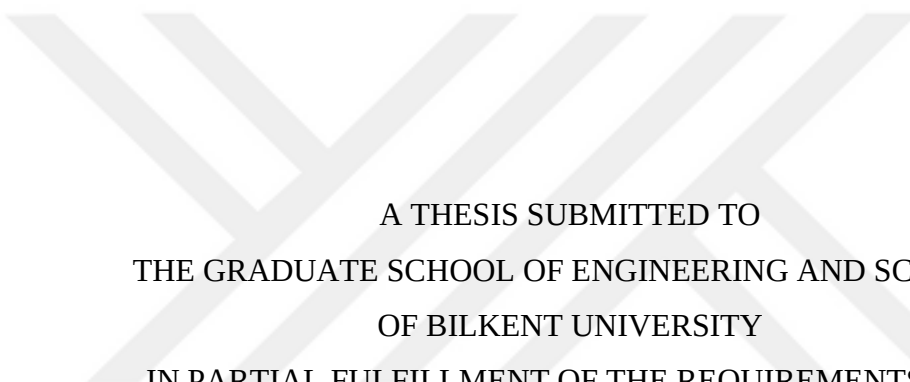


IDENTIFICATION OF THE FIBROGENIC CELLS IN THE LIVER



A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF ENGINEERING AND SCIENCE
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By
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BILKENT UNIVERSITY

IDENTIFICATION OF THE FIBROGENIC CELLS IN THE LIVER

By Müge Bozlar

March 2022

We certify that we have read this thesis and that in our opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Master of Science.

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ABSTRACT

Identification of The Fibrogenic Cells in The Liver

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The liver is an important multitasking organ with diverse functions to maintain body homeostasis. Adjustment of glucose level, detoxification, secretion of blood proteins, and synthesis of bile acids are among these crucial functions. The fibrosis is one of the most common liver injury types. Although liver has a high capacity for regeneration via the proliferation of cells, this regeneration ability is directly dependent on the severity of fibrosis. One of the proliferative cells during fibrosis is cholangiocytes located around the portal triad. The cholangiocytes contain a primary cilium, a non-motile, ciliated organelle. The response of primary cilium to fibrosis is not well understood. The aim of this thesis was to characterize responses of cholangiocytes to liver fibrosis. For this purpose, TAA induced fibrosis model was generated for different weeks of samples, 16 weeks, 20 weeks, and 24 weeks in mice. IHC stainings and data collection from confocal microscopy were performed. According to our study, we observed that around the portal vein, big cell masses are formed and these cells are migrating between portal veins by following a path upon fibrosis. Moreover, several cells are escaping from this path and expanding through the central vein. The main contributors of these giant cell masses are cholangiocytes which keep their primary cilia under fibrotic conditions. Furthermore, we identified a new subpopulation of Arl13b⁺ cholangiocytes which are undergoing partial EMT by expressing mesenchymal markers such as Vimentin, CD34, and Pdgfra in these cell masses. During this process, they gain novel mesenchymal features at the same time they maintain their epithelial character. As a following goal, we wanted to identify components of these big cell masses, so niche components of partial EMT undergoing Arl13b⁺ cholangiocytes. We identified that Vimentin, CD34, and Pdgfra mesenchymal cells and Thy1⁺ portal fibroblasts (minority) are components of these massive cells in addition to homeostatic and partial EMT undergoing Arl13b⁺ cholangiocytes. As a final objective, we identified novel, possible markers for partial EMT undergoing Arl13b⁺ cholangiocytes and their niche

components. To conclude, the novelties of this thesis were the demonstration of the primary cilium response against fibrosis, the identification of a new Arl13b⁺ cholangiocyte subpopulation undergoing partial EMT, and the characterization of components of the migratory big cell masses under fibrotic conditions.

Keywords: liver, primary cilia, fibrosis, cholangiocytes, partial EMT, IHC, portal vein, TAA



ÖZET

Fibrotik Hücrelerin Karaciğerde Tanımlanması

Müge Bozlar

Moleküler Biyoloji ve Genetik, Yüksek Lisans

Tez Danışmanı: Bahar Değirmenci Uzun

Mart 2022

Karaciğer vücut iç dengesini sabit tutma gibi işlevleriyle, sindirim sisteminin kayda değer ve çok görevli bir organıdır. Glukoz miktarının ayarlanması, kan proteinlerinin salgılanması, safra asidinin sentezlenmesi karaciğerin en temel görevleri arasındadır. Karaciğerde en yaygın görülen yaralanmalardan bir tanesi fibrozistir. Hücrelerin çoğalması sayesinde karaciğer yüksek bir yenilenme kapasitesine sahip olmasına rağmen, bu yenilenme kapasitesi aslında fibrozisin şiddetine bağlıdır. Fibrozis durumunda karaciğerde çoğalan hücre tiplerinden bir tanesi portal üçlüsü çevresinde bulunan kolanjiositlerdir. Bu hücre tipi hareketsiz, silyalı organel olan primer silyaya sahiptir. Primer silyanın fibroze karşı reaksiyonu tam olarak anlaşılamamıştır. Bu yüzden, bu tezin ana odağı kolanjiositlerin fibroze karşı olan tepkilerini karakterize etmektir. Bu amaç doğrultusunda, tiyoasetamid (TAA) ile oluşturulmuş farklı haftalık (16 haftalık, 20 haftalık ve 24 haftalık) fibrozis fare modelleri elde edilmiştir. İmmunohistokimya boyamaları ve konfokal mikroskoptan veri analizleri yapılmıştır. Çalışmamıza göre, fibrozis ile portal damar çevresinde büyük hücre topluluklarının oluştuğu ve bu hücre topluluklarının farklı portal damarlar arasında belirli bir yol takip ederek taşındıkları gözlemlenmiştir. Ayrıca, bazı hücrelerin bu yoldan saptıkları ve merkezi damara doğru yayıldıkları tespit edilmiştir. Bu büyük hücre topluluklarına katkıda bulunan temel hücre grubu kolanjiositlerdir ve fibrozis koşulunda primer silyalarını muhafaza ettikleri görülmüştür. Bunlara ek olarak, bahsedilen büyük hücre topluluklarında fibrozis ile görülen yeni bir alt tür hücre grubu tanımlanmıştır. Bu yeni alt tür hücre grubunun kolanjiositlere ait olduğu ve Vimentin, CD34 ve Pdgfra gibi mezenkimal proteinleri üreterek kısmi epitel-mezenkimal değişimi geçirdiği gözlemlenmiştir. Bu değişim sırasında, alt tür kolanjiositler kendi epitel karakterlerini koruyarak yeni mezenkimal özellikler kazanmaktadırlar. Bir sonraki hedefimiz, bu büyük hücre topluluklarının hangi hücrelerden oluştuğunu yani kısmi epitel-mezenkimal değişim geçiren Arl13b+ kolanjiositlerin niş üyelerinin hangi hücreler olduklarını bulmak

olarak belirlenmiştir. Bu yoğun hücre topluluklarının kısmi epitel-mezenkimal değişim geçiren ve homeostatik Arl13b+ kolanjiositlere ek olarak Vimentin, CD34 ve Pdgfra üreten mezenkimal hücrelerden, ve de Thy1+ portal fibroblastlardan (azınlık) oluştukları tespit edilmiştir. Ayrıca, kısmi epitel-mezenkimal değişim geçiren Arl13b+ kolanjiositleri ve de bu hücre grubunun niş üyelerini tanımlamak için yeni ve olası proteinler tespit edilmiştir. Kısacası, bu tezin yenilikleri primer silyanın fibrozise karşı olan reaksiyonunun gösterilmesi, yeni bir hücre alt grubu olan ve fibroziste görülen, kısmi epitel-mezenkimal değişim geçiren Arl13b+ kolanjiositlerin tanımlanması ve de fibrozis koşulu altında gözlemlenen hareketli ve yoğun hücre topluluklarının katılımcılarının karakterize edilmesidir.

