \) inhibitors like pirferidone or ALK5, or injection of an adenovirus expressing a truncated type II TGF-beta receptor resulted in a decrease or even resolution of hepatic fibrosis (Nakamura et al., 2000; García et al., 2002; de Gouville et al., 2005). Taken together, the results of the
many studies investigating the pathogenesis of fibrosis or treatment options have shown that the DMN-induced rat model is a particularly well characterized and valuable model. Recently, Japanese medaka fish (Oryzias latipes) exposed to DMN have been shown to develop morphological hepatic changes similar to those observed in human fibrotic livers and the DMN-induced rat model of hepatic fibrosis (Ala-Kokko et al., 1987; Jezequel et al., 1987; Ohara et al., 2007; Hobbie et al., 2011; Hyon et al., 2011).

7. Microcystin LR Induced Hepatic Injury

a. Microcystins – General Information

Cyanobacteria (blue-green algae) are photosynthetic prokaryotes that are among the oldest forms of life and are widespread in aquatic environments, especially in freshwater lakes and reservoirs (Ferrao-Filho Ada and Kozlowsky-Suzuki, 2011). Cyanobacteria produce many types of cyanotoxins that are usually classified into two broad categories based on 1) their mechanism of action (e.g. hepatotoxins, neurotoxins) or 2) their chemical structure (e.g. cyclic peptides, alkaloids or lipopolyssacharides)( Stewart et al., 2006; Ferrao-Filho Ada and Kozlowsky-Suzuki, 2011).

The principal hepatotoxins are the microcystins, nodularins and cylindrospermopsins. Among the cyanotoxins, microcystins are particularly well characterized hepatotoxins. Microcystins are produced by Microcystis, Oscillatoria, Anabaena, Plankthotrix and Nostoc cyanobacteria species and are among the most lethal toxins known with a median lethal dose (LD 50) of only 43 µg/kg of bodyweight in mice for microcystin LR (MCLR) (Gupta et al., 2003; Ferrao-Filho Ada and Kozlowsky-Suzuki, 2011). They are a well-known cause of
poisoning in animals and humans (Stewart et al., 2006; Ferrao-Filho Ada and Kozlowsky-Suzuki, 2011). More than 60 microcystin variants have been described; they differ by the amino acids in positions 2 and 4, and by the methylation states of amino acids in positions 3 and 7 (Codd et al., 1997; Malbrouck and Kestemont, 2006; Ferrao-Filho Ada and Kozlowsky-Suzuki, 2011). Microcystins are monocyclic heptapeptides with the following general structure: cyclo (d-Ala$^1$-X$^2$-d-Asp$^3$-X$^4$-Adda$^5$-d-Glu$^6$-Mdha$^7$) (Codd et al., 1997; Malbrouck and Kestemont, 2006). In MCLR the variable amino acids are L-leu and L-arg. Natural routes of microcystin exposure in fish include water immersion and ingestion. In addition, IP exposure is also commonly performed in the laboratory. Exposure by immersion is considerably less harmful than by ingestion or IP exposure (Phillips et al., 1985; Tencalla et al., 1994; Malbrouck and Kestemont, 2006). Experimental studies in fish and mammals have demonstrated that microcystins are absorbed across the intestine, gill, or lung membranes and preferentially accumulate in hepatocytes (Runnegar et al., 1995a; Fischer et al., 2005). Absorption across the intestine and accumulation in hepatocytes is mediated through the bile salt organic anions transport polypeptides (OATP) (Eriksson et al., 1990; Runnegar et al., 1991; Falconer et al., 1992; Runnegar et al., 1995a; Fischer et al., 2005). Alternatively, some microcystins that are less hydrophilic may be able to pass through cell membranes by diffusion or through use of other transport systems (Kotak et al., 1996). With variation between species, microcystins can also accumulate in kidney, intestine, and skeletal muscle of mammals and fish suggesting the presence of OATPs in these organs (Kotak et al., 1996; Fischer and Dietrich, 2000; Ito et al., 2001; Mohamed et al., 2003; Xie et al., 2005; Meier-Abt et al., 2007). Yet, the presence of OATPs able to transport microcystin have only
been definitely established in liver and brain of a few species such as mice, Xenopus laevis and the little skate (*Leucoraja erinacei*) for example (Fischer et al., 2005; Meier-Abt et al., 2007). Once inside hepatocytes, microcystins do not require microsomal activation and they inhibit protein phosphatases 1 and 2A, leading to hyperphosphorylation of cytosolic and nuclear proteins, cytoskeletal organization and signaling pathway disruption, and ultimately cell death (Runnegar and Falconer, 1986; Eriksson et al., 1990; Khan et al., 1996). Initially, phosphatase inhibition results from a noncovalent interaction and is reversible, but subsequently becomes irreversible due to covalent link formation with cys-273 on protein phosphatase 1 and cys-266 on protein phosphatase 2A of their catalytic sub-unit (Toivola et al., 1994; MacKintosh et al., 1995; Runnegar et al., 1995b; Guzman et al., 2003). In addition microcystins cause cell damage by inducing the formation of reactive oxygen species and decreasing antioxidant mechanisms, thus causing oxidative stress, cellular and DNA oxidative damage and cell death (Ding et al., 2001; Zegura et al., 2006). There is also some evidence that other enzymes such as mitochondrial aldehyde dehydrogenase, ATP-synthase and acetylcholinesterase may be inhibited by microcystins as well (Lehtonen et al., 2003; Mikhailov et al., 2003; Chen et al., 2006). Microcystins also have a tumor-promoting activity that has been demonstrated in several organs (e.g. skin, stomach and liver) (Fujiki and Suganuma, 2009). Metabolism and elimination of microcystins takes place by conjugation to glutathione (GSH) via glutathione S-transferase and excretion in urine and bile (Kondo et al., 1992; Kondo et al., 1996; Sahin et al., 1996; Pflugmacher et al., 1998).
b. Microcystin LR in Fish

Microcystin toxicity has been investigated in multiple fish species including rainbow trout (Oncorhynchus mykiss), brown trout (Salmo trutta), Tilapia fish (Oreochromis sp.), hardhead catfish (Arisu felis), gulf killifish (Fundulus grandis), roach (Rutilus rutilus), goldfish (Carassius auratus), common carp (Cyprinus carpio) and silver carp (Hypophthalmichthys molitrix) and Japanese medaka (Tencalla et al., 1994; Kotak et al., 1996; Fournie and Courtney, 2002; Malbrouck et al., 2003; Atencio et al., 2008; Djediat et al., 2010; Djediat et al., 2011; Trinchet et al., 2011; Marie et al., 2012). The effect of short term dietary and intraperitoneal exposures, and short and medium term aqueous exposures were evaluated. Medaka in particular have been used as a model to study the effect of MCs in embryos and adults relatively frequently in the last decade (Jacquet et al., 2004; Huynh-Delerme et al., 2005; Malecot et al., 2009; Deng et al., 2010; Djediat et al., 2010; Djediat et al., 2011; Malécot et al., 2011; Trinchet et al., 2011; Marie et al., 2012). Although there are differences in MCLR susceptibility between species, fish are less susceptible than mammals with a LD50 likely above 500 µg/kg for most species (Råbergh et al., 1991; Andersen et al., 1993; Kotak et al., 1996; Fischer and Dietrich, 2000). Interestingly, despite fish have been used to study MCLR toxicity frequently, a literature search failed to reveal published studies determining specific LD50 for microcystins in fish species.

After oral ingestion or IP exposure, the main histological lesion induced by MCs in fish is hepatocyte dissociation, necrosis and apoptosis (Kotak et al., 1996; Fischer and Dietrich, 2000; Fischer et al., 2000; Fournie and Courtney, 2002; Malbrouck et al., 2003; Li et al., 2005; Djediat et al., 2010). The prominent hemorrhages often observed in mammalian livers
do not seem to occur in fish species. When the hepatic necrosis event is non-lethal, complete hepatic regeneration can occur within days post exposure (Fournie and Courtney, 2002). Elevation in enzyme activity classically associated with liver damage such as aspartate aminotransferase, alanine aminotransferase, L-lactate dehydrogenase and alkaline phosphatase were observed (Råbergh et al., 1991; Malbrouck et al., 2003; Li et al., 2004; Atencio et al., 2008). Microcystins can also induce degenerative lesions and necrosis in other organs such as the kidney, intestine, spleen and gonads (Kotak et al., 1996; Fischer and Dietrich, 2000; Ernst et al., 2006; Atencio et al., 2008; Djediat et al., 2010; Trinchet et al., 2011). In aqueous exposure of zebrafish embryos, uptake of MCLR through the chorion was associated with activation of detoxification enzymes (Wiegand et al., 1999). Injection of MCLR in the vitellus of medaka embryo resulted in embryo mortalities and embryo-larval digestive tract developmental abnormalities (Jacquet et al., 2004; Huynh-Delerme et al., 2005).

8. Study Significance and Specific Aim(s)

Small aquarium teleost fish have been used to study genetic regulation of development, carcinogenesis, high-throughput screening of chemicals, and environmental monitoring as well as models of human diseases (Hinton et al., 2009). The process of chronic hepatic injury, regeneration and carcinogenesis are closely linked but our understanding of the fish liver regenerative response and response to chronic injury remains limited (Wolf and Wolfe, 2005; Sadler et al., 2007; Hinton et al., 2008; Alison et al., 2009; Kan et al., 2009). To accurately extrapolate findings obtained using aquatic models, it is essential to understand
the similarities as well as the differences in organ structure, response to injury and relevant molecular mechanisms among fish and mammalian models, and humans. Therefore, the overall goal of this dissertation is to examine the regenerative and fibrotic liver response in the Japanese medaka. Chapter 2 focuses on the pathogenesis of hepatic fibrosis following chronic hepatic injury induced by DMN while chapters 3 and 4 focus on the process of hepatic regeneration and more specifically, hepatic progenitor cells. The identification of conserved cellular response and molecular pathways will strengthen the rationale for using laboratory fish as a model of human disease, as well as for toxicity testing and environmental monitoring.

a. Molecular Mechanism of Hepatic Fibrosis in Medaka

When studying the effect of differences in the mutagenicity of DMN between medaka and rats, hepatic fibrosis/cirrhosis was incidentally induced in medaka (Hobbie et al., 2009; Hobbie et al., 2011). They demonstrated by immunohistochemistry an increase in muscle specific actin (MSA), TGF-β1 and SMAD 3 in the fibrotic liver, thus suggesting that myofibroblastic transdifferentiation of HSCs, and up-regulation of the TGF- β pathways, occurs in fibrotic medaka liver (Hobbie et al., 2011). In this study, we refined the DMN-exposure parameters to reliably induce severe hepatic fibrosis/cirrhosis in medaka and determine if the main cellular and molecular events associated with development of hepatic fibrosis are conserved between fish, rodents and humans.

Our research hypothesis states that hepatic stellate cell activation and upregulation of the canonical TGF-β pathways, relevant MMP and TIMP genes are critical steps in the
development of hepatic fibrosis after DMN-induced hepatic injury in Japanese medaka. To address this hypothesis we anchored the expression of key genes involved in the pathogenesis of fibrosis to the activation of medaka hepatic stellate cells and deposition of collagenous matrix.

b. Differentiation Processes of Fish Hepatic Progenitor Cells

In studies evaluating hepatic regeneration, response to toxic injury and hepatocarcinogenesis in various fish species, proliferation of cells resembling rodent oval cells has been documented, and differentiation towards hepatocytes inferred (Couch and Courtney, 1987; Hinton et al., 1988; Nunez et al., 1990; Okihiro and Hinton, 2000; Fournie and Courtney, 2002; Kan et al., 2009; Hobbie et al., 2011). Histologically, the fish liver reaction to injury appears similar to that in mammals but it has not been determined if the oval-like cells (BPDECs in fish), are the biological equivalent to the human HPCs and rodent oval cells.

Our research hypothesis was that the oval-like cells observed histologically in the injured medaka liver are HPCs and are capable of differentiation along hepatocyte and biliary cell lineages. To test the hypothesis, the cell lineages and differentiation process of these putative progenitor cells were determined using histology, immunohistochemistry and ultrastructural characteristics after acute and chronic toxic hepatic injury.

c. Localization of the Hepatic Progenitor Cell in the Japanese Medaka

Despite the importance of HPCs in tissue homeostasis, repair and carcinogenesis, very little is known about their specific spatial and temporal characteristics in fish models. Given the
morphological similarities and spatial location between the mammalian HPC/oval cells and the fish BPDECs, it has been suggested that the BPDECs are likely the piscine HPC (Hardman et al., 2007; Hinton et al., 2008).

We hypothesized that the medaka liver contains a population of HPCs that can be detected by cell label retention techniques and are found in anatomical locations that correlate with BPDECs. To test this hypothesis, we performed a BrdU label retention cell assay during embryologic development and after acute liver injury in medaka and identified the label retaining cell type by histology and immunohistochemistry.
REFERENCES


activities and pathological changes in intraperitoneally exposed tilapia fish (Oreochromis sp.). *Toxicol Pathol* **36**, 449-58.


CHAPTER 2

Anchoring Gene Expression with the Development of Hepatic Fibrosis and Neoplasia in a Toxicant-Induced Fish Model of Hepatic Injury

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ABSTRACT

Fish have been used as a laboratory model to study hepatic development and carcinogenesis but not in the pathogenesis of hepatic fibrosis. A dimethylnitrosamine-induced fish model of hepatic injury was developed here in Japanese medaka (*Oryzias latipes*) and gene expression was anchored with the development of hepatic fibrosis and neoplasia. Treated livers exhibited mild hepatocellular degenerative changes 2 weeks post-exposure. Within six weeks hepatic fibrosis/cirrhosis was evident with development of neoplasia by 10 weeks. Predictably, gene expression changes preceded the morphological alterations. Stellate cell activation and development of fibrosis was associated with upregulation of *tgfb1*, *tgfb receptor 2*, *smad3 1 of 2*, *smad3 2 of 2*, *ctnmb1*, *c-myc*, *mmp2*, *mmp14 1 of 2*, *mmp14 2 of 2*, *timp2a*, *timp2b*, *timp3*, *colla1 1 of 2*, and *colla1 2 of 2*, and a less pronounced increase in *mmp13* and *col4a1* expression. *Tgfb receptor 1* expression was unchanged.

Immunohistochemistry suggested that biliary epithelial cells and stellate cells were the main producers of TGF-β1. This study demonstrates regulation of a group of candidate genes likely to be involved in the development of hepatic fibrosis, and that the TGF-β pathway likely plays a major role in the pathogenesis. These results support the medaka as a potentially useful fish model of hepatic fibrosis.
INTRODUCTION

Small aquarium teleost fish have been increasingly used in the last 4 decades to study genetic regulation of development, carcinogenesis, high-throughput screening of chemicals, and environmental monitoring (Hinton et al., 2009). In order to accurately extrapolate findings obtained from aquatic models to humans, it is critical to understand the differences and similarities in organ structure, response to injury and molecular mechanisms between these phylogenetically distant species. Given its fundamental function in metabolizing chemicals, the liver is an important target organ of toxicity and has received considerable attention. However the information available regarding the fish hepatic cellular response to injury and molecular mechanism(s) involved remain limited (Wolf and Wolfe, 2005).

In mammals, irrespective of the cause, chronic liver injury results in hepatic fibrosis, the end-stage of which is called cirrhosis, and is associated with chronic liver functional impairment. In the United States, chronic liver disease was the 12th leading cause of death in 2006 (Heron et al., 2009). Abusive alcohol consumption, hepatotoxins (e.g. Aflatoxin B1), autoimmunity, nonalcoholic fatty liver disease (NAFLD) and infections with Hepatitis B-(HBV) and Hepatitis C-viruses (HCV) are the main cause of chronic hepatitis (Sherman, 2010). Chronic liver injury, ultimately predisposes to liver cancer (Wallace et al., 2008). Hepatocellular carcinoma (HCC) alone is the fifth most common cause for cancer and the third cause of cancer-related death world-wide (Parkin et al., 2001; Wallace et al., 2008). Given the very limited therapeutic options, understanding the intricate cellular interactions implicated in liver fibrogenesis and subsequent progression to neoplasia is crucial.
Major events in the development of hepatic fibrosis in mammals involve activation of the TGF-β pathway and hepatic stellate cells (HSC) that results in an increased production of collagen, altered balance between metalloproteinases (MMP) and tissue inhibitors of metalloproteinases (TIMP) and, as an end result, excessive deposition of collagenous extracellular matrix in the hepatic parenchyma (Hernandez-Gea and Friedman, 2010).

Dimethylnitrosamine (DMN) is a potent carcinogenic hepatotoxin that has previously been used in rodents to model human hepatic fibrosis, cirrhosis and hepatocellular carcinoma (HCC), (Ala-Kokko et al., 1987; Jezequel et al., 1987; George et al., 2001; Tada et al., 2001; Hyon et al., 2011). In a comparative model, Hobbie et al. (2011) reported that Japanese medaka fish (*Oryzias latipes*) exposed to DMN developed morphological hepatic changes similar to those observed in human fibrotic liver and the well-established DMN-induced rat model of hepatic fibrosis (Ala-Kokko et al., 1987; Jezequel et al., 1987; Ohara et al., 2007; Hobbie et al., 2011; Hyon et al., 2011). To date, however, knowledge of the mechanism of hepatic fibrosis and progression to neoplasia in fish is very limited. Using immunohistochemical approaches, Hobbie et al. (2011) demonstrated that an increase in muscle specific actin (MSA), TGF-β1 and SMAD 3 labeled cells occurred in the liver of DMN exposed medaka. These results suggested that myofibroblastic transdifferentiation of HSCs, and up-regulation of the TGF-β pathways occur in medaka liver fibrosis similar to that observed in rodent models and humans (Hobbie et al., 2011).

We have refined the DMN-exposure parameters to reliably induce severe hepatic fibrosis/cirrhosis in medaka and hypothesized that activation of hepatic stellate cells and upregulation of the canonical TGF-β pathways, MMPs and TIMPs genes are critical steps in
the development of hepatic fibrosis after DMN-induced hepatic injury in Japanese medaka. In this study, we anchored the expression of key genes involved in the pathogenesis of fibrosis in mammals in relation to the activation of medaka hepatic stellate cells and deposition of collagenous matrix. Our overarching aim achieved in a fish model, was to enhance our knowledge of the comparative vertebrate pathogenesis of hepatic fibrosis.

MATERIALS and METHODS

Chemicals

Dimethylnitrosamine (DMN, C2H6N2O; 99.9%, CAS 62-75-9, MW 74.08 g/mol) was obtained from Sigma-Aldrich (St. Louis, MO). The air tight brown bottle of DMN was stored within a metal container at 4°C. All chemicals and reagents used in this study were of the highest purity available from commercial resources.

Medaka and DMN Exposures

Three-month-old, male and female, orange-red Japanese medaka (Oryzias latipes) were obtained from in-house stock population maintained under recirculating freshwater aquaculture conditions at the Department of Molecular and Environmental Toxicology, North Carolina State University (NCSU), Raleigh, NC. Fish between 6-8 months in age were acclimated for 2 weeks in 10 gallon aquarium tanks filled with reconstituted (0.5 g/liter Instant Ocean® salts, Aquarium System Inc. Mentor, OH) reverse osmosis–purified (RO) water within a recirculating, freshwater culture system under an artificial light photoperiod (16 hours light/8 hours dark) at a temperature of 26°C ± 0.5°C, a pH of ~ 6.5 and a
conductivity of 600 to 800 µS. Dry food (Otohime B1, Reed Mariculture, Campbell, CA) was fed several times per day through automated feeders. Animal care and use were in conformity with protocols approved by the NCSU Institutional Animal Care and Use Committee in accordance with the National Academy of Sciences Guide for the Care and Use of Laboratory Animals.

One hundred and seventy medaka (male and female) were exposed to 100 µg/L (ppm) DMN for 2 weeks in the ambient water. Ninety medaka (male and female) were used as controls. All fish were exposed in 4-liter glass beakers containing 3 liters of reconstituted RO water as described above. For all exposures, medaka were randomly distributed among 4-liter glass beakers, 10–12 fish per beaker. The beakers were placed within a recirculating, heated water bath to maintain temperature at 26°C ± 0.5°C throughout the exposures. DMN was replaced every 3 days to allow for photodegradation of the compound (Hobbie et al., 2011). DMN dilutions were made from the DMN stock solution (in H₂O) and prepared new prior to each treatment. Water quality was maintained with 95% water changes prior to each DMN treatment. Ammonia levels remained under 0.25 mg/liter throughout the entire study. Fish were fed once daily with dry fish food (Otohime B1) and observed twice daily for behavioral responses and signs of overt toxicity. Following exposure durations of 2-weeks, fish were removed from the exposure beakers, gently rinsed in RO water, and replaced in their 10 gallons aquarium tanks for up to 12 weeks (40 fish per tank).
**Sampling Method and Tissue Processing**

Twenty four exposed and 14 control fish were euthanatized with an overdose of tricaine methanesulfonate (300 mg/liter; MS-222, Argent Laboratories, Redmond, WA) at 2, 4, 6, and 10 weeks post exposure. The liver of 20 exposed and 10 control fish were inspected macroscopically, harvested and cut into two approximately equal-sized pieces. One was snap frozen in liquid nitrogen for RNA isolation and quantitative PCR (qPCR) analysis. The other piece was fixed in freshly made 4% paraformaldehyde solution for 24 hours, and transferred to 70% ethanol for histopathology. A few small samples (1 x 1 mm) of 4 exposed and control livers were fixed in 4F:1G fixative (4% formaldehyde and 1% glutaraldehyde buffered in monobasic sodium phosphate, pH 7.2-7.4) for transmission electron microscopy (McDowell and Trump, 1976). To detect infectious disease or background lesions in organs other than the liver that could have influenced the study results, a subset of fish was examined whole by histology. The coelom of four control and exposed fish was incised along the ventral midline to enhance fixative penetration and the fish were fixed whole in 4% paraformaldehyde solution for 24 hours, demineralized in 10% formic acid for 24 hours, and transferred to 70% ethanol for histopathology.