Anahtar sözcükler: Karaciğer, primer silyum, fibrozis, kolanjiosit, kısmi epitel-mezenkimal değişim, immunohistokimya, portal damar, tiyoasetamid

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List Of Abbreviations

A-HSC	Activated Hepatic Stellate Cell
ALD	Alcoholic Liver Disease
ARL13B	ADP- Ribosylation Factor Like Protein 13B
BDL	Bile Duct Ligation
BEC	Biliary Epithelial Cell
DDC	3,5-diethoxycarbonyl-1,4-dihydrocollidine
DR	Ductular Reaction
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor
EMT	Epithelial to Mesenchymal Transition
EV	Extracellular Vesicle
FABP4	Fatty Acid Binding Protein 4
FGF	Fibroblast Growth Factor
GPCR	G Protein-Coupled Receptor
HC	Hepatocyte
HCC	Hepatocellular Carcinoma
HFD	High Fat Diet
HGF	Hepatocyte Growth Factor
HHIP	Hedgehog Interacting Protein
HIF1α	Hypoxia Inducible Factor 1 Alpha
HNF4α	Hepatocyte Nuclear Factor 4 Alpha
HSC	Hepatic stellate Cell
IGFBP7	Insulin Growth Factor Binding Protein 7
IHC	Immunohistochemistry
LPC	Liver Progenitor Cell
LSEC	Liver Sinusoidal Endothelial Cell
LYVE1	Lymphatic Vessel Endothelial Hyaluronan Receptor 1
MAPK	Mitogen-Activated Protein Kinase
MET	Mesenchymal to Epithelial Transition
MF	Myofibroblast
MMP	Matrix Metalloprotease
mTORC	Mammalian Target of Rapamycin Complex 1

NAFLD	Non-Alcoholic Fatty Liver Disease
NASH	Non-Alcoholic Steatohepatitis
OPN	Osteopontin
PBC	Primary Biliary Cholangitis
PCNT	Pericentrin
PDGFRA	Platelet-derived Growth Factor Receptor Alpha
PF	Portal Fibroblast
PHx	Partial Hepatectomy
PI3K	Phosphoinositide-3 Kinase
PKA	Protein Kinase A
PPAR	Peroxisome Proliferator-Activated Receptor
PSC	Primary Sclerosing Cholangitis
Q-HSC	Quiescent Hepatic Stellate Cell
RNF43	Ring Finger Protein 43
RSPO1	R-spondin 1
RTK	Receptor Tyrosine Kinase
SMO	Smoothed, Frizzled Class Receptor
SOX9	SRY-Box Transcription Factor 9
TAA	Thioacetamide
TGFβ	Transforming Growth Factor Beta
TIMP	Timp Metalloprotease Inhibitor
TNFα	Tumor Necrosis Factor Alpha
VEGFA	Vascular Epithelial Growth Factor A
WD	Western Diet
YAP	Yes Associated Transcriptional Regulator
ZEB1	Zinc Finger E-Box Binding Protein
ZNRF3	Zinc and Ring Finger 3

1. INTRODUCTION

1.1 Liver Physiology

The liver is one of the multitasking organs of the gastrointestinal tract. It performs diverse functions in order to maintain homeostasis (Ben-Moshe & Itzkovitz, 2019). The liver can be accepted as a glucostat of the body since it adjusts glucose levels according to fasting and refeeding states. During fasting, it can release glucose to the body whereas, it stores glucose in the form of glycogen under refeeding state (Ben-Moshe & Itzkovitz, 2019; Jungermann, 1986). One of the important functions of the liver is the synthesis of bile acids. After the production of bile acids, they are accumulated into the bile duct and transferred to the intestine for cholesterol and lipid catabolism (Staels & Fonseca, 2009). The liver has a capacity to perform drug detoxification to protect the body from some toxins and chemicals (Ben-Moshe & Itzkovitz, 2019). In addition, it takes roles in the urea metabolism to eliminate nitrogenous waste from the body (Trefts et al., 2017). The liver can perform the synthesis and release of hormones and proteins into the blood such as albumin, complement proteins, and clotting factors (Ben-Moshe & Itzkovitz, 2019).

Liver tissue is consisted of parenchymal and non-parenchymal cell types. Parenchymal cells, hepatocytes, contribute to the majority of liver mass. Cholangiocytes, hepatic stellate cells, portal fibroblasts, liver endothelial cells, and Kupffer cells constitute nonparenchymal cell types in the liver (Ben-Moshe & Itzkovitz, 2019).

The liver is a heterogeneous organ. It is built up of repeating and anatomical subunits which are called lobules. Size of lobules changes between 0.5 mm and 1mm in diameter (Ben-Moshe & Itzkovitz, 2019). Lobules are hexagonal-shaped. Each hexagon is composed of central vein and portal triads. The central vein sits at the center of the hexagon whereas the portal triad which is composed of the hepatic artery, bile duct, and portal vein is located at each corner of the hexagon (Kalra et al., 2021). The hepatic artery takes oxygenated blood from the general circulation. Nutrient containing blood from the small intestine reaches the portal vein. This oxygenated and nutrient-containing blood flows from the portal triad to the central vein. Bile acids are synthesized around the central vein, drain into bile canaliculi and flow in the opposite direction to the blood to accumulate into the bile duct (Lautt et al., 2019).

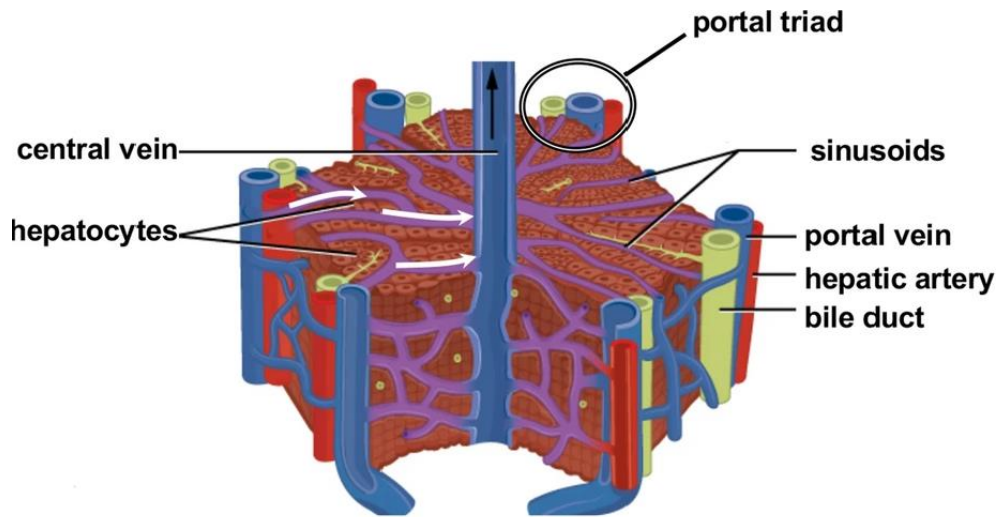


Figure 1: Anatomical structure of liver: Lobule (Wang et al., 2021).

1.2 Liver Zonation

The liver is made up of millions of lobules. Each lobule contains almost 12-15 layers of cells and is separated into three different zones (Droin et al., 2021). The closest zone to the central vein is called the pericentral zone, whereas the region adjacent to the portal triad is termed the periportal zone. The area between the pericentral and periportal zone is known as the midzone (Cunningham & Porat-Shliom, 2021). The direction of blood flow creates a gradient for oxygen, nutrient, and hormone level along the porto-central axis. Also, differences in the expression of signaling factors and morphogens exist through periportal to pericentral zones. This spatial expression of diverse factors leads to a graded microenvironment and this phenomenon is called liver zonation. Thanks to this zonation, various liver tasks can be performed by different layers of cells in the lobule (Ben-Moshe et al., 2021; Gebhardt, 1992; Jungermann & Keitzmann, 1996).

Because of the high oxygen level around the periportal zone, energy demanded functions such as gluconeogenesis, lipid oxidation, ureagenesis, protein catabolism, cholesterol biosynthesis are allocated by cells at the periportal zone. Glycolysis, alcohol detoxification, and lipogenesis are required zero or low levels of oxygen, so cells at the pericentral region have a capacity to fulfill these hypoxia-related tasks (Ben-Moshe & Itzkovitz, 2019).

Along the pericentral to the periportal axis, Wnt and Hedgehog pathways are highly complementary to each other to balance zonation. Components of Wnt pathways are distributed at the pericentral zone whereas the Hedgehog pathway is periportalized. Antagonists of the Wnt pathway show high expression around the periportal zone and repression of the Hedgehog pathway is observed at the pericentral zone by its inhibitors (Kolbe et al., 2019). In addition,

the Ras signaling pathway shapes activities at the periportal zone (Halpern et al., 2017). Generally, cells at the midzone are accepted as transitioning cells and specialized in sequential tasks between periportal and pericentral areas. For instance, bile acids are produced from the pericentral zone and transferred into the bile duct at the periportal zone. During its overall biosynthesis and flow processes, several enzymes are found at the midzone (Halpern et al., 2017; de Aguiar Vallim et al., 2013).

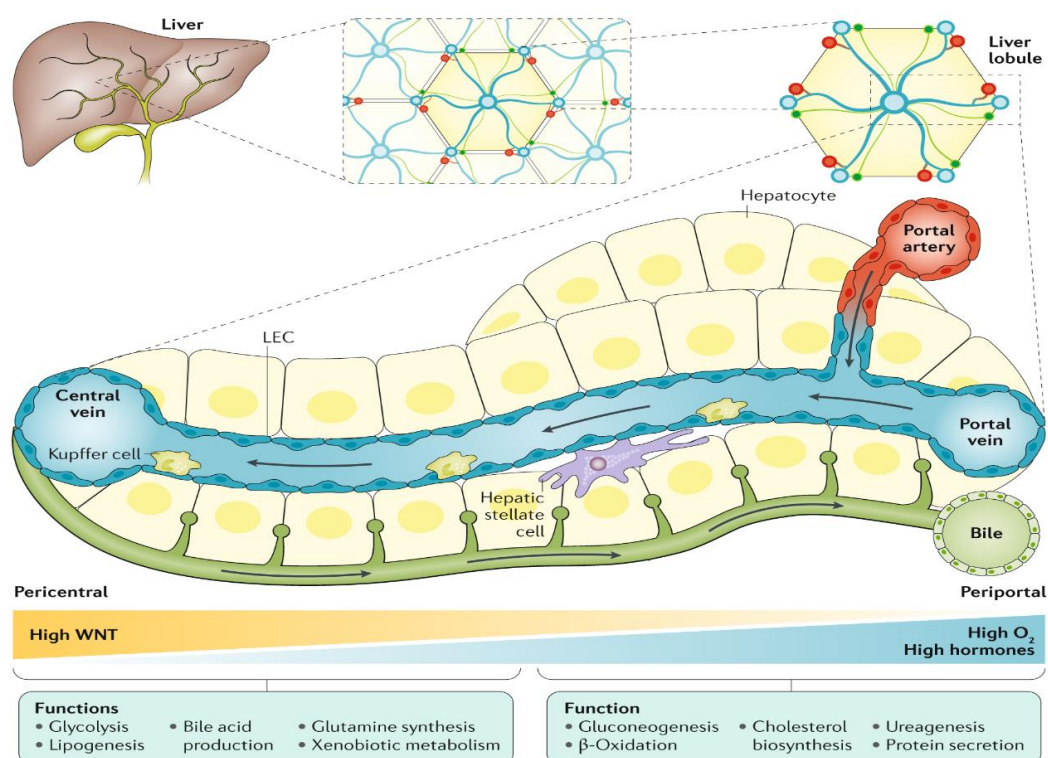


Figure 2: Liver Zonation. It shows lobule, pericentral, and periportal regions and cell types around these regions (Ben-Moshe & Itzkovitz, 2019).

Parenchymal cells, hepatocytes, show spatial expression profiles to accomplish diverse liver functions according to their region. Pericentral hepatocytes mainly express Glul, Cyp2e1, Axin2, Cyp1a2, Rnf43, Znfr3, Lgr5 whereas Ass1, Alb, Cyp2f2, E-cadherin, Arg1 are markers for periportal hepatocytes. Midzone hepatocytes are associated with expressions of Hamp, Igfbp2, Mup3, and Cy8b1 (Halpern et al., 2017, Valle-Encinas & Dale, 2020).

Non-parenchymal cells also are key to show liver zonation. Liver sinusoidal endothelial cells (LSECs) are one of the non-parenchymal cell types that develop spatial zonation. LSECs at the pericentral zone express Wnt9b, Wnt2, Wnt11, Fabp4, and Rspo3. LSECs at this zone create a niche for the pericentral hepatocytes to activate the Wnt pathway by secreting Wnt ligands. On the other hand, periportal LSECs are able to express Dll4, Efnb2, and Ltbp4. In the LSECs located at the midzone, Ecm1 and Pcdhgc5 expressions are mainly observed (Halpern et al., 2018) (Valle-Encinas & Dale, 2020).

Another non-parenchymal cell type, hepatic stellate cells (HSCs) maintain spatial zonation as LSECs. Pericentral HSCs have expression mainly for Adamstl2 which colocalizes with markers of pericentral hepatocyte and LSECs. Ngfr expression is limited into periportal HSCs (Dobie et al., 2019).

1.3 Liver Cell Types

As mentioned previously, the liver is consisted of hepatocytes, cholangiocytes, hepatic stellate cells, endothelial cells, sinusoidal endothelial cells, portal fibroblasts, myofibroblasts, progenitor cells (stem cells), Kupffer cells, and immune cells (Ding et al., 2016). In this section, every cell type will be examined one by one.

1.3.1 Hepatocytes

Hepatocytes which constitute 80% of liver mass are the primary cell type of the liver. Their size changes around 20um to 30um and they have a polygonal shape. LSECs are located adjacent to basal surfaces of hepatocytes where microvilli of hepatocytes are extending through the Space of Disse. With the help of microvilli, hepatocytes are capable of taking nutrients, oxygen from the blood and releasing secreted hormones, proteins into the blood via LSECs (Qin & Crawford, 2018). Generally, more than 50% of hepatocytes are polyploid which occurs due to failure in cytokinesis. During liver regeneration, polyploid hepatocytes proliferate more than diploid hepatocytes. However, by the proliferation of these hepatocytes, ploidy-reduced hepatocytes namely diploid hepatocytes are obtained. Division of diploid hepatocytes gives rise to re-polyploidized hepatocytes. This ploidy plasticity contributes to liver regeneration by both polyploid and diploid hepatocytes (Matsumoto et al., 2020.)

Hepatocytes can perform a high number of liver tasks. Pericentral hepatocytes synthesize bile acids and release them from the apical surface to bile canaliculi (Tabibian & LaRusso, 2014).

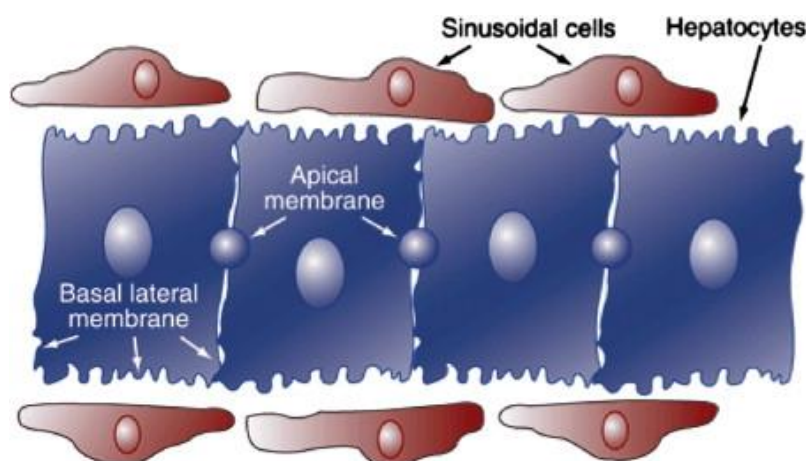


Figure 3: Hepatocytes possess two different membrane surfaces: Apical and basolateral surfaces (Tabibian & LaRusso, 2014).

In addition to bile acids, hepatocytes can secrete distinct blood proteins such as albumin, transferrin, fibrinogen, alpha-fetoprotein. Also, they can store vitamin A. Lipid and glucose metabolisms are maintained mainly by hepatocytes. According to fasting, refeeding states, hepatocytes can perform glycolysis and gluconeogenesis. Under homeostasis, they can synthesize, store, break down and release fatty acids. Dietary lipids are taken up by hepatocytes in the form of chylomicron remnants. They are stored in hepatocytes as lipid droplets. After their catabolism, they are released into circulation very-low-density lipoprotein (VLDL) particles (Schulze et al., 2019). However, because of high dietary intake into the body, an excessive amount of lipids is accumulated in hepatocytes and adipose tissue. This high level of lipid in the liver causes various diseases such as steatosis (Devi, 2010).