**Histology**

Paraformaldehyde–fixed livers were processed, and embedded in paraffin according to routine histologic techniques. Sections, 5-µm thick, were stained with hematoxylin and eosin (HE) and examined by light microscopy. Sections of liver were also stained with Masson’s trichrome stain according to standard methods. Medaka liver lesions were identified based on
criteria set by a consensus of the US National Toxicology Program pathology working group and the International Harmonization of Nomenclature and Diagnostic Criteria for Lesions in Rats and Mice (INHAND) project (Boorman et al., 1997; Thoolen et al., 2010).

**Immunohistochemistry**

Immunohistochemical studies were performed using single and double staining techniques. Five-micrometer thick paraffin sections from all livers were used for MSA and pancytokeratin (CK) immunohistochemistry. The MSA primary antibody used was a mouse monoclonal antibody that recognizes a 42-kD protein specific for actin in skeletal, cardiac, and smooth muscle (BioGenex Laboratories, San Ramon, CA. Catalogue # MU090-UC). The CK AE1/AE3 antibody used is a cocktail of 2 mouse monoclonal antibodies that recognize several acidic (10, 14 to 16, 19), and all basic (1-8) human cytokeratins (BioGenex Laboratories, San Ramon, CA. Catalogue # AM0751-5M). The TGF-β1 antibody was a rabbit polyclonal antibody that recognizes the human TGF-β1 C-terminus (Santa Cruz Biotechnology Inc., Santa Cruz, CA. Catalogue # sc 146). Tissue sections were deparaffinized in xylene, rehydrated in a graded series of ethanol and rinsed in distilled water. Antigen retrieval was performed for CK staining by heating the slides at 99°C in a 10 mM sodium citrate solution at pH 6 for 10 minutes in a vegetable steamer (Oster 5712 food steamer, Maitland, FL). Following antigen retrieval a cool down period of 10 minutes in the warm sodium citrate buffer preceded rinsing of the slides in 1X phosphate-buffered saline (PBS). Slides were treated with 3% hydrogen peroxide for 10 minutes to block activity of endogenous peroxidases. Goat serum was applied for 20 minutes to prevent nonspecific
binding of the secondary antibody (BioGenex San Ramon, CA). For single staining, avidin and biotin blocks were applied successively for 15 minutes each (BioGenex San Ramon, CA). Tissues were incubated with the MSA antibody (1/100 dilution) or CK antibody (sold prediluted) for 30 minutes at room temperature, or with the TGF-β1 antibody (1/500) overnight at 4°C. For double staining, tissues were incubated with a primary antibody cocktail of MSA and TGF-β1 or CK and TGF-β1 antibodies overnight at 4°C. The slides were rinsed in 1X PBS. For single staining, the slides were incubated with the secondary antibody for 20 minutes (BioGenex San Ramon, CA). After a wash in 1X PBS, tissue sections were then treated for 20 minutes with streptavidin peroxidase (BioGenex San Ramon, CA). For double staining the slides were incubated with a polymer cocktail detection system for 30 minutes at room temperature (anti-mouse/horseradish peroxidase (HRP) + anti-rabbit/alkaline phosphatase (AP), MultiVision Polymer Detection System, Thermo Fisher Scientific, Lab Vision Corporation, Fremont, CA). Following a final wash in 1X PBS, development was achieved by treatment of tissue with liquid 3,3-diaminobenzidine (DAB) chromogen for 15 second to 1 minutes (Vector Lab, Burlingame, CA). For double staining, development was achieved using first LVBlue (Thermo Fisher Scientific, Lab Vision Corporation, Fremont, CA) for AP activity followed by DAB for HRP activity. After a wash in tap water, the slides with single staining were counterstained with Mayer’s hematoxylin for 20–40 seconds and cover slipped. The slides with double staining were counterstained lightly with methyl green and cover slipped. For each immunohistochemical reaction, appropriate controls were run. The primary antibody was omitted and replaced by non-
immune serum of the same animal source in the negative control. Tissue serving as positive control was included in each slide (normal intestine and liver).

**Morphometry**

The number of activated stellate cells was determined by measuring the percentage area occupied by MSA immunoreactivity. For each liver histology section, 15 randomly selected sites were photographed at 600X using an Olympus DP 25 digital microscope camera with the CellSens® digital imaging software mounted on an Olympus BH 51 microscope (Olympus Corporation, Tokyo, Japan). The images were manually thresholded to highlight the MSA immunoreactive cells and background was subtracted using Adobe Photoshop CS4 (Photoshop CS4; Adobes Systems, Park Avenue, San Jose, CA, USA). The same setting was used for all images in a given data set. The best setting for background subtraction was determined empirically by testing images with the lowest and highest amount of MSA immunopositivity. Finally, the area fraction covered with the MSA positive cells, expressed as a percentage of the picture surface area, was measured using automatic image analysis system (ImageJ, NIH, Bethesda, MD).

The extent of liver fibrosis was determined semi-quantitatively and assigned a 0 to 3 score using Masson’s trichrome stained slides. The scale used was as follows: 0: no fibrosis; 1, mild fibrosis (small amount of fibrous tissue dissecting between the hepatocyte tubules); 2, moderate fibrosis (moderate amount of fibrous tissue dissecting in between hepatocytes tubules with occasional presence of hepatocellular nodules; and, 3, marked fibrosis/cirrhosis
(frequent large fibrous tissue septa dissecting and isolating variably sized aggregates of hepatocytes, and frequent hepatocellular nodule).

**Electron Microscopy**

Liver samples fixed in 4F:1G fixative were processed routinely for transmission electron microscopy, dehydrated in alcohol, and embedded in Spurr resin (Dykstra, 1993). To determine whether structures of interest were in planar sections of the block face, semithin sections, 0.5 μm thick, stained with 1% toluidine blue in 1% sodium borate, were examined under a light microscope. Ultrathin sections, 90 nm thick, of appropriate blocks were stained with uranyl acetate and lead citrate and examined with a FEI/Philips EM 208S transmission electron microscope (Laboratory for Advanced Electron and Light Optical Methods, NCSU).

**Reverse transcription and real-time PCR**

Liver samples were homogenized using the Bullet Blender (Next Advance, Averill Park, NY) with 0.5 mm zirconium oxide beads (setting 6 for 3 minutes) and total RNA was extracted using RNA Bee (Tel-Test Inc., Friendswood, TX) according to the manufacturer's instructions. RNA purity (260/280 ratio) and quantity were determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). RNA quality was determined using RNA Nano chips with an Agilent Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA). Only RNA samples with RNA integrity number > 7 and a 260/280 ration between 1.75 and 2.05 were used for qPCR analysis and these were stored at −80°C to minimize degradation.
RNA was reverse transcribed into cDNA using 1 µg total RNA and the high-capacity cDNA master kit from Applied Biosystems (Foster City, CA) as per the manufacturer's instructions with random hexamer as primer and processed using quantitative, real-time PCR (qPCR). Selected genes were identified in the medaka genome (http://www.ensembl.org/Oryzias_latipes/Info/Index) and specific primers were designed for: *tgfb1*, *tgfbr1*, *tgfbr2*, *smad3 1 of 2*, *smad3 2 of 2*, *ctnnb1*, *c-myc*, *mmp2*, *mmp13*, *mmp14 1 of 2*, *mmp14 2 of 2*, *timp2a*, *timp2b*, *timp3*, *colla1 1 of 2*, *colla1 2 of 2*, and *col4a1* (Table 1). The efficiency of each primer was tested to fall between 90 and 110%. cDNAs were PCR amplified separately in triplicate using a 96-well PCR plate and an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). Relative levels of the genes transcripts of interest listed above were measured using qPCR and normalized to ribosomal protein L7 (RPL-7) (Zhang and Hu, 2007). Each 25 µl real-time PCR reaction consisted of 2.5 µl (1 ng/µl) first-strand cDNA, 8 µl RNase-free water, 1 µl of 10µM forward primer, 1 µl of 10µM reverse primer, and 12.5 µl of SYBR® Green PCR Master Mix (AB applied biosystems). Real-time PCR reaction conditions were 50° for 2 minutes, 95°C for 10 minutes followed by 41 cycles of 95°C for 15 s, 60°C for 1 minute, and a last cycle at 95°C for 15 s and 60°C for 15 seconds. Relative quantitation of gene expression within each reaction was calculated following the method of Livak and Schittgen (2001).

**Data analysis and statistics**

Liver phenotype, MSA area data, and fibrosis score were compiled in spreadsheets, organized by fish case number using Microsoft Excel software (Microsoft; Redmond, WA).
The fish were grouped by phenotypes and the MSA percentage area and fibrosis score mean ± SEM calculated for each phenotype. All qPCR data were expressed as the mean mRNA level ± SEM. The difference in gene expression levels or MSA positive areas between the phenotypes was determined using the Mann–Whitney U test. The difference in fibrosis scores between phenotypes was determined using Fisher’s exact test. A P value less than .05 was considered significant. Correlation between MSA percentage area and fibrosis score or gene expression and fibrosis score was determined using the Spearman rank correlation test. The Fisher’s exact test was performed using SAS Version 9.1.3 for Windows (SAS institute, Cary, NC). The Mann–Whitney U tests and Spearman rank correlation tests were performed using Sigma Stat 3.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

Among the 170 fish exposed to DMN, 22 (12.9%) fish died or were euthanized for humane reasons during the grow out period. Given that autolysis occurs rapidly in tank water maintained at 26°C, 14 fish were moderately to severely autolysed at the time they were found and were discarded. All of the remaining 8 fish evaluated by histology had severe hepatic fibrosis/cirrhosis. None of the control fish died or were euthanized on ethical grounds during the duration of the study. For quality control, four additional non-treated and four exposed fish at each time point were processed for whole animal histology to evaluate for the presence of an undetected infectious disease or background lesion in organs other than the liver that could influence the study results. We found no evidence of infectious disease or
significant morphological change in one or two whole body sagittal sections in all animals examined, except in the liver of exposed fish.

**Gross findings**

Among the exposed fish, no macroscopic lesions were noted in the fish with hepatocellular degeneration or bile preductular epithelial cell (BPDEC) hyperplasia, although some of the livers were subjectively paler than the majority of the control livers. The fish with hepatic fibrosis had a mildly to markedly bosselated surface and their overall size was subjectively within normal limits or rarely reduced (Fig.1a). Among the 32 livers diagnosed with hepatic neoplasia on histology, 15 livers had a discrete hepatic tumor that was grossly visible. The overall liver size was markedly increased and filled most of the coelomic cavity in 2 fish.

**Histology**

Ninety-five half livers from the exposed fish and 56 half livers of control fish were evaluated by histology. Morphological changes and categorization of the liver was performed using H&E and Masson’s trichrome-stained liver sections. The control fish showed normal hepatic architecture with the exception of 2 fish collected at 10 weeks post-exposure that had multifocal aggregates of macrophages within the hepatic parenchyma. A Ziehl-Neelsen stain did not reveal the presence of acid-fast bacteria. The cause of the macrophage aggregates in these 2 fish was not determined. They were treated as outliers for the remainder of the study. Histologic lesions observed in the liver of exposed fish included hepatocellular vacuolar degeneration, Mallory body-like inclusion, hepatocyte hypertrophy, karyomegaly, altered
foci, necrosis, collapse of the hepatic architecture, histiocytic inflammation, BPDEC hyperplasia (presumed fish liver bipotential stem cell), HSCs hyperplasia, biliary hyperplasia, fibrosis, hepatocellular regeneration, and multinodular reorganization of the liver architecture. Cholangiocarcinoma (CC), HCC, combined hepatocellular-cholangiocarcinoma (CHC) and spindle cell neoplasm so-called hemangiopericytoma in fish (Fig. 1). A summary of the histologic lesions is presented in Table 2.

The morphological liver changes were classified in 5 different phenotypes: 0) normal liver, 1) hepatocellular degeneration, 2) BPDEC hyperplasia, 3) hepatic fibrosis and 4) hepatic fibrosis and neoplasia. A normal liver phenotype was defined as a liver with a normal architecture and an absence to moderate amount of hepatocellular vacuolization (Fig. 1b) (Hardman et al., 2007). The hepatocellular degeneration phenotype was characterized by an increase in hepatocellular vacuolization, presence of globular eosinophilic intra-cytoplasmic inclusions and scattered apoptotic hepatocytes (Fig. 1c). The BPDEC hyperplasia phenotype was characterized by proliferation of small elongated cells with a hyperchromatic round to elongated nuclei that separated the hepatocyte tubules and sometimes surrounded individual hepatocytes (Fig. 1d). The BPDEC hyperplasia phenotype closely resembled the oval cell hyperplasia observed in rodents (Thoolen et al., 2010). The hepatocytes were often variably sized, hypereosinophilic and with reduced amounts of glycogen. The hepatic fibrosis phenotype was characterized by increased fibrous connective tissue separating hepatocyte tubules. In advanced fibrosis stages, the collagenous stroma separated and individualized single or small groups of hepatocytes and hepatocyte tubules (fig. 1e). The hepatic fibrosis with neoplasia phenotype was defined as a fibrotic liver with the presence of a hepatic
neoplasm (hepatocellular carcinoma or cholangiocellular carcinoma) (Fig. 1f). Within treatment groups there were 41 (43%) males and 54 (56%) females and 1 (1%) medaka of undetermined gender. In the control group we identified 31 (55%) males and 25 (45%) females. Four fish (4%) in the treatment group had no hepatic lesions (Phenotype 0); 22 (23%) had evidence of degenerative hepatocellular changes (phenotype 1); 19 (20%) had marked BPDEC hyperplasia (phenotype 2); 34 (36%) had hepatic fibrosis (phenotype 3); and 16 (17%) developed liver neoplasia (phenotype 4). Among the livers with hepatic neoplasms, 10 fish (63%) were identified with HCC (2 males and 8 females), 3 (19%) with CC (1 male and 2 female), 2 (12%) fish with HCC and a CC (females), and 1 (6%) fish with hemangiopericytoma (unknown sex). The liver of 1 fish (1%) collected at 2 weeks post exposure was lost during processing for histology. The rate of occurrence of the 5 histologic phenotypes observed at 2, 4, 6 and 10 weeks post-exposure are presented in figure 2. At 2 weeks post exposure, 3 (13%) male fish had no hepatic lesions, 3 (13%) male and 13 (57%) female had hepatocellular degenerative changes, and 4 (17%) male had BPDEC hyperplasia. At 4 weeks post exposure, 1 (4%) male fish had a normal liver, 2 (8%) female had evidence of hepatocellular degeneration, 7 (29%) male and 1 (4%) female had BPDEC hyperplasia and 13 (54%) female had hepatic fibrosis. At 6 weeks post exposure, 4 (17%) female had hepatocellular degenerative changes, 4 (17%) male had BPDEC hyperplasia, 2 (8%) male and 12 (50%) female had hepatic fibrosis and 2 (8%) female had hepatic neoplasm on background of hepatic fibrosis. At 10 weeks post exposure, 3 (12%) male had BPDEC hyperplasia, 4 (17%) male and 3 (12%) female had hepatic fibrosis, and 5 (21%) male and 9 (38%) female had hepatic neoplasms.
Overall, there was a defined progression with time of the hepatic lesions following DMN treatment towards fibrosis and neoplasia. First hepatocellular degeneration was observed, followed by BPDEC hyperplasia, hepatic fibrosis, and ultimately hepatic fibrosis with neoplasm overtime. The females progressed faster to hepatic fibrosis and neoplasia than did the males (i.e., lagging approximately 2 weeks, data not shown) as reported previously following diethylnitrosamine exposure (Teh and Hinton, 1998).

**Transmission electron microscopy**

To confirm that the HSC acquired fibroblast-like characteristics and that collagen was the ECM deposited, control and fibrotic liver were examined by TEM. In control liver cells with HSC morphologic characteristics were extremely rare and no cells demonstrated fibroblast-like features. However, in fibrotic liver, HSC were more frequent, adjacent to collagen fibrils and had acquired myofibroblast characteristics such as cytoplasmic intermediate filament bundles, loss of lipid vacuoles and occasional potential collagen secretion granules (Fig. 3 to 5). Collagen fibrils in space of Disse were very rare in control liver but frequently observed in fibrotic liver.

**Morphometry**

*Muscle specific actin*: Morphometric quantitation of MSA immunolabeling was used as a measure of stellate cell myofibroblastic transdifferentiation (Fig. 6). Liver sections from 22 controls, 12 phenotype 1, 8 phenotype 2, 16 phenotype 3 and 12 phenotype 4 had adequate MSA immunohistochemical labeling for morphometric analysis. The relationship between
phenotype and the percent area occupied by the MSA immunohistochemically-reactive cells is presented in figure 7. The percent area of MSA staining was significantly different between phenotype except between control and phenotype 1 (hepatic degeneration) \((P < .05)\). All MSA-positive cells were included for the morphometric analysis although a significant number where likely myofibroblasts. Cells located in the hepatic parenchyma and immunoreactive for MSA were interpreted as activated stellate cells. Cells closely surrounding bile ducts were interpreted as myofibroblasts. In control liver, MSA-positive cells were only observed closely surrounding bile ducts.

*Masson’s trichrome staining*: Morphometric quantitation of Masson’s trichrome staining was performed as a measure of collagen deposition and fibrosis (Fig. 8). Liver sections from 22 controls, 12 phenotype 1, 9 phenotype 2, 17 phenotype 3 and 12 phenotype 4 samples had adequate Masson’s trichrome staining for morphometric analysis. The relationship between phenotype and the fibrosis score is presented in figure 8. The fibrosis score were significantly different between all phenotypes. The MSA percentage area was significantly correlated with the fibrosis score \((P < .05, r = .903)\)

*Gene expression*

Gene expression levels were determined only in liver samples that had an adequate mRNA quality matching with adequate tissue slides for morphometric analysis: 25 controls, 12 phenotype 1, 9 phenotype 2, 18 phenotype 3 and 12 phenotype 4 samples were evaluated. Messenger RNA expression levels of multiple fibrosis related genes were evaluated in relation to the liver phenotypes (Fig. 9 to 13). Change in expression of the following genes
significantly correlated with the fibrosis score in phenotype 1 and 2: \textit{tgfb1, tgfbr2, mmp2, mmp14 1 of 2, mmp14 2 of 2, timp2a, timp3, colla1 1 of 2, and colla1 2 of 2} ((P < .05, r > .99). At later time points, when fibrosis was established and continued to progress (phenotype 3 and 4), the expression levels of the genes listed above remained elevated but were lower than observed in phenotype 2, and therefore, correlation between gene expression level and fibrosis score was lost. No correlation was observed between gene expression of \textit{tgfbr1, smad3 1 of 2, smad3 2 of 2, col4a1, mmp13, timp2b, c-myc, ctnnb1} and fibrosis score in phenotype 1 and 2 livers.

\textbf{TGF-β1 localisation}

Immunohistochemistry was used to localize cells producing TGF-β1. In control liver and exposed liver with phenotype 1, TGF-β1-immunolabelling was present diffusely in the cytoplasm of cholangiocytes forming bile ducts, the BPDECs that are scattered along the biliary canaliculi and few scattered macrophages (Fig. 14 and 15). In exposed liver displaying phenotype 2 to 4, the number of cells immunolabeled with TGF-β1 was markedly increased due mainly to hypertrophy and hyperplasia of BPDECs lining biliary canaliculi and bile duct hyperplasia. Cholangiocytes and BPDECs were strongly immunoreactive. Weak to moderate positivity was present in a small number of macrophages. Occasional intermediate hepatocytes were weakly positive. No immunoreactivity for TGF-β1 was detected in the population of small elongated cells resembling rodent oval cells in the BPDEC hyperplasia phenotype. TGF-β1 immunoreactivity was detected in all neoplasm. Most neoplastic hepatocytes had weak to moderate cytoplasmic immunoreactivity while neoplastic
cholangiocytes were consistently strongly positive (Fig. 16 to 18). In order to confirm the identity of the non-hepatocyte cells expressing TGF-β1 in the hepatic parenchyma, labeling of consecutive tissue sections as well as double immunolabelling for CK, MSA and TGF-β1 was performed. Most cells immunolabeled with TGF-β1 and located in between hepatocytes were positive for CK but negative for MSA, thus supporting the notion that they were BPDECs. A smaller number of MSA-positive cells, often located along sinusoids, and interpreted as activated HSCs, were also positive for TGF-β1 (Fig. 14 and 15).