Hepatocyte self-renewing under both homeostasis and injury is one of the contradictory topics in the liver. According to studies, diploid Axin2⁺ hepatocytes show stem cell property to maintain Axin2⁺ hepatocyte pool under homeostasis. Axin2⁺ hepatocytes are located at the pericentral zone. By proliferation, these Axin2⁺ cells maintain the majority of hepatocytes into the pericentral region and expand through the midzone, but they cannot replace with the periportal hepatocytes which are Axin2⁻. The reason behind this is explained by the Wnt ligand secreting endothelial cells which are located in pericentral area and midzone. Axin2 is a component of the Wnt signaling pathway. Endothelial cells secreting Wnt ligands constitute a niche for Axin2⁺ hepatocytes. Because of the absence of these endothelial cells around the periportal zone, Axin2⁺ hepatocytes cannot expand and proliferate there (Wang et al., 2015). However, in another study, they show that Axin2⁺ hepatocytes show limited proliferation and cannot be present beyond the pericentral region under homeostasis. Hepatocytes in all zones have equal participation for maintenance of the hepatocyte population during homeostasis (Sun et al., 2020). As opposed to these studies, another research demonstrates that hepatocytes located in the midzone are the main source for overall hepatocyte pool under homeostasis (Wei et al., 2021). The same research is performed under different injury conditions by another group. During different injuries affecting different zones, hepatocytes at the midzone always reveal greater contribution for regeneration of hepatocytes at other zones because of the close proximity of the midzone to other zones (He et al., 2021).

The effect of liver damage on hepatocytes is dependent on damage level. If damage is low, hepatocytes can maintain their population by proliferating from their existing counterparts. However, hepatocytes cannot self-renew due to the remaining low number of hepatocytes under high damage. Therefore, cholangiocytes differentiate into hepatocytes according to damage type (Deng et al., 2018).

During liver injury and regeneration, hepatocytes can communicate with other liver cell types in a direct way or through secreting vesicles. Kupffer cells secrete IL-6, TNF, TGFB1 and with these immune morphogens, they can interact with hepatocytes (Michalopoulos & Bhushan, 2020). At the same time, hepatocytes start secreting extracellular vesicles (EVs) for macrophages. These EVs contain CXCL10 which leads to macrophage migration to the injury area. In addition to macrophages, hepatocytes release evs carrying miR-128-3p to contact with hepatic stellate cells (Ipsen & Tveden-Nyborg, 2021; Povero et al., 2015).

1.3.2 Cholangiocytes

Cholangiocytes in other words biliary epithelial cells (BECs) are another epithelial cell type apart from hepatocytes. They constitute almost 3-5% of all liver cells. They are located in periportal region by lining the intrahepatic bile duct and Canal of Hering. The main function of cholangiocytes is the modification of bile acids under homeostasis (Tulasi et al., 2021). This modification is performed by conjugation of taurine and glycine to bile acids via ion channels of cholangiocytes and bile salts are obtained to be transferred into the bile duct (Pozniak et al., 2017). Cholangiocytes are a very heterogeneous population in terms of size, function, location, Yap and Sox9 expression. Generally, small cholangiocytes sit around small ductules, and small intrahepatic bile ducts whereas large ones reside around large intrahepatic and extrahepatic bile ducts. (Tulasi et al., 2021). As another significant aspect of cholangiocytes, they possess primary cilium which is a tubular organelle extending from the apical membrane of BECs. Every cholangiocyte has one primary cilium (Tabibian et al., 2013).

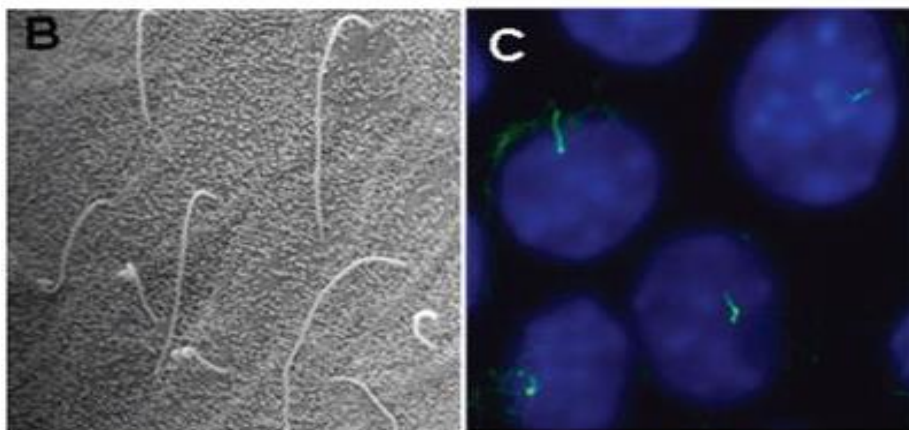


Figure 4: Primary cilia extruding from cholangiocytes demonstrating via B) Scanning electron microscopy and C) Fluorescence microscopy (Tabibian et al., 2013).

Cholangiocytes are one of the most responsive cell types against an injury in the liver. As a main response, they start to proliferate and expand into the periportal region. This physiologic event is called ductular reaction (DR) (Sato et al., 2018). Three different types of DR are present

according to the level of liver damage. Type I DR is explained as the self-renewal of cholangiocytes. This means under a low level of injury, cholangiocytes proliferate from pre-existing ones. Type II DR is observed by hepatocyte metaplasia. Due to severe injury, periportal hepatocytes begin differentiating into cholangiocytes and extending towards the pericentral zone. Type II DR also can be seen in hypoxic areas in the midzone. Type III DR happens against most severe injuries. Liver progenitor cells activate and differentiate into cholangiocytes (Desmet, 2011).

During the proliferation of cholangiocytes after damage, some mesenchymal populations around cholangiocytes control this proliferation. In this regulation, the distance between cholangiocytes and mesenchymal cells is the fundamental determinant. During injury, cholangiocytes are proliferating and the distance between these two cell types is increasing gradually. Thanks to this distance, secreted factors from mesenchymal cells contribute to the proliferation of cholangiocytes. However, during regeneration, both mesenchymal cells and BECs are under growth, so they begin to approach each other. The close proximity leads to contact between them and stops the proliferation of BECs. This control system proves that surrounding mesenchymal cells provide a niche for BEC proliferation (Cordero-Espinoza et al., 2021).

As mentioned in hepatocytes, cholangiocytes can interact with other cell types and their own cell type. Under disease conditions, cholangiocytes can secrete exosomes via their primary cilia and control the proliferation of other cholangiocytes. Thus, released exosomes in the extracellular matrix are increasing depending on the injury (Masyuk et al., 2010). Moreover, cholangiocyte-derived vesicles containing lncRNA H19 are taken up by hepatic stellate cells. This lncRNA promotes proliferation, activation, and migration of HSCs through the injury site. This communication directly affects the severity of liver damage (Liu et al., 2019).

1.3.3 Hepatic Stellate Cells (HSCs)

Hepatic stellate cells are the main cell type of the non-parenchymal cells. They constitute almost 5-8% of overall liver cells. They possess stellate shape as seen in the Figure 5 (Weiskirchen & Tacke, 2014; Geerts, 2001). They are distributed periportal, pericentral, and parenchymal regions of the liver by locating Space of Disse where the defined area between LSECs and hepatocytes (Kisseleva & Brenner, 2020). They show mesenchymal features due to their mesodermal origin (Lua et al., 2016; Asahina et al., 2008). Under normal physiological conditions, HSCs are in a quiescent and non-proliferative state. In that state, HSCs are called pericytes or qHSCs (Kisseleva & Brenner, 2020; Iredale, 2007; Geerts, 2001; Senoo et al.,

2007). They contain vitamin A as retinyl ester form which is important for the maintenance of quiescence of HSCs (Lua et al., 2016; Friedman, 2008; Schuppan & Kim, 2013). In addition to vitamin A storage, they can produce different components of extracellular matrix and plasminogen activation system, and erythropoietin (Weiskirchen & Tacke, 2014; Mederacke et al., 2013; Gressner & Weiskirchen, 2006; Tacke & Weiskirchen, 2012).

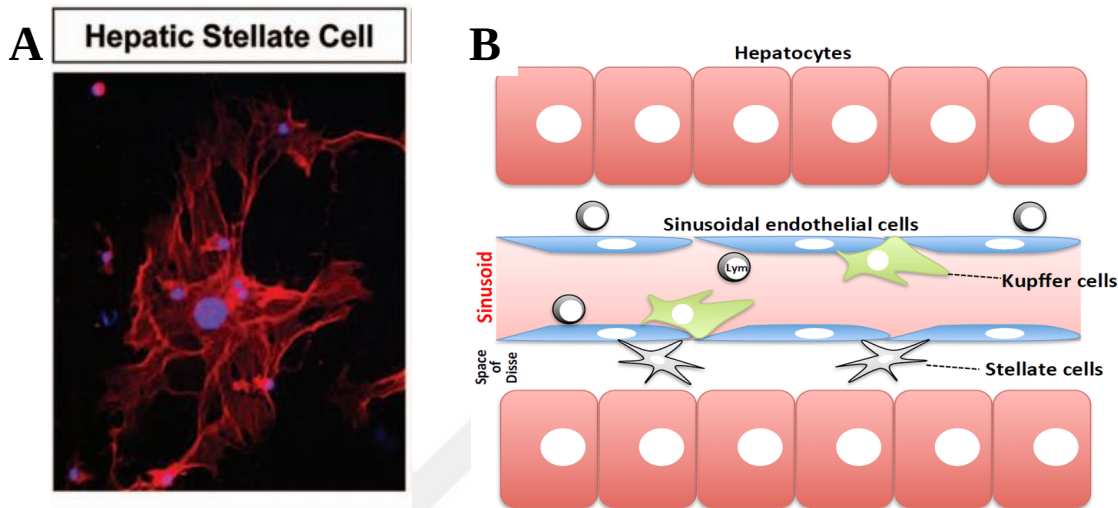


Figure 5: A) Hepatic Stellate Cells in the liver by immunofluorescence staining (Schachtrup et al., 2011). B) Illustration for Space of Disse (Tsutsui & Nishiguchi, 2014).

HSCs are the primary contributors of liver damage, mainly liver fibrosis. During fibrosis, pericytes downregulate or lose their vitamin A storage and become activated HSCs (aHSCs). This type of HSCs migrates to the injury area, proliferates, and transdifferentiates into myofibroblasts. Both aHSCs and myofibroblasts produce collagen and cause fibrosis progression, but myofibroblasts are accepted as a leading factor for collagen production (Kisseleva & Brenner, 2020; Iwaisako et al., 2014; Koyama et al., 2017).

There are numerous differences between pericytes and aHSCs. The most significant ones are the capability of vitamin A storage and collagen synthesis. In addition to these, PPAR γ expression is low in the aHSCs compared to pericytes (Kisseleva & Brenner, 2020; Fallowfield et al., 2007). Furthermore, distinct markers are preferred for these two different types of HSCs to identify them among all liver cell types due to their different gene expression profiles. The main proteins expressed in qHSCs are GFAP, synaptophysin, Lrat, and NGFR1 whereas markers for aHSCs are Vimentin, desmin, alpha-sma, and collagen1a1 (Nishio et al., 2019).

During distortion in the liver, HSCs can contact other HSCs. As seen in other cell types of liver, aHSCs can secrete vesicles that contain PDGFR α -SHP2. These vesicles are taken up by other HSCs which migrate to the damage site and get activated. Thus, scar in tissue progresses. (Kostallari et al., 2018).

Collagen producing cells and agents are eliminated so that fibrosis regression can occur. For this elimination, aHSCs are undergoing apoptosis and senescence generally. However, some populations of aHSCs are dedifferentiated into qHSCs or inactivated. These two processes are very similar to each other, but not identical. The key difference between them is epigenetic memory. Inactivated HSCs have epigenetic memory means that by any fibrogenic stimuli, these cells are rapidly activated and differentiated to myofibroblasts. However, qHSCs don't possess this memory (Kisseleva & Brenner, 2020; Kisseleva et al., 2012).

1.3.4 Portal Fibroblasts (PF)

Portal fibroblasts are another mesenchymal and nonparenchymal cell type of the liver. They are accumulated around the bile duct by surrounding cholangiocytes. Their role in the homeostatic liver is not well defined. They constitute a minority of liver cells. aHSCs and PFs have some common aspects due to their shared mesodermal origin. One of the common points is the expression of the same markers (Vimentin, Desmin, PDGFRA). However, PFs express specific markers as Elastin, NTPDase2, Thy1, and Fibulin (Lua et al., 2016; Uchio et al., 2002; Dranoff et al., 2002; Wells et al., 2004; Lemoine et al., 2015). In addition to markers, PFs are another contributor of myofibroblasts as aHSCs. Under scar, PFs are activated and differentiated into myofibroblasts to produce more collagen. Among differences between HSCs and PFs, vitamin A storage is the most considerable point. As explained previously, qHSCs can store vitamin A as droplets whereas PFs are not able to keep them (Dranoff & Wells, 2009; KNOOK et al., 1982; Friedman & Roll, 1987).

As mentioned above, Thy1+ PFs proliferate and differentiate into myofibroblasts due to damage in liver. Hence, they can contribute to the progression of fibrosis (Katsumata et al., 2017). On the contrary, they can inhibit fibrosis via constituting a niche for liver progenitor cells. Thy1+ PFs surround liver progenitor cells during injury. Thanks to the close proximity, secreted factor FGF7 from the PFs can reach the liver progenitor cells which proliferate and give a response to the damage. Decrease of FGF7 expression directly has negative effects on LPC response (Takase et al., 2013).

Another contribution of PFs to fibrosis is performed via communication with other cell types such as liver sinusoidal endothelial cells. PFs release vesicles which are taken by liver sinusoidal endothelial cells. These vesicles contain VEGFA which is important for LSEC angiogenesis increasing the fibrosis (Ipsen & Tveden-Nyborg, 2021; Lemoine et al., 2015).

1.3.5 Myofibroblasts (MFs)

The most minor population of mesenchymal cells is myofibroblasts. They are characterized by stellate shape as observed in HSCs. However, they don't have a capability to store vitamin A unlike HSCs (Iwaisako et al., 2012; Eyden, 2007). They don't exist in the homeostatic liver. After the injury, alpha-SMA expressing MFs are generated by different sources. Bone marrow-derived mesenchymal cells are one of the contributors to MFs. When damage happens, they migrate to the liver and constitute a low percent of myofibroblasts (Iwaisako et al., 2014; Kisseleva et al., 2006; Kisseleva et al., 2011; Scholten et al., 2011). The main myofibroblast producer cells are HSCs and PFs as mentioned before. However, the source of myofibroblasts changes according to the type of injury. If the pericentral region is mainly affected by the damage, the majority of MFs are derived from HSCs. Nonetheless, when the injury site is located at the periportal zone, mainly PFs are generating MFs (Iwaisako et al., 2014).

1.3.6 Liver Progenitor Cells (LPCs)

One of the controversial topics for the liver is the liver progenitor/oval cells/stem cells, evidently. According to several research, additional cell type is absent for progenitor or stem cells in the liver. Due to the trans-differentiation of hepatocytes and cholangiocytes to each other under injury, they are accepted as bi-facultative stem cells (Michalopoulos & Khan, 2015). However, several studies show that liver progenitor cells are arising under severe liver injury when enough cholangiocytes and hepatocytes for differentiation or self-renewal don't exist (Lin et al., 2017). LPCs are not found in the homeostatic liver (So et al., 2020; Duncan et al., 2009; Miyajima et al., 2014). Under injury, they are proliferating at the Hering Canal, a small area between the bile duct and hepatocytes as seen in Figure 6 (So et al., 2020; Stanger, 2015). After proliferation, oval cells differentiate into hepatocytes or cholangiocytes according to injury type and morphogenic factors coming from their surrounding mesenchymal niche (Huch et al., 2013). When LPCs appear in damaged liver, they start to express both hepatocyte and cholangiocyte markers (So et al., 2020). However, the most important marker for this cell type is the *Lgr5* which is the main stem cell marker for almost all stem cells in the gastrointestinal tract (Huch et al., 2013).

Origin of LPC is not characterized well. However, according to studies, HSCs are subjected as a source of LPCs. During regeneration of the liver, a specific HSC population gives rise to liver progenitor cells as intermediate cell type which expresses *Epcam*, *K19*, and *Vimentin* at the same time. After that, liver progenitor cells generate hepatocytes or cholangiocytes dependent

on surrounding mesenchymal factors and their microenvironment. Nevertheless, this HSC population is so small and the majority of HSCs constitute a mesenchymal niche of LPCs for hepatocyte/cholangiocyte fate (Kordes et al., 2014). On the contrary, another research shows that HSCs, MFs, and PFs are of mesodermal origin whereas LPCs, HCs, and BECs derive from endoderm. Hence, LPCs, HCs, and BECs cannot be derivatives of HSCs (Lua et al., 2014).