DISCUSSION

The use of small fish models in biomedical and environmental research continues to expand. In order to utilize these models to their full potential it is imperative to understand the similarities and differences in disease mechanisms among human, mammalian and fish animal models. The anatomical and histological differences between mammalian and piscine liver have been thoroughly studied in several species and especially the Japanese medaka (Hardman et al., 2007; Hardman et al., 2008). Medaka have a single lobed liver with microvasculature resembling a single mammalian hepatocytic lobule making it an ideal model to study the effect of hepatotoxicant (Hardman et al., 2007). Yet, our understanding of the piscine response to injury at the molecular level is still limited.

Hepatic fibrosis is a common wound-healing response to chronic liver injuries in mammals but is less often observed in fish species (Wolf and Wolfe, 2005). Liver fibrosis has however been described in medaka exposed to DMN and methylazoxymethanol acetate (MAM-Ac) (Hatanaka et al., 1982; Hobbie et al., 2011). In this study, the expressions of key
genes in the pathogenesis of hepatic fibrosis were associated with morphological changes that occur during development of hepatic fibrosis/cirrhosis in DMN-exposed medaka. The DMN exposure protocol used in this study reliably produced morphological pathological liver changes similar to those reported in medaka or observed in rodent models of hepatic fibrosis such as the DMN-induced rat model (Wallace et al., 2008; Hobbie et al., 2011).

Progression to neoplasia was much faster in medaka than in rodents with neoplasia present in 8% and 67% of the fish collected at 6 and 10 weeks post exposure, respectively (Peto et al., 1991).

Given the anatomical and functional differences between medaka and mammalian livers, some lesion patterns expected in mammals were absent, such as centrilobular necrosis and bridging fibrosis (Hinton et al., 2008). Zonal necrosis patterns are not observed in fish as they lack hepatocellular metabolic zonation. As the entire medaka liver is approximately the anatomical equivalent of one mammalian hepatic lobule and their biliary-venous tracts (portal tract equivalent) are mainly located near the hilus and rarely in the parenchyma, it is not expected to see bridging fibrosis similar to that occurring in a mammalian liver (Hardman et al., 2007). The pattern of fibrosis that best characterizes the fibrotic changes observed in medaka is pericellular (perisinusoidal) fibrosis. In pericellular fibrosis collagenous matrix extends along the sinusoids and surrounds single or small groups of hepatocytes resulting in a chickenwire or latticework appearance.

In mammals, liver injury is associated with an increase in HSC number and the acquisition by the quiescent HSC of a myofibroblast-like phenotype. The activated HSCs express smooth muscle α-actin (α-SMA) and acquire contractile properties. In human and
rodent livers, immunohistochemistry for α-SMA is considered the gold standard to identify and localize activated HSC and myofibroblasts, and used as a marker of active fibrogenesis (Hautekeete and Geerts, 1997; Kweon et al., 2001). In medaka, perisinusoidal cells have been shown by IHC to express actin in fibrotic liver induced by DMN and hepatocellular carcinoma induced by DMN, DEN or MAM-Ac (Bunton, 1995; Hobbie et al., 2011). In this study, evaluation of control and fibrotic liver by TEM confirmed HSCs were more numerous in fibrotic liver and had acquired fibroblast-like characteristics, thus supporting the MSA staining results. Additionally, an increased amount of collagen fibrils was observed in the perisinusoidal spaces of fibrotic liver and supported the observations of H&E and Masson’s trichrome stained liver sections. We quantified the amount of actin-positive cells following DMN injury and demonstrated a significant increase in actin-positive cell number that correlated with development and progression of fibrosis determined on H&E and Masson’s trichrome slides. These results indicate that the medaka liver response is similar as observed in mammals where HSCs acquire a fibroblast-like phenotype and proliferate in fibrotic liver.

We did not attempt to separate actin-positive cells (activated HSCs and myofibroblasts/smooth muscle cells) for morphometric analysis. The surface of all MSA positive cells was used for the analysis. Therefore, it was not determined if the increased amount of actin-positive cells results from an increase in activated HSCs alone or in combination with an increase in myofibroblasts. An increase in both activated HSCs and myofibroblasts is most likely.

Teleost fish often have two or more copies of single-copy mammalian genes (Postlethwait, 2007). The reason is presently unresolved but the whole genome duplication
theory appears to be the most concrete explanation for teleostean gene duplication (Postlethwait, 2007). Therefore we evaluated the expression levels of both gene copies when present in this study (coll1a1 1 of 2, and coll1a1 2 of 2; smad3 1 of 2 and smad3 2 of 2, mmp14 1 of 2 and mmp14 2 of 2).

Overall, gene expression pattern over time was similar for all genes evaluated. A moderate increase in transcription level during the early stage of hepatic degeneration (phenotype 1) was followed by a marked increase peaking when BPDEC hyperplasia (phenotype 2) occurred. Subsequently, gene expression decreased but remained significantly elevated when fibrosis (phenotype 3) was established and neoplasm(s) (phenotype 4) were developing.

In mammals, the TGF-β pathway is central in mediating fibrotic responses by regulating ECM production and resorption, and cellular proliferation. TGF-β1 is a potent antiproliferative cytokine that suppresses the proliferation of epithelial cell, including hepatocytes, and regulates the function of HSCs (Gressner et al., 2002). TGF-β signaling stimulates the synthesis of ECM components, such as type I, type III and IV collagen and reduced ECM degradation by up-regulating the expression of antiproteases such as TIMP-1 (Gressner et al., 2002).

In this study, data showed that activation of the TGF-β pathway occurs in medaka liver after hepatic injury. Increased tgfβ1 expression correlated with activation of HSCs and abnormal connective tissue deposition during onset and progression of fibrosis. A significant increase in expression of tgfβ1 and tgfbr2 was accompanied by a delayed and lesser increase in smad3 1 of 2, and smad3 2 of 2 expression. Expression of tgfbr1 remained unchanged.
Since the type II receptor is critical in receptor activation by binding the TGF-β ligand and activating TGFβRI, the increase in \textit{tgfbr2} would presumably amplify activation of the TGF-β pathway. Similarly, \textit{tgfbr2} level is up-regulated in other fibrotic disease such as in glomerulosclerosis and tubulointerstitial fibrosis of diabetic nephropathy, wound healing disorders, or keloid formation (Chin et al., 2001; Hong et al., 2001; Schultze-Mosgau et al., 2003). In patients with chronic liver disease, Calabrese et al., found an increase in expression of TGFBR2 with colocalization of TGF-β1 and SMAD on hepatocytes correlated with increased fibrosis score (Calabrese et al., 2003). Additionally, impairing the TGF-β pathway in rat exposed to DMN using an adenoviral vector expressing a truncated TGFR2 resulted in a marked reduction in hepatic fibrosis (Qi et al., 1999). However, Roulot et al., found that fibrotic livers in humans and rats exhibited a decreased in \textit{Tgfbr2} expression and an increased in \textit{Tgfbr1} expression in correlation with proliferation of HSCs and increased fibrosis (Roulot et al., 1999). These data suggest that there remains an inconsistent expression profile with TGF-β signaling and correlation to fibrosis in mammals.

We also observed a significant increase in \textit{Smad3 1 of 2} and \textit{smad3 2 of 2} levels within BPDEC hyperplasia and fibrotic livers, however, the fold induction was much lower than observed with \textit{tgfb1} or \textit{tgfbr2}. We chose to examine \textit{Smad3} expression due to previous studies in mammals that demonstrated pro-fibrotic activities of TGF-β were mediated by SMAD3 in several organs including liver (Flanders, 2004; Roberts et al., 2006). The significant but lower change in \textit{Smad3} expression is likely due to the fact that TGF-β1 stimulation of \textit{Smad3} is mediated mostly through an increase in SMAD3 phosphorylation rather than a modification in \textit{smad3} mRNA expression.
The TGF-β pathway is implicated in a number of cancers, including hepatic neoplasms. In the early stage of carcinogenesis, the TGF-β pathway acts mainly as a tumor suppressor, but later acts as a tumor promoter (Wakefield and Roberts, 2002; Musch et al., 2005; Mamiya et al., 2010). The results of this study suggest that upregulation of the TGF-β canonical pathway occurs in the fish liver neoplasms like in the spontaneous mouse and human HCC. Immunoreactivity for TGF-β1 was present in the neoplastic cells and the expression of tgfβ1, tgfbr2, and smad3 1 of 2 remained upregulated in the fibrotic liver with neoplasms. We did not separate neoplastic tissue from fibrotic liver tissue during mRNA isolation. Therefore the expression level measured is not representative of the neoplasm alone and a definitive conclusion regarding the upregulation of the TGF-β pathway in these liver carcinomas cannot be made.

Expression and localization of tgfbr2 is also known to be altered in cancer. However, its role remains unclear and expression has been shown to be increased, unchanged or decreased in HCC in human or rodent model (Kiss et al., 1997; Abou-Shady et al., 1999; Mamiya et al., 2010; Hoenerhoff et al., 2011). In this study, tgfbr2 remained upregulated when neoplasms developed in the fibrotic liver.

The trend in expression levels of tgfβ1 and tgfbr2 paralleled those of colla1 1 of 2, colla1 2 of 2, and to a lesser extend of col4a1. Upregulation of tgfβ1, tgfbr2, colla1 1 of 2, and colla1 2 of 2, correlated with activation of HSCs and deposition of ECM. Our findings are in accordance with those in humans and mammalian models, in which increased collagen gene expression and deposition of collagen in hepatic fibrosis accompanies increased TGF-β1 production (Gressner et al., 2002).
TGF-β1 immunohistochemistry in fibrotic livers demonstrated protein expression in BPDECs, cholangiocytes, and in some macrophages, HSCs, and rarely in intermediate hepatocytes. These results are consistent with the one previously reported in medaka, and rat after hepatic injury (Jakowlew et al., 1991; Milani et al., 1991; Tao et al., 2000; Hobbie et al., 2011). They suggest that in fish, like in mammals, resident and recruited inflammatory cells (macrophages and Kupffer cells) and proliferating biliary epithelial cells promote fibrogenesis through increased production TGF-β1 (Roth et al., 1998; Chantal, 2000; Matsuzaki, 2009). Although the antibody used in this study is sold as an anti-TGF-β1 antibody, the manufacturer states that it cross-reacts with TGF-β2. Given that its specificity in fish species is unknown, it is possible that the immunoreactivity observed in the cholangiocytes and BPDECs is due to TGF-β2 and not TGF-β1. Expression of Tgfb2 mRNA is known to occur in cholangiocytes of proliferating bile ducts in rat and human fibrotic liver (Milani et al., 1991). It is interesting to note that Kupffer cells, an important source of TGF-β1 after hepatic injury in mammals, are absent in medaka and in most teleosts (Hardman et al., 2007; Hinton et al., 2008). However, fish livers often have interhepatocytic, perisinusoidal macrophages (IPM) that increases in number and phagocytize cellular debris following hepatic injury (Boorman et al., 1997; Okihiro and Hinton, 1999; Okihiro and Hinton, 2000). Given that immunoreactivity for TGF-β1 of some IPM was observed in this study, it is possible that IPM play a role similar to Kupffer cells in the pathogenesis of hepatic fibrogenesis.

A significant increase in expression of mmp2, mmp14 1 of 2, mmp14(2 of 2, timp2a, timp2b, and timp3 was observed during development of hepatic fibrosis and neoplasia. A
much smaller but significant increase in \textit{mmp13} was also present. Trends in expression levels of the MMP and TIMP genes evaluated were paralleled by the upregulation of other genes typically expressed in fibrosis like \textit{tgfb1} and \textit{colla1}. These results are similar to those reported in human and rodents studies where \textit{Mmp2} and \textit{Mmp14} expression levels gradually increase with disease progression while the increase in \textit{Mmp13} expression is transient, taking place mainly during early phase of fibrosis and during recovery from fibrosis (Watanabe et al., 2000; Lichtinghagen et al., 2003; Hemmann et al., 2007). Expression of \textit{Timp}-1, 2 and 3 also increased following liver injury in human and rodents and persisted as fibrosis progressed (Iredale et al., 1996; Herbst et al., 1997; Kossakowska et al., 1998; Yoshiji et al., 2000). TIMP1 is the collagenase inhibitor most often evaluated in human and rodents studies and is considered an essential player in the development of hepatic fibrosis. Unfortunately, an orthologous gene was not identified in the medaka genome.

The results suggest that this part of the pathogenesis in the medaka model is likely similar to the one in mammals where activation of the TGF-\(\beta\) pathways and HSCs results in alteration in the balance between production and resorption of ECM components. The net accumulation of collagen results predominantly from increase in transcription of collagen genes, mainly type I collagen, and impaired degradation due to changes in the balance between MMPs and TIMPs (Kossakowska et al., 1998; Hemmann et al., 2007). Studies of fibrosis in various organs including the liver indicate that an increase in TIMP/MMP ratio promotes fibrosis (Iredale et al., 1996; Yoshiji et al., 2000; Madtes et al., 2001; Nicholson et al., 2002).
The Wnt signaling pathway has been implicated in organ fibrosis, including liver fibrosis, and neoplasia (Shackel et al., 2001; Kim et al., 2005; Myung et al., 2007). There is evidence that the profibrogenic role of Wnt signaling occurs through HSC activation and promotion of survival (Myung et al., 2007; Cheng et al., 2008). However, the role of the Wnt pathway in hepatic fibrosis is still poorly understood compared to its role in hepatic neoplasia (Thompson and Monga, 2007). β-catenin is the chief downstream effector of the canonical Wnt signaling pathway. Binding of the Wnt ligands to the frizzled family receptor induces activation and accumulation of β-catenin, entry into the nucleus, and activation of target genes (Thompson and Monga, 2007). In this study, there was upregulation of ctnnb1 suggesting upregulation of the Wnt canonical pathways during development and progression of hepatic fibrosis as it is the case in mammals (Shackel et al., 2001; Myung et al., 2007; Cheng et al., 2008). However, we did not evaluate if nuclear translocation of catenin occurred to provide direct evidence and confirm activation of the canonical Wnt pathways in this fish model. Additionally, ctnnb1 upregulation was observed in fibrotic medaka liver with neoplasia suggesting dysregulation of the Wnt pathway may be occurring in medaka liver carcinomas. Dysregulation of the Wnt pathway with overexpression of the β-catenin protein is an important factor in the development and progression of HCCs in zebrafish, rodent and human (Harada et al., 2004; Haramis et al., 2006; Kim et al., 2008). However, HCCs were not separated from the surrounding fibrotic liver and it cannot be determined if this increase is part of the fibrotic process, HCCs or both.

MYC is a ubiquitous transcription factor regulating the transcription of numerous genes that are involved in many functions including the control of cell proliferation and
differentiation (Oster et al., 2002). Up-regulation of Myc occurs following activation of various mitogenic signaling pathways including the Wnt pathway. In this study, myc was up-regulated in all phenotypes evaluated including livers with neoplasia, thus suggesting possible roles in hepatocyte proliferation and neoplasia in medaka. Temporary up-regulation of Myc is known to occur during hepatic regeneration while sustained up-regulation is linked to neoplasia in rodent and human (Santoni-Rugiu et al., 1996; Michalopoulos and DeFrances, 1997). Sustained myc expression in hepatocytes of transgenic zebrafish and medaka overexpressing myc resulted in hyperplasia and, in zebrafish only, possible hepatocellular adenoma (Gong et al., 2011; Menescal et al., 2012).

In conclusion, the results of this study demonstrate that the main cellular and molecular events in the pathogenesis of hepatic fibrosis in mammals and medaka fish exposed to DMN are conserved. Hepatocellular injury is followed by activation of HSCs, TGF-β pathway activation, change in the balance between MMPs and TIMPs and increase in collagen production with the end result of excessive deposition of collagenous ECM. These data also support the medaka as a potentially useful alternative animal model of hepatic fibrosis and improves the comparative understanding of the liver’s response to chronic injury across taxa.

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### TABLES

Table 1. Sequences for primers used in quantitative, real-time PCR

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<td>CGCCAGATCTTCAAGGTTGTAT</td>
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<tr>
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* (http://www.ensembl.org/index.html)
Table 2. Degenerative and Proliferative Hepatic Lesions in Medaka exposed to 100 ppm Dimethylnitrosamine for 2 weeks.

<table>
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<tr>
<th>Week post exposure</th>
<th>Cellular degeneration</th>
<th>Spongiosis hepatis</th>
<th>Necrosis / apoptosis</th>
<th>Architectural change</th>
<th>BPDEC(^a) proliferation</th>
<th>Bile duct hyperplasia</th>
<th>Fibrosis</th>
<th>Cellular dysplasia</th>
<th>Neoplasia</th>
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<td>2 weeks</td>
<td>21/23</td>
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<td>22/24</td>
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<td>22/24</td>
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\(^a\) Bile preductular epithelial cell
Figure 1. Gross appearance and histology of control and DMN exposed liver A: Gross appearance of a normal medaka liver (left) and 2 livers with severe hepatic fibrosis (center and right). The liver on the right has a grossly visible neoplasm diagnosed as a hepatocellular carcinoma by histology. Bar = 1 mm. B: Histology of a normal female medaka liver (phenotype 0). C: Hepatocellular degeneration (phenotype 1) characterized by increased hepatocellular vacuolization, presence of globular eosinophilic intra-cytoplasmic inclusions (arrow) and scattered apoptotic hepatocytes. Insert: Close-up of an eosinophilic intra-cytoplasmic inclusion. 100X. D: Biliary preductular epithelial cell hyperplasia (phenotype 2) characterized by proliferation of small spindle cell with a hyperchromatic elongated nuclei that separated the hepatocytes tubules and sometime surrounded individual hepatocytes. E: Hepatic fibrosis (phenotype 3) characterized by increase connective tissue separating hepatocytes tubules and individualizing single or small group of hepatocytes. F: The hepatic fibrosis and neoplasia phenotype (phenotype 4) was defined as hepatic fibrosis with presence of a hepatic neoplasm (Hepatocellular carcinoma or cholangiocellular carcinoma). Hematoxylin and eosin. Bar = 50 µm 40X
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Figure 3. Transmission electron microscopy of an activated hepatic stellate cell. An activated hepatic stellate cell (HSC) is adjacent to extracellular collagen fibrils (C) and a macrophage (M) in the space of Disse. This HSC has lost its lipid vacuole(s) and a bundle of intermediate filaments (arrow head) is present in the cytoplasm. E: endothelial cell. RBC: red blood cell. Bar = 1 µm. X 8900.
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Figure 5. Presumptive collagen secretion granules in the cytoplasm of an activated HSC. Bar = 100 nm. X 71000.
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* Statistical difference (P < 0.05) in gene expression level between phenotypes.
Figure 10. Relationship between the liver phenotypes observed following DMN exposure and the mRNA expression level of TGF-β1, TGF-β receptor 1 and 2, and SMAD3 genes for each phenotype. Mean ± SEM.

* Statistical difference (P < 0.05) in gene expression level between phenotypes.
Figure 11. Relationship between the liver phenotypes observed following DMN exposure and the mRNA expression level of matrix metalloproteinase 2, 13 and 14 genes for each phenotype. Mean ± SEM.

* Statistical difference (P < 0.05) in gene expression level between phenotypes.
Figure 12. Relationship between the liver phenotypes observed following DMN exposure and the mRNA expression level of tissue inhibitor of matrix metalloproteinase 2 and 3 genes for each phenotype. Mean ± SEM.

* Statistical difference (P < 0.05) in gene expression level between phenotypes.
Figure 13. Relationship between the liver phenotypes observed following DMN exposure and the mRNA expression level of β-catenin and Myc genes for each phenotype. Mean ± SEM.