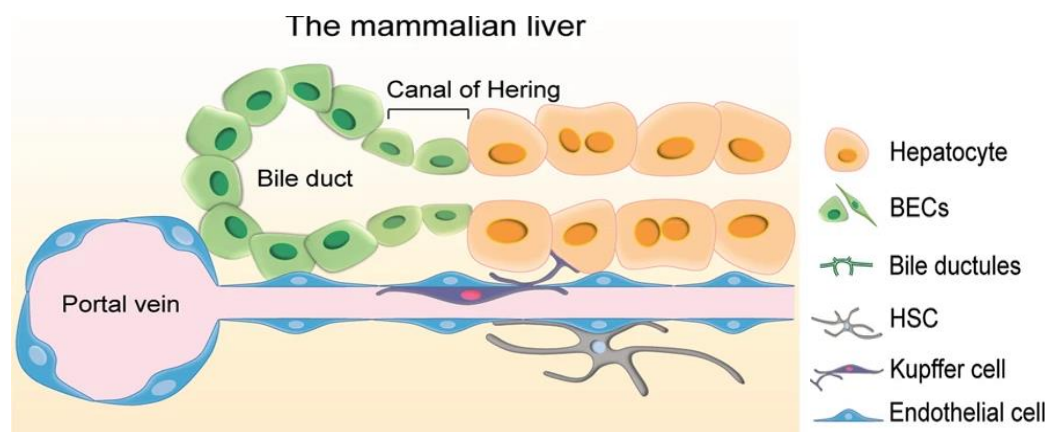


Figure 6: Different cell types in the liver and the exact position of the Canal of Hering (So et al., 2020).

1.3.7 Liver Endothelial Cells and Liver Sinusoidal Endothelial Cells

Liver endothelial cells constitute a major population of non-parenchymal cells. Their task is to provide maintenance of liver homeostasis (Sun & Harris, 2020). Liver sinusoidal endothelial cells (LSECs) are a special type of LECs. They are located between blood cells and hepatocytes/HSCs as an interface role. They constitute 15-20% of all liver cells. They don't have a basement membrane which distinguishes LSECs from other endothelial cell types. This feature makes LSECs the most permeable ECs in the human body, so their endocytosis capacity is high (Poisson et al., 2017; Maslak et al., 2015). Another important point of LSECs is the ability to control qHSCs. Under homeostasis, LSECs have a role on the continence of HSC quiescence. However, capillarization occurs in LSECs due to distortion in the liver. Capillarization is defined as the formation of basement membrane and loss of fenestration completely in LSECs. By capillarization, LSECs lose their identity and ability to maintain HSC quiescence, so HSCs can be activated. Thus, LSECs are accepted as gatekeepers of liver fibrosis by this task (Lee et al., 2018; Iwakiri et al., 2014; DeLeve et al., 2008). In addition to capillarization, angiogenesis which is the formation of new blood vessels happens for all LECs as a contributory factor to fibrosis progression. (Poisson et al., 2017).

1.3.8 Kupffer Cells and Other Immune Cells

Although the liver is accepted as a metabolic organ due to its metabolic tasks, it has various immune cell repertoires. These immune cells are Kupffer cells, macrophages, monocytes, dendritic cells, T and B cells, NK cells, neutrophils, eosinophils, which constitute part of the non-parenchymal cell population. They are responsible for the maintenance of liver homeostasis with the help of different recognition systems when foreign molecules such as bacteria, viruses occupy the liver (Robinson et al., 2016). Among immune cells in the liver, Kupffer cells are liver specific. They are specialized resident macrophages and make up almost 80% of the whole macrophage populations of the liver (Roy-Chowdhury & Roy-Chowdhury, 2010). Kupffer cells take roles in the elimination of dead cells, phagocytosis, and inhibition of pathogen spreading through general circulation from the liver (Campana et al., 2021; Blériot & Ginhoux, 2019; Sierro et al., 2017).

As other cell types in the liver, immune cells are also responding to liver damage. They cause inflammation and increase the secretion of some cytokines and chemokines against fibrosis. For instance, macrophages and dendritic cells secrete HMGB1 which provides migration of other immune cells into a damaged area (Campana et al., 2021; Campana et al., 2009; Dumitriu et al., 2006; Schiraldi et al., 2012; Yang et al., 2011). Also, among cytokines secreted by macrophages IL-6 is important for liver regeneration by increasing hepatocyte proliferation (Campana et al., 2021; Zhou et al., 2017).

1.4 Liver Fibrosis

Fibrosis is a kind of healing response to injury in the liver. It aims at the regeneration of the liver and wound healing for acute diseases which occur rapidly and last for a short time. Thus, fibrosis happens in a fast process. However, the several times occurrence of injury in the liver leads to chronic diseases and uncontrollable fibrosis. At that point, fibrosis acts in an opposite way of regeneration and distorts the whole liver structure and function. Progression of fibrosis causes a more severe type of liver disease such as cirrhosis and hepatocellular carcinoma as a final stage of fibrosis (Acharya et al., 2021; Iredale et al., 2013).

There are several causes for fibrosis in the liver. Different genetic defects and mutations in highly expressed, specific genes in the liver promote fibrosis. Generally, genetic problems related to fibrosis are seen in people during their childhood (Acharya et al., 2021; Pinto, 2015). The big impact on fibrosis belongs to hepatic viruses such as hepatitis A, B, C, D. These viruses damage hepatocytes directly causing fibrosis (Acharya et al., 2021; Zuckerman, 1996). Another

contributor to liver fibrosis is non-alcoholic steatosis (NAFLD). It's a metabolic disease seen in obese patients as a side effect on the liver and damages almost all cell types in the liver (Acharya et al., 2021; Rosselli et al., 2014). Last but not least, high-level alcohol consumption results in alcoholic liver disease (ALD) and causes fibrosis in the liver (Acharya et al., 2021; Stickel et al., 2017). These listed causes for fibrosis can be categorized as reasons for hepatotoxic injury because all of them mainly affect hepatocytes and impair their functions. The second category for the reason of fibrosis is cholestasis which is explained by obstruction of bile acids flow to the bile duct and restriction of bile acid production. Generally, cholestasis caused fibrosis is seen in primary/secondary biliary cholangitis (PBC), primary sclerosing cholangitis (PSC), and biliary atresia which have negative effects on factors on periportal region (Kisseleva & Brenner, 2020; Bataller & Brenner, 2005).

Fibrosis affects all cell types, functions, and subunits of the liver. The main indicator of fibrosis is the high accumulation of ECM components such as collagen type I and III which are crosslinked (Kisseleva & Brenner, 2020; Friedman, 2003). The balance between extracellular matrix metalloproteases (MMPs) and their inhibitors (TIMPs) are significant for scar formation because MMPs are degrading ECM whereas their inhibitors cause accumulation of them. Therefore, levels of MMPs are falling and TIMPs are increasing during fibrosis (Campana et al., 2021; Iredale, 1997). Collagen accumulation is performed by the generation of myofibroblasts from the differentiation of HSCs and PFs. At the same time, almost all cell types in the liver mainly hepatocytes and cholangiocytes proliferate. This proliferation is called hyperplasia (Campana et al., 2021). Extreme collagen accumulation and hyperplasia lead to narrowing of blood vessels, so increasing the pressure in the portal venous system which is called portal hypertension. Because of this narrowing, hypoxia is observed by increasing expression of Hif1 α and angiogenesis occurs to form new blood vessels and carry enough oxygen to cells (Desmet, 2011). In addition to that, the immune system gets activated during fibrosis. Inflammation is observed, immune cells begin to migrate to the injury site and secrete cytokine and chemokines (Kisseleva & Brenner, 2020; Bataller & Brenner, 2005). As a consequence, necrosis, fibrotic septa, fibrous scar formation, and hypertrophy which is the enlargement of an organ appear in the liver structurally (Suk, 2015). Metabolically, glucose and lipid metabolisms change completely. Lipid accumulation increases whereas glucose metabolism drops. Hepatocytes start to accumulate lipids and become ballooned by swelling (Lackner et al., 2008). HSCs lose their vitamin A storage capability due to activation of HSCs. This activation is resulted in by capillarization of LSECs (Kisseleva & Brenner, 2020; Shetty et al., 2018). According to the severity of fibrosis, differentiation of cholangiocytes, hepatocytes

or liver progenitor cells occurs (Deng et al., 2018) (Huch et al., 2013). In addition to these outcomes, different cell types start to communicate with each other by increasing secreted factors and exosomes to progress or eliminate fibrosis (Szabo & Momen-Heravi, 2017).