* Statistical difference (P < 0.05) in gene expression level between phenotypes.
Figure 14. Hepatic fibrosis. Double immunolabelling for MSA (brown) and TGF-β1 (blue), methyl green counterstain. Representative picture of a control liver (left). No immunoreactivity for MSA is observed in the hepatic parenchyma. TGF-β1 immunoreactivity is observed in BPDEC. Representative picture of a liver with hepatic fibrosis (right). BPDEC are increased in number, hypertrophied and strongly positive for TGF-β1. Myofibroblasts (asterisk) and HSCs (arrow head) are immunoreactive for MSA. Some HSCs are immunoreactive for MSA and TGF-β1 (arrow). Bar = 20 µm 100X.
Figure 15. Hepatic fibrosis. Double immunolabelling for MSA (red) and TGF-β1 (brown), hematoxylin counterstain. BPDEC are increased in number, hypertrophied and strongly positive for TGF- β1. HSCs (arrow head) are immunoreactive for MSA. Some HSCs is immunoreactive for MSA and TGF- β1 (arrow). Bar = 20 µm 100X
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CHAPTER 3

Characterization of the Hepatic Progenitor Cell Compartment in Japanese Medaka (*Oryzias latipes*) following Acute and Chronic Hepatitic Injury: an Immunohistochemical and Ultrastructural Study

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ABSTRACT

Hepatic progenitor cells (HPC), called oval cells in rodents, participate in liver regeneration following severe injury or impaired hepatocyte replication, and are precursor of both hepatocytes and cholangiocytes. In fish, oval-like cells are observed in injured and regenerating liver. Here, the process of oval cell differentiation is defined in fish liver using histopathology, immunohistochemistry and transmission electron microscopy. To generate oval cell proliferation, hepatic injury was induced using either microcystin LR or dimethylnitrosamine exposure in Japanese medaka (*Orizia latipes*). A transgenic medaka strain expressing a red fluorescent protein (RPF) exclusively in the hepatocytes was used to label hepatocytes. The morphological response to injury with ductular reaction comprised of cytokeratin AE1/AE3 (CK) positive oval cells progressing to intermediate hepatobiliary cells variably positive for CK and RFP and mature hepatocytes and cholangiocytes supported a differentiation pathway of fish oval cells towards hepatocytes and cholangiocytes. Ultrastructural morphology confirmed the presence of oval cells and differentiation towards hepatocytes. These results demonstrate clear similarities between piscine and mammalian liver patterns of reaction to injury, and support putative bipotential lineage capabilities of the piscine HPC/oval cell.
INTRODUCTION

It is well-known that the liver of mammals has the ability to regenerate following injury (Michalopoulos, 2010; Riehle et al., 2011). Yet, in several diseases such as in chronic hepatitis and cirrhosis, these regenerative mechanisms are impaired or insufficient and restoration of the hepatic mass and function does not occur (Wallace et al., 2008; Riehle et al., 2011). In order to better understand and potentially control hepatic regeneration for therapeutic purposes, the cellular and molecular mechanisms have been studied extensively using a variety of animal models (Palmes and Spiegel, 2004; Sadler et al., 2007; Kan et al., 2009; Michalopoulos, 2010). Although small fish species are considered robust models to study regenerative processes such as fin and heart, the process of hepatic regeneration in fish has received considerably less attention (Poss et al., 2003; Sadler et al., 2007).

In mammals, liver regeneration (compensatory growth) after injury is accomplished primarily by proliferation of mature hepatocytes (Riehle et al., 2011). However after severe hepatic injury or when hepatocyte division is impaired, hepatic progenitor cells (HPC), called oval cells in rodents, participate in the regenerative process and are the alleged precursor cells of hepatocytes and cholangiocytes (Evarts et al., 1989; Fausto and Campbell, 2003; Riehle et al., 2011). These HPCs are also considered a possible cell of origin of hepatic neoplasms, further stressing the importance of understanding their biology (Mikhail and He, 2011). A third pathway of liver regeneration by fusion or transdifferentiation of bone marrow stem cells into hepatocytes has been documented, although this pathway is not considered clinically significant (Oh et al., 2007; Riehle et al., 2011).
Studies investigating the regenerative liver response in fish have demonstrated that the two predominant pathways of mammalian hepatic regenerative response occur in fish (Okihiro and Hinton, 2000; Sadler et al., 2007; Kan et al., 2009; Hobbie et al., 2011). Following partial hepatectomy, compensatory liver growth in zebrafish (Danio rerio) and rainbow trout (Oncorhynchus mykiss) occurs via proliferation of mature hepatocytes, or a combination of hepatocytes and cells presumed to be progenitor cells due to their morphological resemblance to rodent oval cells (Okihiro and Hinton, 2000; Sadler et al., 2007; Kan et al., 2009). In studies evaluating the hepatic response to toxic injury and hepatocarcinogenesis in various fish species, proliferation of cells resembling rodent oval cells has been documented, and their presumptive differentiation towards hepatocytes inferred (Couch and Courtney, 1987; Hinton et al., 1988; Nunez et al., 1990; Okihiro and Hinton, 2000; Fournie and Courtney, 2002; Hobbie et al., 2011).

The terminology used to describe HPC/oval cells in histology sections can be confusing. In humans, the descriptive term “oval cell” is discouraged because rodent oval cells are considered not exactly comparable with their human equivalent; the term “HPC” is favored (Roskams et al., 2004). However, in rodent animal models, “oval cell” remains the recommended term (Thoolen et al., 2010). In fish, these small cells with high nuclear-cytoplasmic ratio have been termed “bile preductular epithelial cells” (BPDEC) by the similarity of location with that of mammalian oval cells/HPCs (Couch and Courtney, 1987; Hinton et al., 1988; Nunez et al., 1990; Okihiro and Hinton, 2000; Fournie and Courtney, 2002; Hobbie et al., 2011). The fish liver has numerous bile preductules that are transition canals between the biliary canaliculi formed by adjacent hepatocytes and the bile ductules.
which are entirely lined by biliary epithelial cells (Hardman et al., 2007). Bile preductules are formed by BPDECs and hepatocytes (Hardman et al., 2007). Hence, BPDECs reside in a location equivalent to mammalian oval cells in the canal of Hering. Further supporting their probable equivalence fish “BPDECs”, rodent “oval cells” and human “HPC” share common ultrastructural morphology. They have junctional complexes with hepatocytes and/or biliary cells. They have a round to oval nucleus, scant cytoplasm, few organelles, variable amounts of intermediate filaments and no basal lamina (Factor et al., 1994; Hardman et al., 2007; Sobaniec-Lotowska et al., 2007). Moreover, in trout liver primary cultures, the longest living cells shared morphologic features and immunoreactivity (CK AE1/AE3) with BPDECs (Ostrander et al., 1995), which further supports the hypothesis that BPDECs are the HPC in fish, since this longevity is evocative of a “stem cell like” state. In the present study, the terminology defined by the International Harmonization of Nomenclature and Diagnostic Criteria for Lesions in Rats and Mice (INHAND) project and the descriptive term oval cell in fish will be applied. Although fish HPC/oval cells might be derived from BPDEC, use of the term oval cell will allow distinction between quiescent BPDECs and the activated transient amplifying population of HPC that resemble rodent oval cells. The latter will be defined as elongated to round cells with a high nuclear-cytoplasmic ratio and round to oval hyperchromatic nuclei that are often organized in a single or double row in linear arrays and pseudo-duct-like structures (Fig. 1 and 2) which are called “ductular reaction” in humans and “oval cell hyperplasia” in rodents (Roskams et al., 2004; Thoolen et al., 2010). Histologically, the fish liver reaction to injury appears similar to their mammalian counterpart. However, it has not been determined if the oval cells observed in the fish liver
are the biological equivalent to the HPC and rodent oval cells. It is hypothesized that the fish oval cells are the piscine hepatic progenitor cells and have bipotential differentiation abilities in the hepatocyte and biliary cell lineages. The aim of the present study is to determine the immunohistochemical and ultrastructural characteristics of the putative piscine progenitor cell compartment after acute and chronic toxic hepatic injury. The putative fish progenitor cell compartment was studied in the medaka fish model using two chemical hepatotoxicants, MCLR and DMN to induce acute and chronic hepatic injury, respectively. The HPC population response after hepatic injury was characterized using histology, single and double immunohistochemistry, and transmission electron microscopy (DMN exposure only) to characterize the cells lineages and differentiation process. The effects of microcystins have been evaluated in several fish species and they are known be potent hepatotoxins (Malbrouck and Kestemont, 2006). Dimethylnitrosamine (DMN) is a potent carcinogenic hepatotoxin that has previously been used in rodents and fish to model human hepatic fibrosis, cirrhosis and hepatocellular carcinoma (Ala-Kokko et al., 1987; Jezequel et al., 1987; George et al., 2001; Tada et al., 2001; Hyon et al., 2011) (Ala-Kokko et al., 1987; Jezequel et al., 1987; Ohara et al., 2007; Hobbie et al., 2011; Hyon et al., 2011).

MATERIALS and METHODS
To label hepatocytes, we employed a transgenic orange-red medaka fish line Tg(zfL-fabp:DsRed) which expressed a red fluorescent protein (RFP) under the regulatory control of the 2.8-kb fragment of the zebrafish liver fatty acid binding protein (L-FABP) promoter (Korzh et al., 2008). The founder L-FABP transgenic medaka for our colony were originally
obtained from Drs. Joerg Renn and Christoph Winkler, Department of Biological Sciences, National University of Singapore. This stable transgenic line exhibited standard Mendelian inheritance from F2 generation onwards. The offspring were screened by observation of embryos or larvae for RFP protein expression in the liver. The colony of Tg(zfL-fabp:DsRed) medaka was housed under recirculating freshwater aquaculture conditions at the Department of Molecular and Environmental Toxicology, North Carolina State University (NCSU), Raleigh, NC. Water temperature and pH were monitored daily and maintained at 23–26 ◦C and ∼ 7.2, respectively, and the fish were kept under a defined light-dark cycle (16 h light, 8 h dark). Dry food (Otohime B1, Reed Mariculture, Campbell, CA) was fed several times per day through automated feeders, and newly hatched Artemia nauplii were fed once per day.

**DMN and MCLR exposures**

Three-month-old male and female medaka from the stock colony were acclimated for 2 weeks in 10 gallon aquarium tanks filled with reconstituted reverse osmosis–purified (RO) water (0.5 g/liter Instant Ocean® salts, Aquarium System Inc. Mentor, OH) within a static, freshwater culture system. The artificial light photoperiod was 16 hours light/8 hours dark. The water temperature was maintained at 26°C ± 0.5°C, the water pH at ∼ 6.5 and the water conductivity between 600 to 800 µS. Dry food (Otohime B1) was fed throughout the day using automated feeders. Animal care and use conformed with protocols approved by the NCSU Animal Care and Use Committee in accordance with the National Academy of Sciences Guide for the Care and Use of Laboratory Animals. For the DMN and MCLR exposures, medaka were randomly distributed among 4-liter glass beakers, 10–12 fish per
beaker, containing 3 liters of reconstituted RO water as described above. The beakers were placed within a recirculating, heated water bath to maintain temperature at 26°C ± 0.5°C throughout the exposures. Fish were fed once daily. Animals were observed twice daily for behavioral responses and signs of overt toxicity.

**DMN exposure**

Eighteen Tg(zfL-fabp:DsRed) medaka were exposed to 100 μg/L (ppm) of dimethylnitrosamine (DMN) for 2 weeks in the ambient water. Nine fish were used as controls. DMN was replaced every 3 days to compensate for photodegradation of the compound (Hobbie et al., 2011). DMN dilutions were made new from the DMN stock solution before each renewal. Water quality was maintained with 95% water changes prior to each DMN treatment. Ammonia levels remained under 0.25 mg/liter at all times. After the 2-week exposures, fish were returned to the 10 gallon aquarium tanks for up to 8 weeks after a gentle rinse in RO water.

**MCLR exposure**

Twenty one, twenty seven and twelve Tg(zfL-fabp:DsRed) medaka received a single intracoelomic injection of microcystin-LR (MCLR) dosed at 100, 250 and 500 ±25 ug/kg, respectively. The MCLR stock solution was dissolved in sterile phosphate-buffered saline (PBS) (pH 7.2) at a concentration of 0.025 μg/μL. Fish were briefly anesthetized using tricaine methanesulfonate (120 mg/liter; MS-222, Argent Laboratories, Redmond, WA), weighed, placed in right lateral recumbency, and injected intraperitoneally using a glass 25
µL Hamilton syringe (PB-600 Repeating Dispenser, Hamilton, Reno, NV), equipped with a 32 gauge needle (Hamilton, Reno, NV). Twenty control fish were anesthetized and injected with 4µL of sterile PBS.

**Sampling Method and Tissue Processing**

Six DMN-exposed and 3 control fish were euthanized with an overdose of tricaine methanesulfonate (300 mg/L) at 4, 6 and 8 weeks post exposure. Individual fish livers were harvested and fixed for 24 hours in freshly made 4% paraformaldehyde solution for histopathology. Samples of each liver were also fixed in 4F:1G fixative (4% formaldehyde and 1% gluteraldehyde buffered in monobasic sodium phosphate, pH 7.2-7.4) for transmission electron microscopy (McDowell and Trump, 1976).

A subset of at least 2 MCLR-exposed and 1 control medaka were euthanized with an overdose of tricaine methanesulfonate (300 mg/L) at 1, 2, 4, 6 and 8 days post exposure for whole body histology. The coelom of control and exposed fish was incised along the ventral midline to enhance penetration of the fixative solution. The fish were fixed whole in 4% paraformaldehyde solution for 24 hours, demineralized in 10% formic acid for 24 hours, and transferred to 70% ethanol for histopathology.

Paraformaldehyde-fixed livers and whole fish were processed using standard histological techniques, embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin (HE). All liver sections as well as paramedian and midsagittal sections of whole fish were evaluated by light microscopy and liver lesions were classified based on criteria set by a
consensus of the US National Toxicology Program pathology working group and the INHAND guide (Boorman et al., 1997; Thoolen et al., 2010).

Liver samples fixed in 4F:1G fixative were processed routinely for transmission electron microscopy, dehydrated in alcohol, and embedded in Spurr resin (Dykstra, 1993). Semithin sections, 0.5μm thick, were stained with 1% toluidine blue in 1% sodium borate. Ultrathin sections, 90nm thick, were stained with uranyl acetate and lead citrate and examined with a FEI/Philips EM 208S transmission electron microscope (Laboratory for Advanced Electron and Light Optical Methods, NCSU, Raleigh, NC).

**Immunohistochemistry**

Five-micrometer thick paraffin serial sections of the liver were used for single and double immunohistochemistry. Primary antibodies used included anti-cytokeratin AE1/AE3 (CK), anti-muscle-specific actin (MSA), anti-red fluorescent protein (RFP) and anti-proliferating cell nuclear antigen (PCNA) (Table 1). Biotinylated goat anti-rabbit and goat anti-mouse immunoglobulin G were used as secondary antibodies (BioGenex San Ramon, CA). Tissue sections were deparaffinized in xylene and rehydrated in a graded series of ethanol and rinsed in distilled water. For antigen retrieval the slides were heated at 99°C in a 10 mM sodium citrate solution at pH6 for 10 minutes using a vegetable steamer (Oster 5712 food steamer, Maitland, FL). Slides were left for an additional 10 minutes in the warm sodium citrate buffer and then rinsed in 1X PBS. Endogenous peroxidases activities were blocked by exposing the tissues to 3% hydrogen peroxide for 10 minutes. Goat serum, avidin and biotin blocks were applied successively for 20 and 15 minutes, respectively, to prevent nonspecific
binding of the secondary antibody and streptavidin conjugates (BioGenex San Ramon, CA). Tissues were incubated with a primary antibody for 30 minutes at room temperature, rinsed in 1X PBS, and then incubated with the secondary antibody for 20 minutes. After a wash in 1X PBS, tissue sections were then treated for 20 minutes with streptavidin peroxidase. Following a final wash in 1X PBS, development was achieved by treatment of tissue with liquid 3,3-diaminobenzidine (DAB, Vector Lab, Burlingame, CA) chromogen for 30 second to 2 minutes or NovaRED (Vector Lab) for 1 to 5 minutes. Subsequently the slides were washed in tap water, counterstained with Mayer’s hematoxylin for 20–40 seconds and cover slipped. For double immunohistochemistry tissues were incubated with a primary antibody cocktail made of CK and RFP, MSA and RFP, or CK and MSA antibodies at room temperature for 30 minutes. The slides were rinsed in 1X PBS and incubated with a polymer cocktail detection system for 30 minutes at room temperature (anti-mouse/horseradish peroxidase (HRP) + anti-rabbit/alkaline phosphatase (AP), MultiVision Polymer Detection System, Thermo Fisher Scientific, Lab Vision Corporation, Fremont, CA).

After rinsing in 1X PBS, development was achieved using first DAB (Vector Lab) for HRP activity followed by NovaRED (Vector Lab) for AP activity. Following a wash in tap water, the slides were counterstained with Mayer’s hematoxylin for 20–40 seconds and cover slipped. For negative controls, the primary antibody was omitted and non-immune serum of the same species as the primary antibody was applied. For positive controls, sections of intestine and liver were included in each slide.
RESULTS

Histopathology and immunohistochemistry: DMN exposure

The morphological changes in the liver of DMN exposed fish were similar to those previously described by Hobbie et al., 2011 (Table 2). Histologic lesions included hepatocellular vacuolar degeneration, Mallory body-like inclusions, altered foci, necrosis, collapse of the hepatic architecture, histiocytic inflammation, oval cell hyperplasia, hepatic stellate cell hyperplasia, biliary hyperplasia, fibrosis, hepatocellular regeneration, and multinodular reorganization of the liver architecture. No remarkable microscopic abnormalities were seen in the liver of the control medaka other than occasional small areas of spongiosis hepatis in 3 of 9 (33%) controls (3/6 female and 0/3 male). Among the 18 fish exposed to DMN, 4 (22% - 3 males and 1 female) were sampled at the time their liver displayed a distinct phenotype where oval hyperplasia was a prominent feature (Fig. 1). The remaining fish (67% - 10 females and 2 males) had moderate hepatic fibrosis. Two of the 12 (17%) fish, both female had hepatic fibrosis with neoplasia; one hepatocellular carcinoma and one cholangiocellular carcinoma. Two fish (11%) died during the grow out period; one of them was evaluated by histology and had severe hepatic fibrosis/cirrhosis. The other one was autolyzed and not evaluated.

Oval cell hyperplasia phenotype was characterized by diffuse loss of hepatocellular glycogen, scattered individual hepatocellular apoptosis or necrosis, and marked proliferation of thin, elongated cells (oval cell) with oval hyperchromatic nuclei that extended and sometimes surrounded hepatocytes. Hepatocytes were variably sized, often hypertrophied, with a variable degree of hydropic degeneration (hyalinization) (Fig. 1). In the fibrotic livers,
deposition of collagenous extracellular matrix was mild to moderate, separated hepatocyte

tubules and individualized single or small groups of hepatocytes. In livers with oval cell

hyperplasia and to a lesser extent in the fibrotic livers, individualized or small groups of cells

with a phenotype intermediate between oval cell and hepatocytes (intermediate hepatobiliary
cells - IHBC) were observed (Fig. 1).

Immunostaining of serial liver sections revealed that the oval cells had intense, diffuse
cytoplasmic immunoreactivity for CK and were negative for RFP and MSA (Figs. 2).

Intermediate hepatobiliary cells had variable cytoplasmic immunoreactivity for CK and RFP

and were negative for MSA (Figs. 2 and 3). Double immunohistochemistry for CK and RFP

revealed occasional double cytoplasmic immunoreactivity in IHBCs (Fig. 2). No cells
demonstrated double immunoreactivity for CK and MSA or RFP and MSA.

Immunohistochemistry for MSA showed cytoplasmic immunoreactivity in activated stellate
cells present along sinusoids and in spindle cells surrounding mature bile ducts (Fig. 3).

Similar MSA immunoreactivity was present in spindle cells that formed a fin rim

surrounding clusters of IHBCs, some of which had a small central lumen or where organized

in distinct immature bile ducts (Fig. 3). These MSA-positive spindle cells were interpreted to

be myofibroblasts surrounding clusters of IHBCs that were differentiating into

cholangiocytes and organizing into bile ducts. Hepatocytes, cholangiocytes and

myofibroblasts surrounding bile ducts and blood vessels served as internal positive controls

and had diffuse intense cytoplasmic immunoreactivity for RFP, CK and MSA, respectively.

Identification of the proliferating cells was done using PCNA immunohistochemistry.

Immunoreactivity was observed in the hepatocytes, cholangiocytes, IHBC and in the oval
cells (Fig. 1). Immunoreactivity for PCNA of hepatocytes or cholangiocytes was rare in control livers. Positive PCNA immunoreactivity in the transient amplifying cells of the intestinal crypts served as positive controls. Results from immunohistochemical staining are summarized in table 4.

**Histopathology and immunohistochemistry: MCLR exposure**

Among the fish exposed to MCLR, 4 of 21 (19%), 5 of 27 (19%) and 3 of 12 (25%) exposed to 100, 250 and 500 ± 25 µg/kg respectively showed massive hepatic necrosis with evidence of hepatic regeneration. At the exception of 2 fish, all fish that were euthanized had no morphological liver changes. One fish exposed to 250 µg/kg and euthanized 8 days after exposure had a small focus of hepatocytes resembling IHBCs admixed with a moderate number of macrophages filled with cell debris. MCLR exposure results are presented in Table 3.

In the fish that presented morphological hepatic changes, the reaction to injury was similar in character and time independent of the MCLR dose administered. Diffuse massive coagulative hepatocellular necrosis with few scattered clusters and short cords of polygonal basophilic cells were observed throughout the parenchyma at 14 to 36h post exposure (Fig. 4). These cells were interpreted as proliferating oval cells. By 36 to 48h post exposure, cords and tubules of basophilic cells were more numerous, longer and started to merge with adjacent cords. In between the cords of regenerating cells, macrophages where numerous and filled with cell debris (Fig. 4). At 48 to 72h post exposure, the number and size of the basophilic cells had further increased and they resembled IHBC or small hepatocytes (Fig.
4). The number of macrophages had subjectively decreased. At 96h post exposure, the morphology and organization of the basophilic cells was progressing toward a hepatocyte phenotype and restoration of normal liver architecture (Fig. 4). The macrophage numbers had further decreased. Surprisingly, mitotic figures were rarely observed in the oval cells (less than 1 per 40X field). However, most cells between 24 to 96h post exposure displayed strong nuclear immunoreactivity for PCNA, signifying that very active cell proliferation was occurring. Increase in PCNA nuclear positivity was observed in the focus of IHBCs and small hepatocytes. present in the liver of the fish exposed to 250 µg/kg and euthanized at 8 days post exposure. No remarkable microscopic abnormalities were seen in the liver or other organs of the control animals.

At 24 and 48h post exposure, the basophilic proliferating oval cells displayed strong diffuse cytoplasmic immunoreactivity for CK. However CK immunoreactivity subsided and was markedly decreased or absent by 96h post exposure (Figs. 5). These oval cells were negative for RFP at 24 and 48h post exposure. However livers of the fish collected at 96h post exposure had rare IHBCs cells with faint cytoplasmic RFP immunoreactivity. In one liver mild to moderate immunoreactivity for RFP was present in IHBCs. Extracellular cell debris and the cytoplasm of macrophages were immunoreactive for RFP at 24 and 48h post exposure but immunoreactivity was weaker or occasionally absent at 96h post exposure. Results from immunohistochemical staining are summarized in table 4.
Transmission electron microscopic findings

The oval cells observed in the liver of the fish exposed to DMN showed ultrastructural characteristics similar to rodent oval cells or human HPC (Figs. 6). These fusiform to polygonal cells were located next to or in between differentiated or intermediate (immature) hepatocytes. They were located consistently away from sinusoids and, occasionally, contact with BPDEC was evident (Fig. 7). The fish oval cells had scant to moderate amounts of organelle-poor cytoplasm and no basal lamina. Few mitochondria and occasional scant endoplasmic reticulum and presumptive lysosomes were present. The adjacent intermediate (immature) hepatocytes showed features of hepatocyte differentiation. They had increased amounts of cytoplasm with more numerous mitochondria; increased amounts of glycogen; and showed development of rough endoplasmic reticulum. The livers of the MCLR-exposed fish were not evaluated by transmission electron microscopy.

DISCUSSION

Animal models of human diseases are essential to elucidate their molecular mechanisms and develop diagnostic and therapeutic approaches. Detailed knowledge of the model’s biology and its tissue response to injury is crucial to select an appropriate model and interpret study results accurately. We investigated the putative fish progenitor cell compartment and its response to acute or chronic injury in the medaka fish model using histology, single and double immunohistochemistry, and transmission electron microscopy (DMN exposure only). Use of cytokeratin AE1/AE3 antibody was selected because it reliably labels many epithelial cell types including cholangiocytes and BPDECs but not hepatocytes in fish, including
medaka (Bunton, 1993; Bunton, 1994; Okihiro and Hinton, 2000). Although in mammals cytokeratin 18 labels hepatocytes, an anti-human cytokeratin 18 antibody (Cytokeratin 18, clone Ks 18.04, Cat. #: 10R-C161A, Fitzgerald Industries International, Inc, Concord MA.) has been used to label the biliary tree in zebrafish. (Van Eyken and Desmet, 1993; Lorent et al., 2004). However, immunoreactivity for CK18 using the antibody listed above was not observed in medaka hepatocytes or cholangiocytes (AVW unpublished data). To overcome the lack of a fish hepatocyte marker, we used a transgenic medaka fish that expressed an RFP exclusively in mature hepatocytes. In rodents and human, antibodies to cytokeratin 7 and 19, and HepPar1 can be used to characterize cell lineages in tissue sections and are well accepted biliary epithelium/oval cell marker and hepatocyte marker respectively (Vessey and de la Hall, 2001; Zhou et al., 2007; Bird et al., 2008). Unfortunately, attempts at immunohistochemical staining for CK7 (Cytokeratin 7, clone OV-TL 12/30, Cat. #: M7018, Dako, Carpinteria, CA), and Hep Par 1 (Hepatocyte, Clone OCH1E5, Cat. #: M7158, Dako) in medaka were unproductive (AVW unpublished data). Immunolabeling with MSA is known to react with smooth muscle actin in medaka and was selected to show that the presumptive oval cells were not in fact activated stellate cells or myofibroblasts (Bunton, 1995; Hobbie et al., 2011).

Microcystins are known to be potent hepatotoxins (Malbrouck and Kestemont, 2006). In this study, exposure to MCLR was performed to induce severe acute hepatic necrosis followed by regeneration and evaluate the immunoreactivity of the resulting regenerative cell population as these new cells likely represent the transient amplifying population derived from the HPCs (Fournie and Courtney, 2002; Fausto and Campbell, 2003; Riehle et al.,
Unfortunately, the response to MCLR was highly variable between fish and its use did not prove to be a reliable method to induce a non-lethal event of hepatic necrosis followed by regeneration. Approximately 80% of the fish did not show any hepatic response to MCLR exposure while in the other 20% the hepatic necrosis induced was very severe and lethal. It is unclear why the fish that survive the acute hepatic necrosis phase and had histologic evidence of hepatic regeneration died 2 to 4 days after exposure. No histologic lesions were observed in the other organs. The range of microscopic regenerative changes observed was similar to the changes described in other fish species like the hardhead catfish (*Arius felis*), gulf killifish (*Fundulus grandis*) or rainbow trout (Kotak et al., 1996; Fournie and Courtney, 2002). In the subset of fish that developed hepatic necrosis and survived long enough to show evidence of a regenerative response, liver regeneration occurred by proliferation of CK positive oval cells that organized in tubules and resembled human ductular reaction type 3 (Desmet, 2011). Acquisition of hepatocyte morphology at a later time was concomitant with loss of CK immunoreactivity and acquisition of RFP immunoreactivity. Our findings are in accordance with studies in humans and rats where oval and hepatobiliary cells are positive for CK7/19 but CK7/19 expression is lost as hepatocytic differentiation progresses (Desmet, 2011). Surprisingly mitotic figures were rarely observed in this transit amplifying oval cell population. However, PCNA immunohistochemistry confirmed that these cells were actively proliferating.

Interestingly, extracellular cell debris and the cytoplasm of macrophages were immunoreactive for RFP in the peracute phase of hepatic necrosis but immunoreactivity was slowly lost overtime as the extracellular debris were phagocytized and processed by the
macrophages. This observation demonstrated that phagocytosis of the necrotic hepatocellular debris was occurring and that the phagocytized cellular debris and RFP protein were degraded by macrophages overtime.

Exposure to DMN was used to induce chronic hepatic injury and oval cell hyperplasia as reported by Hobbie et al. (Thoolen et al., 2010; Hobbie et al., 2011). Dimethylnitrosamine is a potent carcinogenic hepatotoxin that has been used to induce hepatic fibrosis and neoplasia in a number of fish species, including medaka, and rodents (Jenkins et al., 1985; Swenberg et al., 1991; Hasegawa et al., 1998; Hobbie et al., 2011). The subset of DMN exposed liver that exhibited prominent oval cell hyperplasia was selected and evaluated by immunohistochemistry and TEM. The CK immunoreactivity and ultrastructural features of the fish oval cell were consistent with that reported in mammals (Factor et al., 1994; Tan et al., 2002; Sobaniec-Lotowska et al., 2007; Desmet, 2011). The variable immunoreactivity and occasional double reactivity for CK and RFP of the cells with a histologic morphology transitional between HPC and hepatocytes supported that they were IHBCs (Tan et al., 2002; Desmet, 2011). The observation of intermediate hepatobiliary cells organizing in ductules was indicative of differentiation towards a biliary epithelial cell phenotype. Immunohistochemistry for MSA revealed to be useful in highlighting the IHBCs differentiating towards cholangiocytes. A thin rim of MSA positive spindle cells surrounded small clusters of intermediate hepatobiliary cells with sometimes a visible small central lumen as well as immature bile ductules. These spindle cells were usually not discernible on H&E slide alone and interpreted as being likely myofibroblasts. The lack of oval cell MSA immunoreactivity was supportive that the cells similar to rodent oval cells in fish liver were
not activated stellate cells or myofibroblasts. A mix of oval cells, IHBCs, hepatocytes and cholangiocytes were positive for PCNA, thus suggesting that replication of all cell types occurred during hepatic regeneration after DMN injury. It was expected that most of proliferating cells would be oval cells and IHBCs but a similar number of hepatocytes were positive for PCNA. Since PCNA has a long half-life and is also involved in DNA repair, it is possible that some of the immunoreactivity observed was not indicative of active cell division.

Although, the double immunoreactivity for CK and RFP of IHBCs must be interpreted with caution, the immunohistochemistry results support a bipotential lineage relationship of the oval cells towards hepatocytes and cholangiocytes (Fig. 8). Observations made here support differentiation of oval cells into intermediate hepatobiliary cells and then hepatocytes and cholangiocytes. However, the dedifferentiation of hepatocytes into proliferating progenitor-like cells cannot be excluded (Braun and Sandgren, 2000; Chen et al., 2012). Cell lineage tracing studies would be necessary to confirm this postulate.

In order to provide additional evidence that the oval cells observed on histology are liver progenitor cells, TEM was performed on the DMN exposed liver displaying an oval cell hyperplasia phenotype. The ultrastructural features of the oval cells and intermediate hepatobiliary cells were similar to those in rodents and human (Factor et al., 1994; Hardman et al., 2007; Sobaniec-Lotowska et al., 2007). The lack of distinguishing features (i.e. cell morphology, and type and amount of specific organelles) of the oval cells was supportive of a non-differentiated / stem cell-like state. The observation of larger cells with hepatocytes features, adjacent to oval cells, correlated well with intermediate hepatobiliary cells
differentiating towards hepatocytes. The close association and shared characteristics of oval cells and BPDEC supported the hypothesis that BPDECs are the bipotential HPC in the fish liver.

Collectively, this study demonstrates that fish oval cells share similar morphology and immunoreactivity with rodent oval cells and human HPC, and have similar bipotential lineage pathways. It also provides additional evidence of the striking morphological similarities in the hepatic regenerative process between fish and mammals.

ACKNOWLEDGEMENTS
We are indebted to Drs. Joerg Renn and Christoph Winkler, Department of Biological Sciences, National University of Singapore, for providing the founder transgenic medaka for our colony. We are grateful to Sandra Horton, Monica Matmeuller, Laura Shewmon, and the staff of the Histopathology Laboratory as well as Jeanette Shipley-Phillips from the Laboratory for Advanced Electron and Light Optical Methods at the NCSU-CVM for their expertise. We also thank Shashi Gadi for help with the medaka exposure and maintenance.
REFERENCES


### TABLES

Table 1. Function and Cellular Activity of Immunohistochemical marker in Toxicant Exposed Medaka Fish

<table>
<thead>
<tr>
<th>Name</th>
<th>Company</th>
<th>Type</th>
<th>Antibody dilution</th>
<th>Function</th>
<th>Reactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytokeratin AE1/AE3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>BioGenex Laboratories, San Ramon, CA.</td>
<td>Mouse monoclonal</td>
<td>Prediluted antibody</td>
<td>Recognizes high- and low-molecular-weight cytokeratins</td>
<td>Bile duct epithelium, BPDECs and intermediate cells</td>
</tr>
<tr>
<td>Muscle Specific actin (MSA)</td>
<td>BioGenex Laboratories, San Ramon, CA</td>
<td>Mouse monoclonal (HHF35), IgG1</td>
<td>1/100</td>
<td>Reacts with 42-kD protein specific for actin in skeletal, cardiac, and smooth muscle</td>
<td>Periductal smooth muscle cells, activated stellate cells</td>
</tr>
<tr>
<td>Proliferating Cell Nuclear Antigen (PCNA)</td>
<td>BioGenex Laboratories, San Ramon, CA</td>
<td>Mouse monoclonal (PC10), IgG2a</td>
<td>1/250</td>
<td>React with an 36 kD non-histone nuclear protein auxiliary of DNA polymerases δ and ε enzymes necessary for DNA synthesis</td>
<td>Cell in the G1, S and G2/M-phases of cell cycle</td>
</tr>
<tr>
<td>Red fluorescent protein</td>
<td>Abcam, Cambridge, MA.</td>
<td>Rabbit polyclonal</td>
<td>1/500</td>
<td>Reacts with RFP protein - full length amino acid sequence (234aa) from the mushroom polyp coral Discosoma</td>
<td>Transgenic hepatocytes expressing the RFP</td>
</tr>
</tbody>
</table>

<sup>a</sup>AE1 recognizes 10,14, 15, 16, and 19; AE3 recognizes 1, 2, 3, 4, 5, 6, and 8.
Table 2. Degenerative and Proliferative Hepatic Lesions in Medaka exposed to 100 ppm Dimethylnitrosamine for 2 weeks.

<table>
<thead>
<tr>
<th>Week post exposure</th>
<th>Cellular degeneration</th>
<th>Spongiosis hepatis</th>
<th>Necrosis / apoptosis</th>
<th>Architectural change</th>
<th>BPDEC&lt;sup&gt;a&lt;/sup&gt; proliferation</th>
<th>Bile duct hyperplasia</th>
<th>Fibrosis</th>
<th>Cellular dysplasia</th>
<th>Neoplasia</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 weeks</td>
<td>5/5</td>
<td>4/5</td>
<td>5/5</td>
<td>5/5</td>
<td>5/5</td>
<td>4/5</td>
<td>4/5</td>
<td>5/5</td>
<td>0/5</td>
</tr>
<tr>
<td>6 weeks</td>
<td>6/6</td>
<td>5/6</td>
<td>6/6</td>
<td>6/6</td>
<td>5/6</td>
<td>5/6</td>
<td>4/6</td>
<td>6/6</td>
<td>1/6</td>
</tr>
</tbody>
</table>

<sup>a</sup> Bile preductular epithelial cell
Table 3. Microcystin LR exposure: histopathology results.

<table>
<thead>
<tr>
<th>Time post exposure</th>
<th>100 µg/kg (n = 21)</th>
<th>250 µg/kg (n = 27)</th>
<th>500 µg/kg (n = 12)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Died</td>
<td>Euthanized</td>
<td>Died</td>
</tr>
<tr>
<td>14h</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24h</td>
<td>NL: 5</td>
<td>HNR: 1</td>
<td>HN: 2</td>
</tr>
<tr>
<td>36h</td>
<td></td>
<td>HNR: 1</td>
<td></td>
</tr>
<tr>
<td>48h</td>
<td>NL: 2</td>
<td>HNR: 1</td>
<td>HN: 1</td>
</tr>
<tr>
<td>72h</td>
<td></td>
<td>HR: 1</td>
<td>U: 1</td>
</tr>
<tr>
<td>96h</td>
<td>NL: 2</td>
<td>HR: 2</td>
<td></td>
</tr>
<tr>
<td>144h</td>
<td></td>
<td>NL: 6</td>
<td></td>
</tr>
<tr>
<td>192h</td>
<td>NL: 8</td>
<td></td>
<td>NL: 4</td>
</tr>
</tbody>
</table>

U: Unknown due to marked autolysis; NL: no lesion; HN: hepatic necrosis; HNR: hepatic necrosis with regeneration (diffuse hepatic necrosis with multifocal tubules and/or aggregates of polygonal basophilic cells); HR: hepatic regeneration (liver composed of small hepatocytes/IHBCs and numerous individual and aggregate of macrophages). FHR: Focus of hepatic regeneration (normal liver with a focal area of small hepatocytes/IHBCs and moderate number of macrophages filled with cell debris).
Table 4. Summary of Immunohistochemical Expression Analysis

<table>
<thead>
<tr>
<th>Name</th>
<th>Hepatocyte</th>
<th>Cholangio-</th>
<th>Oval cell</th>
<th>Intermediate hepatobiliary cell</th>
<th>Myofibroblast and activated stellate cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytokeratin AE1/AE3</td>
<td>-</td>
<td>+++</td>
<td>+++</td>
<td>(+++)</td>
<td>-</td>
</tr>
<tr>
<td>RFP(^a)</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>(+)</td>
<td>-</td>
</tr>
<tr>
<td>MSA(^b)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+++</td>
</tr>
<tr>
<td>PCNA(^c) (DMN(^d) exposure)</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>PCNA(^c) (MCLR(^e) exposure)</td>
<td>+</td>
<td>+</td>
<td>+++</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

\(^a\) Red fluorescent protein  
\(^b\) Muscle specific actin  
\(^c\) Proliferating cell nuclear antigen  
\(^d\) Dimethylnitrosamine  
\(^e\) Microcystin LR
Figure 1. Histology of control liver, and liver with oval cell hyperplasia, and intermediate hepatobiliary cells. A: Histology of a normal female medaka liver. 100X Bar = 20um. B: Oval cell hyperplasia characterized by proliferation of small spindle cell with a hyperchromatic elongated nuclei that separated the hepatocytes tubules and sometime surrounded individual hepatocytes. Hematoxylin and eosin, 100X Bar = 20um. C: Intermediate hepatobiliary cells characterized by a phenotype in between hepatocytes and oval cell. Hematoxylin and eosin, 100X Bar = 20um. D: Liver, oval cell hyperplasia after DMN exposure. Oval cells, intermediate hepatobiliary cells, hepatocytes and cholangiocytes demonstrate nuclear immunoreactivity for PCNA of variable intensity. Anti-PCNA antibody, hematoxylin counterstain. 40X Bar = 50um.
Figure 2. Single and double immunolabelling for CK AE1/AE3 and red fluorescent protein of liver with oval cell hyperplasia and intermediate hepatobiliary cells. A: Liver, oval cell hyperplasia following DMN exposure. Oval cells are positive for CK (brown). CK AE1/AE3 immunohistochemistry, hematoxylin counterstain. 100X Bar = 20um. B-D: Liver, intermediate hepatobiliary cells (IHBC) admixed with hepatocytes after DMN exposure. B: Hepatocytes are positive for RFP (red). Absent or weak to occasionally moderate immunoreactivity is present in the IHBCs. RFP Immunohistochemistry, hematoxylin counterstain. 100X Bar = 20um. C and D: IHBCs positive for CK (brown) and hepatocytes positive for RFP (red) are admixed with IHBCs positive for RFP and CK (arrow). CK positive IHBCs are forming immature bile ducts (arrow). Double immunohistochemistry for CK and RFP, hematoxylin counterstain. 100X Bar = 20um.
Figure 3. Single immunolabelling for MSA and double immunolabelling for MSA and red fluorescent protein of liver with oval cell hyperplasia and intermediate hepatobiliary cells. A: Liver, oval cell hyperplasia after DMN exposure. Activated stellate cells (Arrow) and myofibroblasts (arrow head) are positive for MSA. MSA Immunohistochemistry, hematoxylin counterstain. 40X Bar = 50um. B: Liver, intermediate hepatobiliary cells (IHBC) admixed with hepatocytes after DMN exposure. Two immature bile duct formed by RFP negative IHBCs are surrounded by a thin rim of MSA positive cell (brown – arrow). Other RFP negative IHBCs are surrounded by a rim of MSA positive cells but no duct lumen has formed yet. Double immunohistochemistry for MSA and RFP, hematoxylin counterstain. 100X Bar = 20um.
Figure 4. Histology of the regenerative liver response following microcystin LR exposure.  
A. Liver, severe diffuse peracute hepatic necrosis 14h after MCLR exposure. The hepatic parenchyma is composed of dissociated rounded hepatocytes with pyknotic nuclei (arrow). Hematoxylin and eosin, 60X Bar = 30um. B. Liver, early hepatic regeneration 24h after MCLR exposure. Basophilic oval cells (arrow) organized in clusters and tubules resembling human ductular reaction are scattered within the necrotic hepatocellular parenchyma. Hematoxylin and eosin, 60X Bar = 30um. C: Liver, early hepatic regeneration 36h after MCLR exposure. Tubules formed by basophilic oval cells often have a visible slit like lumen (arrow) and are interconnected. In between the oval cells, numerous macrophages have infiltrated the parenchyma (asterisk). They are filled with bright red cell debris due to ingestion of the hepatocytic RFP. Hematoxylin and eosin, 60X Bar = 30um. D: Liver, hepatic regeneration 48h post exposure. The hepatic parenchyma is mostly composed of intermediate hepatobiliary cells and macrophages (asterisk) infiltrate is reduced. Hematoxylin and eosin, 60X Bar = 30um. All figures: red blood cells (arrow head).
Figure 5. Hepatic regeneration following microcystin LR exposure, double immunolabelling for CK AE1/AE3 and red fluorescent protein. A: Liver, early hepatic regeneration 24h after MCLR exposure. Basophilic oval cells (arrow) organized in clusters and tubules resembling human ductular reaction are scattered within the necrotic hepatocellular parenchyma. Hematoxylin and eosin, 60X Bar = 30um. B: Liver, early hepatic regeneration 24h after MCLR exposure. Oval cells (arrow) are strongly positive for CK (brown). Surrounding cell debris are variably immunoreactive for RFP (red). Double immunohistochemistry for CK and RFP, hematoxylin counterstain. 60X Bar = 30um. C: Liver, hepatic regeneration 96h post exposure. The hepatic parenchyma is entirely composed of intermediate hepatobiliary cells that begin to acquire hepatocyte features. Hematoxylin and eosin, 60X Bar = 30um. D: Liver, hepatic regeneration 96h post exposure to MCLR. The hepatic parenchyma is composed of intermediate hepatobiliary cells (IHBC) that are occasionally weakly positive for RFP (arrow). Almost all of IHBCs have lost their CK immunoreactivity. Cholangiocytes and bile preductular epithelial cells are positive for CK (asterisk). Macrophages display variable cytoplasmic staining for RFP (arrow head). Double immunohistochemistry for CK and RFP, hematoxylin counterstain. 100X Bar = 20um.
Figure 6. Transmission electron microscopy of an oval cell in the liver of a DMN exposed medaka. An oval cell (HPC) is adjacent to 3 hepatocytes (H). Note the organelle poor cytoplasm and lack of distinguishing morphological features. See Fig. 1b for the histology correlate. Bar = 2 μm X 5600.
Figure 7. Transmission electron microscopy of a bile preductular epithelial cell and intermediate hepatobiliary cells in the liver of a DMN exposed medaka. A row of oval cells (HPC) is located in between hepatocytes (H) and in contact with a bile preductular epithelial cell (BPDEC) and an intermediate hepatobiliary cells (IH) showing features of hepatocytes differentiation: an increased in cell volume, nuclear size, mitochondria number, glycogen and development of rough endoplasmic reticulum. A biliary canaliculi formed by a BPDEC and hepatocytes is visible (arrow) Junctional complex between BPDECs (arrow head). Bar = 2 µm X 4400.
Figure 8. Proposed piscine oval cell differentiation processes toward hepatocytes and cholangiocytes through stages of intermediate hepatobiliary cells.
CHAPTER 4