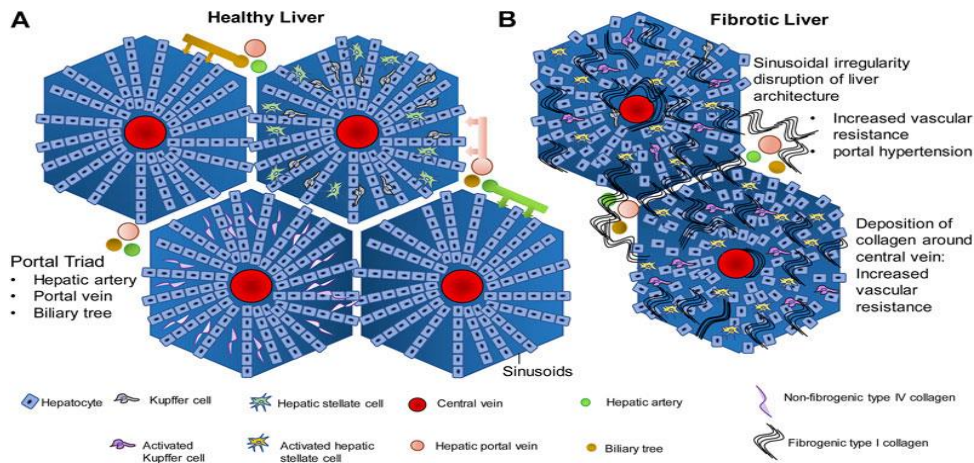


Figure 7: Structural comparison between healthy and fibrotic liver (Acharya et al., 2021).

1.4.1 Main Driver Pathways of the Liver Fibrosis

Liver is affected in many ways due to fibrosis as mentioned previously. Another important consequence of fibrosis in the liver is the changes in the activation of diverse signaling pathways such as Wnt signaling, Hedgehog signaling, Hippo signaling, Notch signaling, and TGF β signaling. These signaling pathways regulate the fibrosis progression and they are also affected by this progression.

1.4.1.1 Wnt Signaling Pathway

Wnt signaling is a significant pathway for liver fibrosis and regeneration as seen in the regeneration of other gastrointestinal organs. Activation of this pathway occurs by binding of Wnt ligands to the Frizzled receptor and migration of β -catenin into the nucleus to promote transcription of several genes. In the absence of Wnt ligands, β -catenin is degraded by a destruction complex including Axin2, Ck1, GSK3 β , and APC (Wild et al., 2020). In homeostasis, Wnt ligands are at the basal level and pericentrally located. Nonetheless, Wnt signaling is activated and expression of Wnt ligands are increased in fibrosis. Generally, Wnt signaling directly affects the proliferation of hepatocytes in all zones whereas cholangiocytes are not affected by Wnt signaling (Planas-Paz et al., 2019).

Inhibitors of Wnt signaling also take significant roles in liver homeostasis and fibrosis. Znf3 and Rnf43 are well-known inhibitors of Wnt signaling. They are ubiquitin ligases that clear Frizzled and other cell surface receptors of Wnt signaling, so they inhibit Wnt signaling. Under their absence in the homeostatic liver, Wnt signaling is constitutively active. This leads to the

formation of hepatocellular carcinoma, extreme hepatocyte proliferation, and impairment of liver zonation by expression of pericentral HC markers in the periportal HCs (Sun et al., 2021). Lack of these inhibitors in the fibrotic liver results in HCC, high hepatocyte proliferation, and decreased regenerative capacity despite of the constitutively active Wnt signaling. Since high HC proliferation and constitutively active Wnt signaling should provide high regenerative capacity to the liver to eliminate fibrosis. However, these proliferative hepatocytes are not undergoing maturation and only expanding. This limits their regenerative capacity and causes persistent fibrosis (Mastrogiovanni et al., 2020).

1.4.1.2 Hedgehog Signaling Pathway

Hedgehog is another important pathway affected in fibrosis. This pathway is initiated by binding of Hedgehog ligands to the patched receptor. This binding causes inhibition of patch receptor and it cannot prevent translocation of SMO from cell membrane to primary cilium. Thanks to this inhibition, Gli proteins can migrate to the nucleus as affecting the transcription of some genes (Wild et al., 2020). Under homeostatic conditions, Hedgehog signaling is silent as other signaling pathways. However, under fibrotic conditions, it increases in hepatocytes, cholangiocytes, and hepatic stellate cells. It affects maintenance of qHSCs and hepatocyte/cholangiocyte proliferation. When stages of fibrosis are increasing, Hedgehog signaling increases, too (Zhu et al., 2020; Machado & Diehl, 2018; Sicklick et al., 2006). Also, its inhibitor, Hhip, is downregulated in myofibroblasts and LSECs to contribute to Hedgehog signaling (Omenetti et al., 2011; Choi et al., 2009; Witek et al., 2009; Yang et al., 2008).

1.4.1.3 Hippo Signaling Pathway

The Hippo pathway is one of the most responsive pathways to fibrosis. Activation of this pathway occurs via Mst1/Mst2 kinases activation which leads to activation of other kinases, Lats1/Lats2, and phosphorylation of Yap. Phosphorylated Yap is degraded by a destructive complex. However, inhibition of this pathway leads to migration of Yap to the nucleus and interaction of Yap with TEAD to promote transcription of some genes (Michalopoulos & Bhushan, 2020; Li et al., 2019). In the homeostatic liver, Hippo pathway is important for bile duct development because its absence results in distortion in the bile duct (Zhu et al., 2020; Zhang et al., 2010). Generally, Yap expression is high in cholangiocytes compared to other cell types and decreasing gradient for the Yap expression exists in the portal to the central axis. Therefore, the Hippo pathway displays opposite zonation with the Wnt signaling pathway (Zhu et al., 2020; Yimlamai et al., 2014; Fitamant et al., 2015). For the fibrotic liver, Hippo pathway

leads to complicated results. Another component of the Hippo pathway, Taz, is overexpressed in fibrotic hepatocytes. This Taz action makes fibrosis severe by increasing injury and inflammation via more Ihh which is one of the Hedgehog ligands and at the same time target of Taz (Zhu et al., 2020; Wang et al., 2016). In addition to this, increasing Yap expression regulates HSC activation (Zhu et al., 2020; Mannaerts et al., 2015). On the other hand, Hippo pathway is important for cholangiocyte expansion during injury and contributes to liver regeneration (Planas-Paz et al., 2019).

1.4.1.4 Notch Signaling Pathway

Notch signaling plays important roles for the liver. It requires contact with neighboring cells for its activation. Notch is a cell surface receptor and when it binds to its target Jagged on another cell membrane, its intracellular domain is cleaved and migrates through the nucleus as a transcription factor (Kopan, 2012). This pathway regulates intrahepatic bile duct formation as the Hippo pathway, so it's located generally in the periportal region of the liver. However, it's quiescent under homeostasis conditions similar to other signaling pathways (Zhu et al., 2020; Zong & Stanger, 2012). Notch signaling is activated under fibrosis by increasing in hepatocytes. It leads to lipid accumulation and lipogenesis in hepatocytes under steatosis with the help of activation of mTORC1 which is the target of the Notch pathway (Zhu et al., 2020; Pajvani et al., 2011; Pajvani et al., 2013). In addition to that, HSC activation is controlled by Notch signaling. If Notch signaling is impaired, Hedgehog signaling is also inhibited because of crosstalk between them. This leads to less formation of myofibroblasts from aHSCs, so liver fibrosis is eased (Zhu et al., 2020; Xie et al., 2013).

1.4.1.5 TGF β Signaling Pathway

Tgf β signaling pathway is another pathway taking roles in both injury and development of the liver. In this pathway, Tgf β superfamily ligands such as BMPs, Activin, Tgf β s, Nodal, GDFs bind their cell membrane receptor. With this binding, Smads are activated by phosphorylation and enter the nucleus for transcription of some specific genes. However, Tgf β signaling which is independent of Smads exists. These are dependent on Erk, MAPK pathways (Massagué, 2012). Tgf β signaling is resting in homeostatic liver, but its expression is upregulated under injury conditions (Michalopoulos & Bhushan, 2020; Webber et al., 1993). Mainly, it's produced in hepatocytes, hepatic stellate cells, and Kupffer cells (Michalopoulos & Bhushan, 2020; Ferdous et al., 2007). It facilitates periportal hepatocyte proliferation and expansion of this proliferation through the pericentral area (Michalopoulos & Bhushan, 2020; Jirtle et al., 1991).