Identification of the Hepatic Progenitor Cell Niche in the Japanese Medaka (*Oryzias latipes*)

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ABSTRACT

Small laboratory fish are considered robust models to study the mechanism of organ regeneration. Surprisingly, the hepatic regenerative process in fish has received considerably less attention than other organs, like the heart and fins. Despite their importance in tissue homeostasis, repair and carcinogenesis, very little is known about the hepatic progenitor cells’ (HPC) specific spatial and temporal characteristics in small aquarium fish models. Here we performed a label retention cell assay (LRCA) to identify the in situ location of the HPCs in the Japanese medaka (Oryzias latipes) liver. We administered a pulse exposure of the nucleotide bromodeoxyuridine (BrdU) to medaka embryos during hepatogenesis and examined the liver for slow-cycling cells after a 10 to 55 day chase period. We also attempted to perform a LRCA in adult medaka using a sublethal dose of microcystin LR (MCLR) to induce hepatic necrosis, regeneration and consequent BrdU uptake in HPCs. Phenotypic characteristics, location and double immunolabelling were used to confirm the identity of the BrdU retaining cells. In larval livers label retention was observed in three possible HPC niches: hepatocytes, bile preductular epithelial cells (BPDEC), and cholangiocytes. In adult medaka, MCLR exposure at the dose administered was either lethal or did not induce any hepatic lesion. Taking into consideration the anatomical differences between medaka and mammalian liver, results of our LRCA analysis further demonstrate similarities between piscine and mammalian liver and the utility of these animals for hepatic injury studies. Overall these data support the hypothesis that the process of hepatic regeneration is a complex system relying on multiple HPC niches.
INTRODUCTION

Acute and chronic liver diseases are important causes of morbidity worldwide (Lim and Kim, 2008). Even though the human liver has a remarkable regenerative capacity, the intricate mechanisms in liver regeneration remain poorly understood (Palms and Spiegel, 2004). Cells that play a major role in tissue regeneration are tissue stem cells or progenitor cells. They reside in specific niches and likely play a crucial role in tissue homeostasis, wound repair and tumorigenesis (Watt and Hogan, 2000; Fuchs et al., 2004).

To study regenerative processes, small laboratory fish are considered robust models due to their natural ability to regenerate organs and/or appendages including the heart, spinal cord, fin, optic nerve and liver (Poss et al., 2003; Sadler et al., 2007). Their strength rests also on the fact that they have been used extensively in developmental biology studies and the mechanisms involved during development and regeneration are often similar (Hata et al., 2007; Chu and Sadler, 2009). The process of hepatic regeneration in fish has received considerably less attention than other organs, like the fins and heart, but a few studies have begun to investigate the piscine hepatic regenerative process. These studies have demonstrated that the 2 main pathways of mammalian hepatic regenerative response are recapitulated in fish hepatocyte and HPC-mediated regeneration (Okihiro and Hinton, 2000; Sadler et al., 2007; Kan et al., 2009; Hobbie et al., 2011). After partial hepatectomy, compensatory liver growth results from proliferation of mature hepatocytes, or a combination of hepatocytes and cells resembling rodent oval cells or human HPC (Okihiro and Hinton, 2000; Sadler et al., 2007; Kan et al., 2009). These oval like cells are called bile preductular epithelial cells (BPDEC) in fish (Hardman et al., 2007). In studies evaluating the hepatic
response to toxic injury and hepatocarcinogenesis, proliferation of oval-like cells/BPDECs is well documented (Couch and Courtney, 1987; Hinton et al., 1988; Nunez et al., 1990; Okihiro and Hinton, 2000; Fournie and Courtney, 2002; Hobbie et al., 2011). A recent study has demonstrated that these oval-like cells/BPDECs consist of a transit amplifying cell population that can differentiate into hepatocyte or biliary cell lineages (Van Wettere et al 2012, see chapter 3). Overall, results from these studies support the existence of a population of hepatic progenitor cell (HPC) in the fish liver. Given their morphological similarities and spatial location with the mammalian HPC/oval cells, it has been suggested that the BPDEC are likely to be the piscine HPCs (Hardman et al., 2007; Hinton et al., 2008). However, to date, the spatial and temporal characteristics of the piscine HPC niche have not been elucidated.

A major obstacle to identify piscine HPCs is the lack of specific tissue stem cell markers. However, location of progenitor cells populations may be identified using a label-retaining approach (Cotsarelis et al., 1990; Oliver et al., 2004; Cervello et al., 2007; Kuwahara et al., 2008; Staszkiewicz et al., 2009). The label-retention cell assay (LRCA) is based on the asymmetrical division property of stem cells that gives rise to a new slow cycling replacement stem cells and the progenitor of a transient amplifying cell population. Following administration of a nuclear label like Bromodeoxyuridine (BrdU) incorporated during cell replication, both daughter cells will retain the label after cell division. The rapidly dividing transit amplifying cells will dilute their label with each cell division during the chase period, but the replacement progenitor cells will retain the label much longer due to infrequent cell cycling. Label retaining cells (LRCs) have been detected in several organs in
mammals but this method has not been previously used in fish (Cotsarelis et al., 1990; Kenney et al., 2001; Tsujimura et al., 2002; Oliver et al., 2004; Cervello et al., 2007; Kuwahara et al., 2008).

The overarching goal of this study was to support the postulate that the BPDECs are the piscine HPCs. We hypothesized that the medaka liver contains a small populations of hepatic progenitor cells that can be detected by the LRC technique and share an anatomical location with previously identified BPDECs. The specific aims were as follows: 1) to locate the LRCs in the medaka liver; 2) to identify LRCs phenotypes using double immunolabeling; 3) compare LRCA techniques using developmental and post-injury labeling of the HPCs.

**MATERIALS and METHODS**

**Medaka culture**

A transgenic orange-red medaka fish line Tg(zfL-fabp:DsRed) which expresses a red fluorescent protein (RFP) under the regulatory control of the 2.8-kb fragment of the zebrafish liver fatty acid binding protein (L-FABP) promoter was used to specifically label hepatocytes (Korzh et al., 2008). The founder Tg(zfL-fabp:DsRed) medaka were obtained from Drs. Joerg Renn and Christoph Winkler, Department of Biological Sciences, National University of Singapore. This stable transgenic line showed standard Mendelian inheritance from F2 generation onwards and offspring were screened by observation of embryos or larvae for RFP protein expression in the liver. A Tg(zfL-fabp:DsRed) medaka colony was housed under recirculating freshwater aquaculture conditions with charcoal filtration and UV treatment used in each passage at the Department of Molecular and Environmental Toxicology, North
Carolina State University (NCSU), Raleigh, NC. Water temperature and pH were monitored daily and maintained at 25 ± 2 °C and ~7.2, respectively. The light-dark cycle was 16 h light, 8 h dark. Dry food (Otohime B1, Reed Mariculture, Campbell, CA) was fed several times per day through automated feeders, and newly hatched *Artemia nauplii* were fed once daily. Animal care and use were in compliance with protocols approved by the NCSU Institutional Animal Care and Use Committee in accordance with the National Academy of Sciences Guide for the Care and Use of Laboratory Animals.

*Chemicals*

5-Bromo-2'-deoxyuridine (BrdU; C₉H₁₁BrN₂O₅; purity 99.9%; CAS 59-14-3; MW 307.13 g/mol) was obtained from Sigma-Aldrich (St. Louis, MO) and diluted to a 10mg/mL solution in distilled water. Aliquots of 22.5mL were stored in polypropylene centrifuge tubes in the dark at -20°C until use. Microcystin LR (C₄₉H₇₄N₁₀O₁₂; purity ≥ 95.5%; CAS 101043-37-2; MW 995.2 g/mol) was obtained from EMD Chemicals, Inc (Gibbstown, NJ) and stored in an airtight brown bottle within a metal container at -20°C. For intraperitoneal injection, the MCLR stock solution was dissolved in sterile 0.9% sodium chloride solution (Aqualite, Abbott Animal Health, Abbott Park, IL) at a concentration of 0.025 µg/µL. All chemicals and reagents used in this study were of the highest purity available from commercial resources.
Label-retention cell assay in developing embryo.

In the culture conditions described above, medaka spawned daily and embryonated eggs (10–30 per mass) were collected between 1 to 3 h after the lights turned on (Stage 1 to 7) (Iwamatsu, 2004). The eggs were separated from each other by gentle rolling on a moistened paper towel surface to disrupt the attachment filaments joining individual eggs. Groups of individual embryonated eggs of specified collection date and developmental stage were suspended in a 2% saline solution made from Instant Ocean (Instant Ocean® salts, Aquarium System Inc. Mentor, OH) in a 100 × 15 mm petri dish and incubated at 26 ± 1°C with 16 h light, 8 h dark light-dark cycle (Iwamatsu, 2004). After 5 days, the 2% saline solution was replaced with 1X embryo rearing medium (ERM) containing 17.1mM NaCl and, in micromolars, 272 CaCl₂·2H₂O, 402 KCl, and 661 MgSO₄·7H₂O through hatching (usually at 9 days post fertilization [dpf]). After hatching the larvae were kept up to 15 dpf in petri dish containing 0.3X ERM. After 15 dpf, the larvae were transferred to 130 x 70 x 120 mm plastic containers filled with water from the recirculating freshwater aquaculture system for the remaining of the grow out period up to 60 dpf. The experiment was not extended beyond 60 days based on results from a preliminary experiment that demonstrated that no BrdU label retaining cells could be detected after a 60 days wash out period. The 0.3X ERM or culture system water filling the petri dish or container holding the larvae was renewed every 2 days. Eighty embryos were exposed to 75mg/L of BrdU for 48 hours during hepatogenesis. Exposures were performed from 1) 3 to 5 dpf, 2) 5 to 7 dpf or 3) 7 to 8 dpf. The embryos were exposed in ambient 2X saline solution or 1X ERM depending upon their age at the time of exposure and then returned to culture conditions as described above. Eighty embryos were
cultured in the same conditions without exposure to BrdU and used as control. After hatching, the larvae were fed once daily with Dry food (Otohime B1). Embryo and larvae were examined once a week under a dissecting microscope to assess normal development. A subset of 10 BrdU and control exposed larvae were euthanatized with an overdose of tricaine methanesulfonate (300 mg/liter; MS-222, Argent Laboratories, Redmond, WA) at 15, 20, 30, 40, 50 and 60 dpf and fixed in 4% paraformaldehyde for 24 hours, and transferred to 70% ethanol for histopathology.

**Label-retention cell assay in adult medaka.**

Three-month-old male and female Tg(zfL-fabp:DsRed) medaka from the stock colony were moved to NCSU College of Veterinary Medicine (Raleigh, NC) and acclimated for 2 weeks in 10 gallon aquarium tanks filled with reconstituted reverse osmosis–purified (RO) water (0.5 g/liter Instant Ocean® salts, Aquarium System Inc. Mentor, OH) within a static freshwater culture system. The artificial light photoperiod was 16 hours light/8 hours dark. The water temperature was maintained at 26 ± 0.5°C, the water pH at 6.5 and the water conductivity between 600 to 800 µS. Dry food (Otohime B1) was fed throughout the day using automated feeders. For the MCLR exposures, medaka were randomly distributed among 4-liter glass beakers, 10–12 fish per beaker, containing 3 liters of reconstituted RO water as described above. The beakers were placed within a recirculating, heated water bath to maintain temperature at 26°C ± 0.5°C throughout the exposures. Fish were fed once daily and observed twice daily for behavioral responses and signs of overt toxicity.
Tg(zfL-fabp:DsRed) medaka were exposed to BrdU (75mg/L) in tank water for 60 hours. Twenty four hours after the start of the BrdU exposure, the fish received a single intracoelemic injection of microcystin-LR (MCLR) and were placed back in the beakers containing the BrdU water for 36 additional hours. Subsequent to exposure, the fish were moved to clean 4L beakers filled reconstituted RO water. Ninety five percent water changes were conducted every 48h to maintain water quality during the length of the study. The fish were briefly anesthetized using tricaine methanesulfonate (120 mg/liter; MS-222), weighed, placed in right lateral recumbency, and injected intraperitoneally using a glass 25 µL Hamilton syringe (PB-600 Repeating Dispenser, Hamilton, Reno, NV), equipped with a 32 gauge needle (Hamilton, Reno, NV). Medaka were dosed at 100 (n=21), 250 (n=27) and 500 ± 25 ug/kg (n=12). Twenty control fish were anesthetized and injected with 4µL of sterile 0.9% sodium chloride solution (Abbott Animal Health, Abbott Park, IL). A subset of at least 2 exposed and 1 control fish were euthanized with an overdose of tricaine methanesulfonate (300 mg/liter; MS-222) at 1, 2, 4, 6 and 8 days post-MCLR injection. The coelom of the fish was incised along the ventral midline to enhance fixative penetration and the fish were fixed whole in 4% paraformaldehyde solution for 24 hours, demineralized in 10% formic acid for 24 hours, and transferred to 70% ethanol for histopathology.

**Histology and Immunohistochemistry**

Paraformaldehyde–fixed embryos and adult fish were processed, and embedded in paraffin according to routine histologic techniques. Sections, 5 µm thick, were stained with hematoxylin and eosin (H&E) and examined by light microscopy. Additional sections were
used for BrdU, RFP, cytokeratin AE1/AE3 (CK AE1/AE3), and cytokeratin 18 (CK18) single and double immunohistochemistry. Immunohistochemistry for BrdU was performed in at least 5 liver sections per larvae/fish per time points to assess for the presence of BrdU retaining cells. The BrdU primary antibody used was a mouse monoclonal antibody, clone Bu20a, that recognizes BrdU in single-stranded DNA, free BrdU or BrdU coupled to a protein carrier (DakoCytomation, Denmark, Catalogue # M 0744). The CK AE1/AE3 antibody used was a cocktail of 2 mouse monoclonal antibodies that recognise several acidic (10, 14 to 16, 19), and all basic (1-8) human cytokeratins (BioGenex Laboratories, San Ramon, CA. Catalogue # AM0751-5M). The CK18 antibody, clone Ks 18.04, was a mouse monoclonal antibody that recognize human CK18 (Fitzgerald Industries International, Inc., Concord, MA). The RFP antibody was a rabbit polyclonal antibody that recognizes full length amino acid sequence of the mushroom polyp coral Discosoma RFP (Abcam Inc, Cambridge, MA. Catalogue # ab34771). Tissue sections were deparaffinized in xylene, rehydrated in a graded series of ethanol and rinsed in distilled water. Antigen retrieval was performed by heating the slides to 99°C in a 10 mM sodium citrate solution at pH 6 for 10 minutes in a vegetable steamer (Oster 5712 food steamer, Maitland, FL). Following a cool down period of 10 minutes in the warm sodium citrate buffer, the slides were rinsed in 1X Tris-buffered saline with 0.05% Tween (TBST) and treated with 3% hydrogen peroxide for 10 minutes to block endogenous peroxidases activity. Goat serum was applied for 20 minutes to prevent nonspecific binding of the secondary antibody (BioGenex San Ramon, CA). Tissues were incubated with the BrdU antibody (1/200 dilution), RFP antibody (1/500 dilution), CK AE1/AE3 antibody (sold ready to use) or CK18 antibody (1/20 to 1/200
dilution), for 30 minutes at room temperature. The slides were rinsed in 1X TBST, and then incubated with the secondary antibody for 20 minutes (BioGenex San Ramon, CA). After a wash in 1X TBST, tissue sections were then treated for 20 minutes with streptavidin peroxidase (BioGenex San Ramon, CA). Following a final wash in 1X TBST, development was achieved by treatment of tissue with liquid 3,3-diaminobenzidine (DAB) chromogen for 15 seconds to 1 minute (Vector Lab, Burlingame, CA). After a wash in tap water, the slides were counterstained with Mayer’s hematoxylin for 20–40 seconds and cover slipped. For double immunohistochemistry, a second immunohistochemistry procedure was repeated starting with the application of the primary antibody, followed by the secondary antibody, streptavidin alkaline phosphatase (BioGenex San Ramon, CA) and VECTOR Red Alkaline Phosphatase Substrate Kit (Vector Lab, Burlingame, CA). For negative controls, the primary antibody was omitted and non-immune serum of the same species as the primary antibody was applied. For positive controls, sections of intestine and liver were included in each slide.

Medaka liver lesions were identified based on criteria set by a consensus of the US National Toxicology Program pathology working group and the International Harmonization of Nomenclature and Diagnostic Criteria for Lesions in Rats and Mice (INHAND) project (Boorman et al., 1997; Thoolen et al., 2010). In this study, an intermediate hepatobiliary cell (IHBC) was defined as a polygonal cell with a large round to oval nucleus sharing morphological characteristics with mature hepatocytes but lacking RFP immunoreactivity. The term hepatocyte was used when the cells presented the classic morphological characteristic and location of hepatocytes and were immunoreactive for RFP.
RESULTS

Label-retention cell assay in developing embryos.

All embryos developed normally, with no anatomical abnormalities detected by examination of live embryos or larvae with a dissecting microscope, or through examination of histologic sections by light microscopy. Evaluation of larval liver sections stained for BrdU showed that dilution of the BrdU label occurred over time independently of the BrdU exposure period (Table 1 and Fig. 1). Ten to fifteen days following BrdU administration (20 dpf), an estimated 10 to 30% of the cell nuclei, mostly hepatocytes, were diffusely immunoreactive for BrdU. A marked decrease in the number of nuclei positive for BrdU occurred between 20 and 30 dpf with less than 5% of nuclei showing punctate staining by 30 dpf. This trend continued over time and, by 60 dpf, all traces of the BrdU label had disappeared with exception of 2 fish in which rare nuclei still contained nominal BrdU immunoreactivity. Cells retaining BrdU at 50 and 60 dpf were considered LRCs. Single and double immunohistochemistry for BrdU and RFP demonstrated that BrdU retention occurred predominantly in the nuclei of hepatocytes, while less often in endothelial cells and biliary epithelial cells, and only occasionally in BPDECs (fig. 1 and 2). Hepatocytes had diffuse cytoplasmic immunoreactive for RFP in control and BrdU exposed larvae. Immunoreactivity for CK AE1/AE3 and CK18 was not observed in any tissue of 15 to 60 dpf control and exposed larvae/fish. Immunoreactivity for BrdU was not observed in control larvae.
**Label-retention cell assay in adult medaka.**

Following exposure to MCLR, forty eight fish (80%) did not display hepatic lesions at the time of collection. In the twelve fish (20%) that presented morphological hepatic changes, the reaction to injury was similar in morphology and independent of the MCLR dose administered. Massive coagulative hepatic necrosis with hepatic regeneration was present in 4 of 21 (19%), 5 of 27 (19%) and 3 of 12 (25%) of the medaka exposed to 100, 250 and 500 ± 25 µg/kg respectively. All of these animals died within 4 days following exposure and before complete hepatic regeneration could occur. One fish exposed to 250 µg/kg and euthanized 8 days after exposure had a small focus of hepatocytes resembling intermediate hepatobiliary cells (IHBC) admixed with a moderate number of macrophages filled with cellular debris. This focus of hepatic regeneration suggested that focal hepatic necrosis had occurred following MCLR exposure. No control fish died and had detectable histologic hepatic lesions. Histology results of the MCLR exposure are summarized in chapter 3, table 3.

In the fish that developed hepatic lesions, hepatocellular necrosis was severe and involved the entire liver mass. At 14 to 36h post-exposure, a few scattered clusters and short cords of polygonal basophilic cells interpreted as proliferating oval cells were observed throughout the parenchyma (Fig. 3 and chapter 3 fig. 4). By 36 to 48h post exposure, cords and tubules of basophilic cells were more numerous, longer and appeared to merge with adjacent cords. Macrophages filled with cellular debris were present between the cords of regenerating cells (Fig. 3 and chapter 3, fig. 4). At 48 to 72h post exposure, the number and size of the basophilic cells were increased and resembled IHBC or small hepatocytes (Fig. 3
and chapter 3, fig. 4). The number of macrophages appeared to be decreased. At 96h post exposure, the morphology and organization of the basophilic cells exhibited more of a hepatocyte phenotype and the number of macrophage were decreased (Fig. 3 and chapter 3, fig. 4). Surprisingly, mitotic figures were rarely observed (less than 1 per 40X field). Immunohistochemistry for BrdU demonstrated that cells between 24 to 96h post exposure had strong, diffuse nuclear immunoreactivity supporting that cell proliferation was evident (Fig.3). Immunoreactivity for BrdU in the normal liver of fish euthanized at 6 and 8 days post exposure was absent or limited to a small number of scattered cells similarly to control liver. Diffuse nuclear BrdU immunoreactivity was observed mainly in hepatocytes, and fewer endothelial cells and BPDECs. In the liver with a regenerative focus, punctate nuclear immunoreactivity was present in more than 60% of the IHBCs and hepatocytes of the regenerative focus only (Fig. 4). BrdU immunoreactivity was present in a small number of scattered cells in the surrounding normal parenchyma as in normal livers (Fig 4). No remarkable microscopic abnormalities were seen in the liver or other organs of the control animals. Additional information regarding other immunohistochemical features of the regenerating cell population observed after MCLR exposure are presented in chapter 3.

DISCUSSION

Hepatic progenitor cell turnover in the adult mammalian liver is relatively low. To perform an LRCA, label exposure must occur when progenitor cells are dividing to ensure label uptake in a sizable number of progenitor cells. In this study, we used 2 experimental approaches to induce label uptake in HPCs (Oliver et al., 2004; Chan and Gargett, 2006;
Kuwahara et al., 2008; Staszkiewicz et al., 2009). A BrdU LRCA was performed in developing embryos and following acute hepatic injury in adult fish.

Embryonic exposure to BrdU was performed within early stages of liver embryogenesis and the fast cellular division occurring during physiological growth diluted the BrdU label. In medaka, liver anlage is initiated at 2 dpf (18-19 somites stage - stage 25) and the liver reaches its adult phenotype by 16 dpf (stage 41) (Iwamatsu, 2004; Hinton et al., 2008). In this study, a 48 hour exposure to BrdU was performed during early and middle stages of hepatogenesis with the aim of labeling dividing hepatoblasts. Subsequent to these exposures, complete dilution of the BrdU label occurred between 50 and 60 dpf independently of the BrdU exposure times between 3 to 5 dpf or later in the development between 8 to 10 dpf. Label retention was observed in hepatocytes, BPDECs, cholangiocytes and endothelial cells, but appeared more frequent in hepatocytes and BPDECs.

The results of this study demonstrate that despite anatomical differences between fish and rodent liver, LRCA yielded similar results (Kuwahara et al., 2008). Conceptually, the entire medaka liver is the anatomical equivalent of one mammalian hepatic lobule with biliary-venous tracts (portal tract equivalent) located at the hilus and an hepatic parenchyma composed of 2 to 3 cell thick muralium rather than the mammalian one cell thick muralium (Hardman et al., 2007). The medaka liver parenchyma has numerous biliary preductules formed by hepatocytes and BPDECs which are the equivalent to the canal of Hering in mammals but are distributed throughout the parenchyma. In this study LRCs were observed in cholangiocytes and in the fish oval cell equivalent, the BPDECs, similarly to results in mice (Kuwahara et al., 2008). Label retaining hepatocytes were observed randomly
throughout the hepatic parenchyma in medaka rather than only in the periportal areas next to canals of Hering as observed in mice (Kuwahara et al., 2008). This difference is likely due to the anatomical differences between medaka and rodent livers. No LRCs were observed in other locations. In mice peribiliary “null” cells in portal tracts showed label retention but no LRCs were detected in the fibrous connective tissue surrounding biliary venous tracts in medaka (Kuwahara et al., 2008). A more detailed examination of the hilus region will be required to draw further definitive conclusion because of the small sampling size available. A BrdU LRCA in adult fish was attempted. A toxin, MCLR, known to cause acute hepatic necrosis in several fish species was selected (Fournie and Courtney, 2002; Malbrouck et al., 2003; Huynh-Delerme et al., 2005; Malbrouck and Kestemont, 2006). Classic hepatotoxicants known to induce acute hepatic necrosis in mammals, such as acetaminophen and carbon tetrachloride, were not used because they do not have the same effect in fish, possibly due to the lack of phase 1 bioactivation of the parent compound, or a more effective phase 2 metabolism (Hinton et al., 2008). At the dose administered in this study, intraperitoneal injection of MCLR in adult medaka did not reliably induce hepatic necrosis followed by regeneration and, therefore, the LRCA could not be effectively performed. Exposure to MCLR did induce severe hepatic necrosis in 20% of the fish exposed, but all fish died within 4 days post exposure despite histological evidence of hepatic regeneration. The range of histologic hepatic response to MCLR was similar to other fish species, such as the hardhead catfish (Arius felis), gulf killifish (Fundulus grandis) or rainbow trout (Kotak et al., 1996; Fournie and Courtney, 2002). It is unclear why the severity of the hepatic lesion was independent of the MCLR dose administered and why the fish that were in the process of
regenerating their livers died. Microcystins do not require microsomal activation and inhibit protein phosphatases 1 and 2A, leading to hyperphosphorylation of cytosolic and nuclear proteins, cytoskeletal organization and signaling pathway disruption, and ultimately cell death (Runnegar and Falconer, 1986; Eriksson et al., 1990; Khan et al., 1996). Conjugation to glutathione (GSH) is the major pathway of elimination and the response variability in this study might have been due to the GSH store present at the time of exposure or perhaps to glutathione S-transferase gene polymorphisms (Kondo et al., 1992; Kondo et al., 1996; Sahin et al., 1996; Pflugmacher et al., 1998; Eaton and Bammler, 1999). Other toxicity mechanisms may have been involved, as microcystins are known to cause cell damage by oxidative stress, and inhibiting enzymes such as mitochondrial aldehyde dehydrogenase, ATP-synthase and acetylcholinesterase (Ding et al., 2001; Lehtonen et al., 2003; Mikhailov et al., 2003; Chen et al., 2006; Zegura et al., 2006).

To identify the LRCs in the liver of larvae and adult, morphology, location and immunohistochemistry were used. Although morphology alone permits identification of hepatocytes, immunohistochemistry for RFP confirmed their identity. BrdU retaining BPDECs were determined by location relative to blood vessels and as smaller cells often without discernible cytoplasm in between hepatocytes and lacking RFP staining. Unfortunately CK immunohistochemistry did not label BPDECs and cholangiocytes in larval medaka and could not be used to confirm the identity of BPDECs. Anti-cytokeratin AE1/AE3 antibodies reliably label many epithelial cell types including cholangiocytes and BPDECs but not hepatocytes in fish, including medaka (Bunton, 1993; Bunton, 1994; Okihiro and Hinton, 2000). Interestingly, CK AE1/AE3 immunoreactivity in BPDECs was not observed in larval
medaka from 15 to 60 dpf. This suggests that the CK expression profile in BPDECs may change over time and that the medaka liver may not be fully mature by 20 dpf as previously thought (Hardman et al., 2008; Hinton et al., 2008).

Collectively, the data presented indicate that hepatocytes, BPDECs, and cholangiocytes are the likely HPCs in the medaka liver. The location of these LRCs is similar in medaka and mice, thus further strengthening the argument that the organization of the piscine and mammalian liver is comparable. They also indicate that the process of hepatic regeneration is a complex system relying on multiple HPC niches that are activated depending on severity, chronicity and location of injury (Kuwahara et al., 2008).

**ACKNOWLEDGEMENTS**

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REFERENCES


**TABLES**

Table 1. Amount of nuclei labeled with BrdU in the medaka fish following exposure during hepatogenesis.

<table>
<thead>
<tr>
<th>BrdU exposure period</th>
<th>15 dpf</th>
<th>20 dpf</th>
<th>30 dpf</th>
<th>40 dpf</th>
<th>50 dpf</th>
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<tr>
<td>3 to 5 dpf</td>
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<td>5 to 7 dpf</td>
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<td>+++</td>
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<td>+</td>
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<tr>
<td>8 to 10 dpf</td>
<td>+++++</td>
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Number of nuclei per liver section: +: 1 to 5 nuclei; ++: 5 to 10 nuclei; +++: 10 to 20 nuclei; ++++: 20 to 40 nuclei; +++++: more than 40 nuclei.
Figure 1. Label retaining cell assay in the liver of medaka embryo. Immunolabelling for BrdU, hematoxylin counterstain. A: at 20 days post fertilization immunoreactivity for BrdU is present in many cell nuclei. Bar = 30μm 60X. B, C and D: at 50 days post fertilization only rare cell nuclei are immunoreactive for BrdU. B: Bar = 30μm 60X. C and D: Bar = 20 μm 100X. Positive hepatocytes nuclei (asterisk), bile preductular cells (arrow) and endothelial cells (arrow head).
Figure 2. Label retaining cell assay in the liver of medaka embryo 50 days post fertilization. Double immunolabelling for BrdU (brown) and red fluorescent protein (red), hematoxylin counterstain. Bar = 20 µm 100X. BrdU retention is observed in the nuclei of hepatocytes (asterisk), endothelial cells (arrow head), bile preductular epithelial cells (up arrow) and biliary epithelial cells (down arrow).
Figure 3. Label retaining cell assay in the liver of adult medaka. Immunolabelling for BrdU (brown), hematoxylin counterstain, bar = 30 µm 60X. At 24h (A) and 96h (B) after exposure to microcystin LR, strong nuclear immunoreactivity for BrdU is present in regenerating liver cells. Cell debris (arrow head). Red blood cells (arrow).
Figure 4. Label retaining cell assay in the liver of adult medaka. Double immunolabelling for BrdU (brown) and red fluorescent protein (red). Liver of the medaka with focal hepatic regeneration. A: Many intermediate hepatobiliary cells and hepatocytes in the regenerative focus are positive for BrdU. Hematoxylin counterstain, bar = 50 µm 40X. B: BrdU retention is observed in the nuclei of few hepatocytes (asterisk), endothelial cells (arrow head), and bile preductular epithelial cells (arrow) in the surrounding normal liver parenchyma like in control liver. Hematoxylin counterstain. Bar = 20 µm 100X.
CHAPTER 5: Conclusions and Future Directions

Laboratory fish have been used for decades as an experimental model to study chemical toxicity including carcinogenesis, and are essential components in ecotoxicological testing. Fish have also been used extensively in developmental biology and are increasingly used as animal model of human diseases (Schmale et al., 2007; Hinton et al., 2009; Cheng et al., 2012). Their small size, ease of housing, small, well annotated sequenced genome, and established techniques to suppress gene expression and create mutant and transgenic lines make these organisms an attractive animal model. However, extrapolation of findings in aquatic models to humans remains a concern, especially in risk assessment. Demonstration of common morphological changes associated with specific molecular mechanisms is one avenue by which data can be compared across divergent phyletic levels (Hobbie et al., 2009). Therefore, the research aims of this dissertation were focused on improving understanding of how the fish liver responds to injury in order to facilitate use, interpretation and extrapolation of data in the context of a relevant mammalian disease.

The following questions were addressed: 1) Where are hepatic progenitor cells (HPCs) located in the medaka liver?, 2) Are the oval-like cells observed in regenerating medaka liver the equivalent to rodent oval cells and human HPCs?, 3) Are the primary cellular and molecular events in the pathogenesis of hepatic fibrosis conserved in fish and mammalian models?

In chapter 2, the study demonstrated that the main cellular and molecular events in the pathogenesis of hepatic fibrosis in mammals and medaka fish exposed to DMN are conserved. Hepatocellular injury is followed by activation of HSCs, TGF-β pathway
activation, a shift in the balance between MMPs and TIMPs, and an increase in collagen production resulting in excess deposition of ECM. These data support the medaka as an alternative animal model of hepatic fibrosis and improves our comparative understanding of the liver’s response to chronic injury across taxa. In chapter 3, the study demonstrated that fish oval cells share similar morphology and immunoreactivity with rodent oval cells and human HPCs, and have similar bipotential lineage pathways. These data provide additional evidence of the striking similarities in the hepatic regenerative process between fish and mammals. In chapter 4, a BrdU label retention cell assay indicated that hepatocytes, BPDECs, and cholangiocytes are likely HPCs in the medaka liver. Collectively, the results support the notion that the organization and response to injury of the piscine and mammalian liver are analogous.

The process of hepatic regeneration, fibrosis and cancer are intimately linked (Alison et al., 2009). In order to better understand and potentially control hepatic regeneration and fibrosis for therapeutic purposes, the cellular and molecular mechanisms have been studied extensively using a variety of animal models (Palmes and Spiegel, 2004; Sadler et al., 2007; Wallace et al., 2008; Michalopoulos, 2010; North et al., 2010). Interestingly, although laboratory fish models are considered robust models to study organ regeneration, the process of hepatic regeneration in fish has received considerably less attention than other organs, such as the heart and fins (Poss et al., 2003; Sadler et al., 2007; Kan et al., 2009). This is because liver regeneration can be studied directly in mammalian models, since it has significant regenerative capacity compared to other organs. However, fish models have some advantages over mammalian models that could help answer fundamental mechanistic
questions. First, fish models have been intensely used in developmental biology studies and the mechanism involved during development and regeneration are often similar (Hata et al., 2007; Chu and Sadler, 2009). Second, powerful mutagenesis and genetics screening studies can be more easily performed in fish and used to uncover molecular pathways. Such studies have been used occasionally to help define the mechanism of hepatic regeneration (Sadler et al., 2007; Goessling et al., 2008). Third, creation of transgenic fish with spatio-temporal cre-lox transgene regulation is easier and faster in fish than in mammalian models (Hans et al., 2009; Hans et al., 2011). Fourth, the zebrafish (Danio rerio) mutant strain casper and the Japanese medaka fish STII and STIII strains are presently the only vertebrate animal models which are transparent in adult stage (Wakamatsu et al., 2001; Hardman et al., 2008b; Hardman et al., 2008a; White et al., 2008). Such transparent fish strains have been used to study the mechanism of hepatobiliary toxicity and are an ideal animal model to study tissue response and gene expression non-invasively in-vivo. (Hardman et al., 2008a).

Given the benefits of fish model systems, the DMN-induced model of hepatic fibrosis characterized in this dissertation is an attractive model to study the mechanism of hepatic regeneration, fibrosis and progression to neoplasia. Generation of transgenic fish expressing a marker protein specific to mature hepatocytes and HPCs will help answer fundamental questions regarding the origin of regenerative nodules and hepatocellular carcinoma. In addition, other lineage tracing studies can be performed to study if epithelial mesenchymal transition occurs during liver fibrosis and, if so, what is the contribution of epithelial mesenchymal transition to the pool of collagen-secreting cells during fibrosis. Use of techniques not relying on the cell cycle to perform label retention cell assay such as
transgenic fish with doxycycline-inducible expression of an H2B-GFP fusion protein would be useful to identify the HPCs and help confirm the data obtained in chapter 4 (Foudi et al., 2009). The studies mentioned above could be performed using a transparent fish strain and the location of the labeled cells observed with high resolution microscopy in-vivo coupled with three-dimensional (3D) reconstructions (Hardman et al., 2008b; Hardman et al., 2008a; Hasegawa et al., 2009).

To conclude, the research presented in this dissertation furthers our understanding of the fish liver response to injury and supports the use of the medaka as a model to study the mechanisms of hepatic disease and environmental toxicity in the liver.
REFERENCES


Appendix A

Effects of Vitamin A Administration on Hepatic Stellate Cells in Japanese Medaka (*Oryzias latipes*)

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ABSTRACT

In the healthy liver of Japanese medaka (*Oryzias latipes*), hepatic stellate cells (HSCs) are challenging to find by histology or transmission electron microscopy (TEM). In contrast to most vertebrate species, medaka’s HSCs are often devoid of lipid or have a single lipid vacuole. In this experiment, we administered a single intraperitoneal injection of vitamin A at 3 different dosages to facilitate detection HSCs and confirm their presence in the medaka liver. Two days after administration of vitamin A, the number of HSCs containing lipid vacuoles increased and were easier to identify in healthy liver by TEM. The HSCs were moderately hypertrophic and contained a variable number of small lipid vacuoles. No difference in the amount of lipid accumulation in HSCs was observed between the 3 different doses administered. This result confirms the presence of HSCs in the medaka liver and the effectiveness of a vitamin A exposure to facilitate their detection using TEM.
INTRODUCTION

Hepatic stellate cells (HSCs) are versatile cells that play an important role in liver development, regeneration, control of sinusoidal blood flow, vitamin A metabolism, inflammatory reaction and response to xenobiotic (Friedman, 2008; Winau et al., 2008; Atzori et al., 2009). They are well known for their central role in liver fibrosis but also are protagonists in most liver pathologies from hepatitis to liver carcinogenesis (Mikula et al., 2006; Hernandez-Gea and Friedman, 2010; Kang et al., 2011; Tsukamoto et al., 2012).

Hepatic stellate cells were first described by von Kupffer in 1876, and called “sternzellen” (“star cells” in German) (Wake, 1980; Aterman, 1986). During the following decades, other investigators described similar “perisinusoidal” cells but they were rediscovered and described by Ito in human livers in 1951 using the name “fat storing cell” (Ito and Nemoto, 1952; Aterman, 1986). Numerous studies have investigated the liver sinusoid microanatomy and many different names have been used by investigators to describe HSCs: perisinusoidal cells, parasinusoidal cells, hepatic pericytes, lipocytes, fat storing cells, and Ito cells. To avoid confusion, in 1996, the name HSCs became the standard terminology to refer to this cell type in the normal liver (Anonymous, 1996; Friedman, 2008; Atzori et al., 2009).

Studies have demonstrated the presence of HSCs in the liver of mammals, birds, reptiles, amphibian and fishes (Ito and Nemoto, 1956; Ito et al., 1962; Wake et al., 1986; Wake et al., 1987; Nagy et al., 1997). In fish, HSCs have been identified in almost all species examined (Ito et al., 1962; Wake et al., 1987; Lauren et al., 1990; Hinton, 1993; Yoshikawa et al., 2006). Interestingly, HSCs were not observed in Japanese medaka (Oryzias latipes) when
first investigated, but later found to be present (Ito et al., 1962; Lauren et al., 1990). In studying the pathogenesis of liver fibrosis in medaka, activated HSCs that had acquired myofibroblast morphological characteristics were observed by transmission electron microscopy (chapter 2). However, in control liver, quiescent HSC were very difficult to find.

Vitamin A storage is one of the characteristic roles of the quiescent HSCs and over-supplementation with vitamin A has been use previously in mammals and fish to highlight the presence of HSCs (Kobayashi and Takahashi, 1971; Kobayashi et al., 1973; Wake, 1974; Takahashi et al., 1978; Yamamoto et al., 1978; Tanuma and Ito, 1980).

In this study we exposed medaka to a high dose of vitamin A to confirm the presence of HSCs in the medaka liver and see if their detection could be facilitated.

**MATERIALS and METHODS**

**Chemicals**

Retinol (vitamin A; C20H30O; purity 99.9%, CAS 68-26-8, MW 286.5 g/mol) and dimethyl sulfoxide (DMSO; (CH3)2SO, purity 99.9%, CAS 67-68-5, MW 78.13 g/mol) were obtained from Sigma-Aldrich (St. Louis, MO). The brown glass bottle containing the vitamin A powder was stored in a metal container at -20°C until use. The brown glass bottle storing the DMSO was stored at room temperature.

**Medaka vitamin A Exposures**

Three-month-old, male and female, orange-red Japanese medaka (Oryzias latipes) were obtained from an in-house stock population maintained under recirculating freshwater aquaculture conditions at the Department of Molecular and Environmental Toxicology,
North Carolina State University (NCSU), Raleigh, NC. Medaka from the stock colony were moved to NCSU College of Veterinary Medicine (Raleigh, NC) and acclimated for 2 weeks in 10 gallon aquarium tanks filled with reconstituted reverse osmosis–purified (RO) water (0.5 g/liter Instant Ocean® salts, Aquarium System Inc. Mentor, OH) within a static freshwater culture system. The artificial light photoperiod was 16 hours light/8 hours dark. The water temperature was maintained at 26°C ± 0.5°C, the water pH at 6.5 and the water conductivity between 600 to 800 µS. Dry food (Otohime B1, Reed Mariculture, Campbell, CA) was fed throughout the day using automated feeders. For the vitamin A exposures, medaka were randomly distributed among 4-liter glass beakers, 3 fish per beaker, containing 3 liters of reconstituted RO water as described above. The beakers were placed within a recirculating, heated water bath to maintain temperature at 26°C ± 0.5°C throughout the exposures. Fish were fed twice daily and observed twice daily for behavioral responses and signs of overt toxicity.

The vitamin A powder was dissolved in DMSO (Sigma-Aldrich) to a concentration of 12.5 IU (3.75 ug) per µL. The fish were briefly anesthetized using tricaine methanesulfonate (120 mg/L; MS-222), weighed, placed in right lateral recumbency, and injected intraperitoneally using a glass 25 µL Hamilton syringe (PB-600 Repeating Dispenser, Hamilton, Reno, NV), equipped with a 32 gauge needle (Hamilton, Reno, NV). Medaka were dosed at 25,000 (n=3), 50,000 (n=3) and 100,000 IU/kg (n=3). Three control fish were anesthetized and injected with 2µL of DMSO solution. After receiving the intraperitoneal injection, the fish were returned to the 4L beakers filled with fresh reconstituted water. Animal care and use were in conformity with protocols approved by the NCSU Institutional
Animal Care and Use Committee in accordance with the National Academy of Sciences Guide for the Care and Use of Laboratory Animals.

**Sampling Method and Tissue Processing**

The 3 fish in each exposure group and the control fish were euthanatized with an overdose of tricaine methanesulfonate (300 mg/liter; MS-222, Argent Laboratories, Redmond, WA) 2 days post exposure. The liver was inspected macroscopically, harvested and cut into two approximately equal-sized pieces. One piece was fixed in freshly made 4% paraformaldehyde for 24 hours, and transferred to 70% ethanol for histopathology. The other piece was cut in small samples (1 x 1 mm) and fixed in 4F:1G fixative (4% formaldehyde and 1% glutaraldehyde buffered in monobasic sodium phosphate, pH 7.2-7.4) for transmission electron microscopy (McDowell and Trump, 1976).

**Histology and Electron Microscopy**

Paraformaldehyde –fixed livers were processed, and embedded in paraffin according to routine histologic techniques. 5 μm sections were stained with hematoxylin and eosin (H&E) and examined by light microscopy. Liver samples fixed in 4F:1G fixative were dehydrated in alcohol, and embedded in Spurr resin for transmission electron microscopy (Dykstra, 1993). To determine whether structures of interest were in planar sections of the block face, semithin sections, 0.5 μm thick, stained with 1% toluidine blue in 1% sodium borate, were examined under a light microscope. Ultrathin sections, 90 nm thick, of appropriate blocks were stained with uranyl acetate and lead citrate and examined with a FEI/Philips EM 208S.
transmission electron microscope (Laboratory for Advanced Electron and Light Optical Methods, NCSU).

RESULTS

Gross findings and histology

No macroscopic or histologic changes were noted in all exposed and control fish livers.

Transmission electron microscopy

In control liver, HSCs where rarely identified and the few observed had a single variably electron dense intracytoplasmic lipid vacuole that occasionally appeared to indent the nucleus. In vitamin A exposed fish, HSCs were easier to find, moderately hypertrophied and contained several intracytoplasmic lipid vacuoles of variable size (Fig. 1). No difference in the number or size of lipid vacuoles in the HSCs between the 3 vitamin A exposure doses was observed. In control and vitamin A exposed liver, the cytoplasm of HSCs was characterized by the small number of mitochondria and sparse rough endoplasmic reticulum. Desmosomal junctions between HSCs and sinusoidal endothelial cells and hepatocytes were observed.

DISCUSSION

The results of this experiment confirm the presence of HSCs in medaka liver and vitamin A exposure can be used to increase their lipid content and facilitate their definition. The ultrastructural morphology of HSCs in control liver in this study is similar to that described
by Lauren et al, 1990, and demonstrates that HSCs in normal medaka liver infrequently contain lipid vacuoles. This sparse lipid content make them difficult to detect by TEM and may explain why it was first concluded that medaka lack HSCs (Ito et al., 1962). This low amount of lipid has been described in other fish species, and vitamin A exposure resulted in a similar lipid accumulation in HSCs (Takahashi et al., 1978; Yamamoto et al., 1978; Tanuma and Ito, 1980).

No difference in the amount of lipid vacuoles was observed among the 3 vitamin A exposure doses. However, a more detailed morphometric analysis might have revealed subtle differences. In carp (*Cyprinus carpio*), for instance, daily intramuscular injection of vitamin A over a period of 10 days lead to a more pronounced lipid accumulation in HSCs than a 5 days course (Takahashi et al., 1978).

To conclude, this experiment confirms the presence of HSCs in the medaka liver and the effectiveness of a vitamin A exposure to facilitate their detection using TEM.

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Figure 1. Liver of a medaka exposed to vitamin A. Two large lipid vacuoles (L) are visible in the cytoplasm of a hepatic stellate cell (HSC) in the space of Disse. H: Hepatocyte. RBC: Red blood cell. M: Macrophage. Bar = 2μm X 5600.
Figure 2. Liver of a medaka exposed to vitamin A. multiple lipid vacuoles (L) are visible in the cytoplasm of a hepatic stellate cell (HSC). H: Hepatocyte. RBC: Red blood cell. Bar = 1µm. X 7100.
Figure 3. Liver of a medaka exposed to vitamin A. One large lipid vacuoles (L) is visible in the cytoplasm of a hepatic stellate cell (HSC) in the space of Disse. H: Hepatocyte. Bar = 2μm X 5600.
Figure 4. Liver of a medaka exposed to vitamin A. A lipid vacuole (L) is visible in the cytoplasm of a hepatic stellate cell (HSC) in the space of Disse. H: Hepatocyte. RBC: Red blood cell. Bar = 1 µm X 11000.
APPENDIX B

Phenotypic Characterization of a Transgenic Japanese medaka (Oryzias latipes) Strain with Red Fluorescent Protein-Labeled Hepatocytes

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ABSTRACT

The demonstration of cell type specific antigen using immunohistochemistry or immunofluorescence is a useful technique to confirm the identity or lineage of cells. Unfortunately no specific cell markers that label fish hepatocytes has been developed. Therefore, we used a transgenic medaka fish strain that expressed a red fluorescent protein (RFP) exclusively in mature hepatocytes to permit hepatocyte labeling in the chapter 3 and 4 studies. Here, we examined the phenotype of this transgenic medaka strain, Tg(zfL-fabp:DsRed), using light microscopy, immunohistochemistry (IHC) and transmission electron microscopy (TEM). Expression of RFP was first detected by IHC in the embryonic liver at 7 days post fertilization (dpf). From 12 dpf, RFP expression was present in and limited to all hepatocytes. By light microscopy, brightly eosinophilic globules and acicular inclusions were observed in hepatocytes with greater frequency in older fish and correlated with membrane-bound electron dense inclusions on TEM. These inclusions were immunoreactive for RFP. These results define the onset of RFP expression in the Tg(zfL-fabp:DsRed) medaka and confirm that RFP expression is specific to hepatocytes. They also identify specific phenotypic changes in hepatocytes related to RFP expression.
INTRODUCTION

The demonstration of cell type specific antigen using immunohistochemistry or immunofluorescence is a useful technique to confirm the identity or lineage of cells. In rodents and human, antibodies against cytokeratin 7 and 19, and HepPar1 are well accepted biliary epithelium/oval cell marker, and hepatocyte marker, respectively (Vessey and de la Hall, 2001; Zhou et al., 2007; Bird et al., 2008). In fish, including medaka, cytokeratin AE1/AE3 is a reliable marker of epithelial cells including cholangiocytes and bile preductular epithelial cells (BPDEC) but not hepatocytes (Bunton, 1993; Bunton, 1994; Okihiro and Hinton, 2000). Unfortunately, no specific marker for fish hepatocytes has been developed and attempts at immunohistochemical staining for CK7 (Cytokeratin 7, clone OV-TL 12/30, Cat. #: M7018, Dako, Carpinteria, CA), and Hep Par 1 (Hepatocyte, Clone OCH1E5, Cat. #: M7158, Dako) in medaka have proven unproductive in our lab. To overcome the lack of a fish hepatocyte marker for the studies in chapter 3 and 4, a transgenic medaka fish was obtained that expressed a red fluorescent protein (RFP) exclusively in mature hepatocytes. Here, phenotypic information regarding this transgenic fish strain were examined.

MATERIALS and METHODS

Medaka culture

A transgenic orange-red medaka fish line Tg(zfL-fabp:DsRed) which expresses a red fluorescent protein (RFP) under the regulatory control of the 2.8-kb fragment of the zebrafish liver fatty acid binding protein (L-FABP) promoter was used to specifically label hepatocytes
The founder Tg(zfL-fabp:DsRed) medaka were obtained from Drs. Joerg Renn and Christoph Winkler, Department of Biological Sciences, National University of Singapore. This stable transgenic line showed standard Mendelian inheritance from F2 generation onwards and offspring were screened by observation of embryos or larvae for RFP protein expression in the liver. Our Tg(zfL-fabp:DsRed) medaka colony was housed under recirculating freshwater aquaculture conditions with charcoal filtration and UV treatment used in each passage at the Department of Molecular and Environmental Toxicology, North Carolina State University (NCSU), Raleigh, NC. Water temperature and pH were monitored daily and maintained at 25 ± 2 °C and ~7.2, respectively. The light-dark cycle was 16 h light, 8 h dark. Dry food (Otohime B1, Reed Mariculture, Campbell, CA) was fed several times per day through automated feeders, and newly hatched Artemia nauplii were fed once daily.

In the culture conditions described above, medaka spawned daily and embryonated eggs (10–30 per mass) were collected daily. The eggs were separated from each other by gentle rolling on a moistened paper towel surface to disrupt the attachment filaments joining individual eggs. Groups of individual embryonated eggs of specified collection date and developmental stage were suspended in a 2% saline solution made from Instant Ocean (Instant Ocean® salts, Aquarium System Inc. Mentor, OH) in a 100 × 15 mm petri dish and incubated at 26 ± 1°C with 16 h light, 8 h dark light-dark cycle (Iwamatsu, 2004). After 5 days, the 2% saline solution was replaced with 1X embryo rearing medium (ERM) containing 17.1 mM NaCl and, in micromolars, 272 CaCl2·2H2O, 402 KCl, and 661 MgSO4·7H2O through hatching (usually at 9 days post fertilization [dpf]). After hatching the
larvae were kept up to 15 dpf in petri dish containing 0.3X ERM. After 15 dpf, the larvae were transferred to 130 x 70 x 120 mm plastic containers filled with water from the recirculating freshwater aquaculture system for the remaining of the growth out period up to 60 dpf. The ERM or culture system water filling the petri dish or container holding the larvae was renewed every 2 days. After hatching, the larvae were fed once daily with Dry food (Otohime B1). At 60 dpf, the fish were transferred to 40L tanks in recirculating freshwater aquaculture conditions. Animal care and use were in conformity with protocols approved by the NCSU Institutional Animal Care and Use Committee in accordance with the National Academy of Sciences Guide for the Care and Use of Laboratory Animals.

**Sampling Method and Tissue Processing**

Embryos were collected at 4, 5, 6, 7, 12, 15 and 20 dpf, euthanatized with an overdose of tricaine methanesulfonate (300 mg/liter; MS-222, Argent Laboratories, Redmond, WA) and fixed in 4% paraformaldehyde for 10 hours. The unhatched embryos were dechorionated using a fine tip scissor under a dissecting microscope, and transferred to 70% ethanol for histopathology. At least 20 embryos in each age group were examined by light microscopy. In addition, whole adult fish and liver from control fish used in other experiments (chapter 3 and 4) and older breeding stock medaka were used for this study. Adult fish were euthanatized with an overdose of tricaine methanesulfonate (300 mg/liter; MS-222, Argent Laboratories) at approximately 3, 6, 12 and 18 months of age. At least 5 fish in each age group were examined. The coelom was incised along the ventral midline to enhance fixative penetration and the fish were fixed whole in 4% paraformaldehyde for 24 hours.
demineralized in 10% formic acid for 24 hours, and transferred to 70% ethanol for histopathology. The liver from 3 additional 6 month old control fish (chapter 3) was inspected macroscopically, harvested and cut into two approximately equal-sized pieces. One piece was fixed in freshly made 4% paraformaldehyde solution for 24 hours, and transferred to 70% ethanol for histopathology. The other piece was cut in small samples (1 x 1 mm) and fixed in 4F:1G fixative (4% formaldehyde and 1% glutaraldehyde buffered in monobasic sodium phosphate, pH 7.2-7.4) for transmission electron microscopy (TEM) (McDowell and Trump, 1976).

**Histology and Electron Microscopy**

Paraformaldehyde–fixed fish livers and whole embryos, larvae and fish were processed, and embedded in paraffin according to routine histologic techniques. Sections, 5 µm thick, were stained with hematoxylin and eosin (H&E) and examined by light microscopy. Randomly oriented sections were cut through the embryos while median and paramedian sections were made through the 3-month-old and older fish.

Liver samples fixed in 4F:1G fixative were, dehydrated in alcohol, embedded in Spurr resin, and processed for TEM (Dykstra, 1993). To determine whether structures of interest were in planar sections of the block face, semithin sections, 0.5 µm thick, stained with 1% toluidine blue in 1% sodium borate, were examined under a light microscope. Ultrathin sections, 90 nm thick, of appropriate blocks were stained with uranyl acetate and lead citrate and examined with a FEI/Philips EM 208S transmission electron microscope (Laboratory for Advanced Electron and Light Optical Methods, NCSU).
**Immunohistochemistry**

To assess for RFP expression in the liver and other organs, immunohistochemistry for RFP was performed in at least 5 liver sections per embryo, larva or adult fish. The RFP antibody was a rabbit polyclonal antibody that recognizes the full length amino acid sequence of the mushroom polyp coral Discosoma RFP (Abcam Inc, Cambridge, MA. Catalogue # ab34771). Tissue sections were deparaffinized in xylene, rehydrated in a graded series of ethanol and rinsed in distilled water. Antigen retrieval was performed by heating (99°C) the slides in a 10 mM sodium citrate solution at pH 6 for 10 minutes in a vegetable steamer (Oster 5712 food steamer, Maitland, FL). Following a cool down period of 10 minutes in the warm sodium citrate buffer, the slides were rinsed in 1X Tris-buffered saline with 0.05% Tween (TBST) and treated with 3% hydrogen peroxide for 10 minutes to block endogenous peroxidases activity. Goat serum was applied for 20 minutes to prevent nonspecific binding of the secondary antibody (BioGenex San Ramon, CA). Tissues were incubated with the RFP antibody (1/500 dilution) for 30 minutes at room temperature. The slides were rinsed in 1X TBST, and then incubated with the secondary antibody for 20 minutes (BioGenex San Ramon, CA). After a wash in 1X TBST, tissue sections were then treated for 20 minutes with streptavidin alkaline phosphatase (BioGenex San Ramon, CA). Following a final wash in 1X TBST, development was achieved by treatment of tissue with VECTOR Red Alkaline Phosphatase Substrate Kit (Vector Lab, Burlingame, CA) for 1 to 5 minutes. After a wash in tap water, the slides were counterstained with Mayer’s hematoxylin for 20–40 seconds and cover slipped. For negative controls, the primary antibody was
omitted and rabbit non-immune serum was applied. For positive controls, sections of liver from adult Tg(zfL-fabp:DsRed) medaka were run at the same time.

Medaka liver lesions were identified based on criteria set by a consensus of the US National Toxicology Program pathology working group and the International Harmonization of Nomenclature and Diagnostic Criteria for Lesions in Rats and Mice (INHAND) project (Boorman et al., 1997; Thoolen et al., 2010).

RESULTS

Gross findings and histology

Livers of the Tg(zfL-fabp:DsRed) medaka had a distinct red color compared to the brown color of the outbred orange red medaka strain (fig. 1). The difference in coloration was most evident in 3 months old and older fish. Except the coloration, no significant macroscopic changes were noted in all embryo, larvae or adult fish. No microscopic lesions were appreciated in the 4 to 20 day old embryo/larvae. In the 3 to 18 months old fish, 2 to 10 µm eosinophilic globules and 2 x 3 to 5 x 20 deeply eosinophilic acicular inclusions were observed in the cytoplasm of hepatocytes (Fig. 2). The amount of eosinophilic material in hepatocytes varied greatly between livers but was more frequently observed and in larger quantity in livers of 12 and 18 months old fish (Fig. 3). Material with similar staining characteristics filled the cytoplasm of a few to a moderate number of scattered interhepatocytic macrophages. Again, the number of macrophages filled with this eosinophilic material was increased in the 12 and 18 months old fish compared to the 3 and 6
months old fish. Occasional small areas of spongiosis hepatis or cystic degeneration were observed in the 12 and 18 months old medaka.

Immunoreactivity for RFP was absent in the embryonic liver of all embryos evaluated at 4, 5 and 6 dpf. At 7 dpf, cytoplasmic immunoreactivity was observed in some but not all hepatocytes of the embryonic liver (Fig. 4). In embryos, larvae or adult fish evaluated at 12, 15 and 20 dpf and 3, 6, 12 and 18 months, all hepatocytes demonstrated diffuse cytoplasmic immunoreactivity for RFP (Fig. 5). Intracytoplasmic immunoreactivity for RFP was also observed in some macrophages in 3, 6, 12 and 18 month old fish; the liver of older fish containing more immunoreactive macrophages. With the exception of macrophages, immunoreactivity for RFP was not observed in cells other than hepatocytes in multiple sections of whole embryos, larvae and adult fish.

Transmission electron microscopy

By TEM, a small to moderate number of hepatocytes contained intracytoplasmic membrane-bound inclusions composed of dark, homogenous moderately electron-dense material (Fig. 6). The form and size were variable and ranged from less than 0.5 x 0.5 µm irregular globules up to 3 x 15 µm rhomboid or acicular inclusions. At high magnification, an approximately 5 nm wide membrane surrounding the electron dense inclusion was visible (Fig. 7). Multifocal electron dense material that distended rough endoplasmic reticulum cisternae was observed exclusively in hepatocytes. No other unusual ultrastructural feature was observed in the hepatocyte, the biliary epithelial cells, bile preductular epithelial cells, endothelial cells, or macrophages examined.
DISCUSSION

To address the lack of a fish hepatocyte marker for our studies in chapter 3 and 4, a transgenic medaka fish was obtained that expressed an RFP in mature hepatocytes as assessed by in vivo fluorescence microscopy. However, exclusive expression in hepatocytes needed to be verified. This study confirmed exclusive expression of the RFP in the hepatocytes using light microscopy, immunohistochemistry, and TEM. The observation by TEM of electron dense RFP material in hepatocytes correlated well with the light microscopy and immunohistochemical findings. This electron dense material was also located in dilated rough endoplasmic reticulum cisternae and very likely represented accumulation of RFP. Immunogold labeling would be needed to confirm that this homogenous electron dense material is RFP. However, correlation between the light microscopic and ultrastructural appearance with the specific RFP immunoreactivity in histologic sections already provides strong indirect evidence that the material observed by TEM is RFP. Accumulation of large amounts of RFP was observed by light microscopy and TEM in some hepatocytes, especially in older fish, and suggests a deficit in RFP degradation and turn over in some cells. It is interesting to note that the hepatic macrophages contained RFP protein which suggests they had phagocytized hepatocellular debris, likely from normal cell turn over. No apparent morphological deleterious effect or reproductive effects resulting from the RFP expression were observed in this study. However, reproductive and hepatocyte metabolic testing were not performed.
The results of this study also define the onset of RFP expression in the medaka fish line Tg(zfL-fabp:DsRed). In medaka the liver anlage occurs at 2 dpf and the liver achieve its adult phenotype at 16 dpf (Iwamatsu, 2004; Hinton et al., 2008). Expression of RFP was first observed at 7 dpf when a small mass of markedly vacuolated cells resembling hepatocytes was already clearly recognizable (Fig. 4). By 12dpf, all hepatocytes expressed RFP. This result indicates that expression of L-FABP in medaka hepatocytes starts around 7dpf and is not expressed in the earlier hepatoblast stage. This result also indicates that this RFP zebrafish L-FABP construct would be useful to create transgenic fish line for use in lineage tracing studies where a marker of mature hepatocytes is needed.

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Figure 1. Gross appearance of the liver of an adult Tg(zfL-fabp:DsRed) medaka (left) and an adult orange red strain medaka (right). Note the red color of the transgenic fish strain liver compared to an outbred non transgenic medaka fish strain. Bar = 1 mm.
Figure 2. Histology of an adult Tg(zfL-fabp:DsRed) female medaka liver. Note the deeply eosinophilic globules and rhomboid inclusions in the cytoplasm of some hepatocytes (arrow). Some macrophages contain similar deeply eosinophilic material (arrow head). Hematoxylin and eosin stain. Bar = 30 μm 60X.
Figure 3. Histology of an adult Tg(zfL-fabp:DsRed) male medaka liver. Note the pronounced accumulation of intracytoplasmic eosinophilic inclusions in hepatocytes. This level of RFP accumulation was mainly observed in the 12 and 18 month old fish and not in all fish. Hematoxylin and eosin stain. Bar = 30 µm 60X.
Figure 4. Histology and immunolabelling for red fluorescent protein of a 7-days-old (stage 37) Tg(zfL-fabp:DsRed) medaka embryo liver. Immunolabelling for RFP, hematoxylin counterstain. Cytoplasmic immunoreactivity (red) is observed in some hepatocytes of the embryonic liver. L: liver. F: foregut. Bar = 50µm 40X.
Figure 5. Immunolabelling for red fluorescent protein of a 30-days-old Tg(zfL-fabp:DsRed) medaka larva liver, hematoxylin counterstain. Diffuse cytoplasmic immunoreactivity for RFP (red) is present in all hepatocytes. Bar = 50µm 40X.
Figure 6. Transmission electron microscopy of adult Tg(zfL-fabp:DsRed) medaka hepatocytes. Large electron-dense inclusions are present in the cytoplasm of a hepatocyte (asterisk). Segments of rough endoplasmic reticulum cisternae are distended by similar electron dense material (arrow). #: artifact. Bar = 2 µm. X 5600