During liver regeneration Tgf β signaling monitors gene expression profiles of hepatocytes and EMT (Michalopoulos & Bhushan, 2020; Oh et al., 2018). Also, it regulates HSC activation in a Smad-dependent manner (Kisseleva & Brenner, 2020; Dooley et al., 2001).

1.4.2 EMT & MET

Epithelial to mesenchymal transition (EMT) is a kind of cellular reprogramming causing the gain of the new identity of a cell. Epithelial cells lose their epithelial character by downregulation of cell junctions and gain mesenchymal features. There are well-known indicators for EMT. First of all, cells that show EMT detach from the epithelial-derived layer and start to migrate towards the mesenchymal regions. Their original shape changes to a spindle which is a fibroblastic shape (Fabris et al., 2016; Fabris & Strazzabosco, 2011). In addition to that, cells with EMT downregulate epithelial markers such as E-cadherin, β -catenin, and upregulate mesenchymal markers including α -SMA, Vimentin, N-Cadherin, S100A4 (Xie & Diehl, 2013; Zeisberg & Neilson, 2009).

There are three types of EMT. Type 1 EMT is observed during development and organogenesis. Type 2 EMT is happening during wound healing, regeneration, and fibrosis. Type 3 EMT is one of the important indicators of cancer. Type 1 and Type 3 EMTs are more similar to each other compared to Type 2 in terms of their process (Choi & Diehl, 2009; Kalluri & Weinberg, 2009).

The generation of EMT is controlled by different transcription factors which are important during embryogenesis. These are Snail, Snail2, Twist1-2, and Zeb1/2 (Fabris et al., 2016; Hanahan & Weinberg, 2011; Craene & Berx, 2013). The expression of these transcription factors is regulated by different signaling pathways that indirectly regulate the EMT process. The master regulatory pathway of EMT is Tgf β signaling. By Tgf β binding to its receptor and Smad activation, Snail, Snail2, and Twist are transcribed and facilitate expression of mesenchymal genes by blocking epithelial genes (Xie & Diehl, 2013; Kaimori et al., 2007; Zavadil & Böttinger, 2005). Also, Tgf β signaling affects EMT through a Smad independent way via MAPK and Pi3K pathways (Xie & Diehl, 2013; Xu et al., 2009). Another fundamental pathway for the EMT is the Wnt pathway. By β -catenin accumulation in the nucleus, Zeb1 and Twist are expressed, and by inhibition of GSK, Snail is stabilized (Fabris et al., 2016; Zhou et al., 2004). The Hedgehog pathway also regulates EMT by expression of Gli transcription factor which promotes transcription of Snail (Xie & Diehl, 2013; Li et al., 2012). Last but not least, the Notch pathway is another key pathway for EMT by expression of Snail (Fabris et al., 2016; Leong et al., 2007; Li et al., 2005). In addition to these main pathways, TNF α , hypoxia, cellular

stress, autophagy, and RTKs as EGF, FGF, HGF, PDGF, are other significant regulators of EMT (Fabris et al., 2016; Lamouille et al., 2014; Lindsey & Langhans, 2014).

EMT requires a process. Cells that are at the midst of the EMT process are called transitioning cells. This process is termed partial EMT. Cells in partial EMT express both mesenchymal and epithelial markers at the same time and show features of both mesenchymal and epithelial characteristics. Generally, partial EMT is observed during cancer and fibrosis (Choi & Diehl, 2009; Zavadil & Böttinger, 2005).

MET is a completely reverse process of EMT, so differentiation of mesenchymal cells to epithelial cells (Xie & Diehl, 2013). For liver fibrosis and regeneration, the balance between EMT and MET processes carries a big importance. If EMT dominates the MET, liver fibrosis occurs whereas if MET is favored to EMT, fibrosis lysis happens and regeneration is obtained (Choi & Diehl, 2009).

1.4.2.1 EMT in Cholangiocytes

One of the controversial topics for liver fibrosis is the occurrence of EMT in cholangiocytes. According to several studies, epithelial markers decrease and mesenchymal markers increase in cholangiocytes by showing EMT in the BDL liver fibrosis model. They showed that especially myofibroblasts contribute to this epithelial to mesenchymal differentiation in BECs by secreting Hedgehog ligands to cells undergoing EMT. In other words, myofibroblasts create an EMT niche for the BECs (Omenetti et al., 2008). Also, in another liver fibrosis model, NASH, cholangiocytes are exposed to EMT by regulation of Hedgehog signaling. (Syn et al., 2009) In addition to these studies, Tgfb stimulation in cholangiocytes leads to the occurrence of EMT by causing fibrosis (Rygiel et al., 2007). When the severity of fibrosis increases, Tgfb expression in the liver increases, too but the overlap of both epithelial and mesenchymal markers in the BECs decreases. Therefore, by increasing doses of Tgfb stimulation in BECs, epithelial markers are downregulated whereas mesenchymal markers are upregulated (Xiao et al., 2014).

Nevertheless, a few studies which deny the EMT in cholangiocytes in fibrotic liver exist. In BDL and CCl4 fibrosis models, no overlap for the mesenchymal and epithelial markers are identified in cholangiocytes (Scholten et al., 2010). Also, another liver fibrosis model, DDC, shows no clue for the co-expression of the mesenchymal and epithelial markers in the BECs. Therefore, they support that fibrosis is not causing EMT in cholangiocytes (Chu et al., 2011). The reason behind the discrepancy between these studies is based on the methods used to prove EMT. Generally, EMT supporter studies conduct immunohistochemistry by showing overlap

of mesenchymal and epithelial markers under fibrosis. However, in the studies against EMT, fibrotic transgenic animals that label cholangiocytes are used. By fluorescent staining of the liver with mesenchymal markers, they show no expression of mesenchymal markers in labeled BECs. Hence, there is no exact information about the occurrence of EMT in BECs. Other important factors for the discrepancy are the non-labeling of all cholangiocytes in the transgenic animal by Cre-LoxP system (success of system) and also usage of different markers for BECs in transgenic animals and IHC, so labeling different BEC populations (Xie & Diehl, 2013; Kretzschmar & Watt, 2012).

1.4.2.2 EMT in Hepatocytes

The same controversy which is discussed in EMT cholangiocytes is valid for hepatocytes. There are different proofs of whether EMT occurs in hepatocytes under fibrosis. According to a study, it was shown that Tgfb β stimulated hepatocytes lead to expression of mesenchymal markers although under normal conditions there is no expression. At the same time, their epithelial markers are downregulated. These EMT undergoing hepatocytes have more migration capacity compared to normal hepatocytes. Hence, it is believed that these changes are consequences of EMT (Xie & Diehl, 2013; Nitta et al., 2008). In addition to that, another group demonstrates that hepatitis C induced fibrosis results in EMT in hepatocytes (Xie & Diehl, 2013; Akkari et al., 2012). Nevertheless, some studies show pieces of evidence against EMT in hepatocytes under injury. One of them displays that in the CCl₄ induced fibrosis model, transgenically labeled hepatocytes don't express mesenchymal markers, so there is no EMT (Taura et al., 2016; Chu et al., 2011).

1.4.2.3 MET in Hepatic Stellate Cells

MET in hepatic stellate cells is a much more complicated issue compared to EMT in cholangiocytes or hepatocytes, since whether the origin of hepatic stellate cells is epithelial or mesenchymal is not known exactly. Generally, adult hepatic stellate cells are characterized as mesenchymal cells (Xie & Diehl, 2013; Michelotti et al., 2013; Yang et al., 2008). However, according to some sources they are classified as epithelial cells because of their predicted origins, mesothelial cells which express both epithelial and mesenchymal markers at the same time (Xie & Diehl, 2013; Mutsaers, 2004). Several studies show activated HSCs undergo MET by expressing epithelial markers rather than mesenchymal markers under fibrosis (Xie & Diehl, 2013; Michelotti et al., 2013). Contradictorily, another group shows that in transgenic animals labeling HSCs, CCl₄ induced fibrosis model demonstrates no MET in HSCs since there is no

expression of epithelial proteins in labeled HSCs. However, in that model HSCs and epithelial cells are in close proximity to each other (Scholten et al., 2010). By supporting this study, another research explains that HCS cannot undergo MET by contributing to LPCs, HCs, and BECs. The main reason behind this is the discrepancy between the origins of HSCs and LPCs. It was shown that HSCs are derived from mesoderm with the expression of *Mesp1* transcription factor specific to mesoderm whereas LPCs have an endodermal origin (Lua et al., 2014).

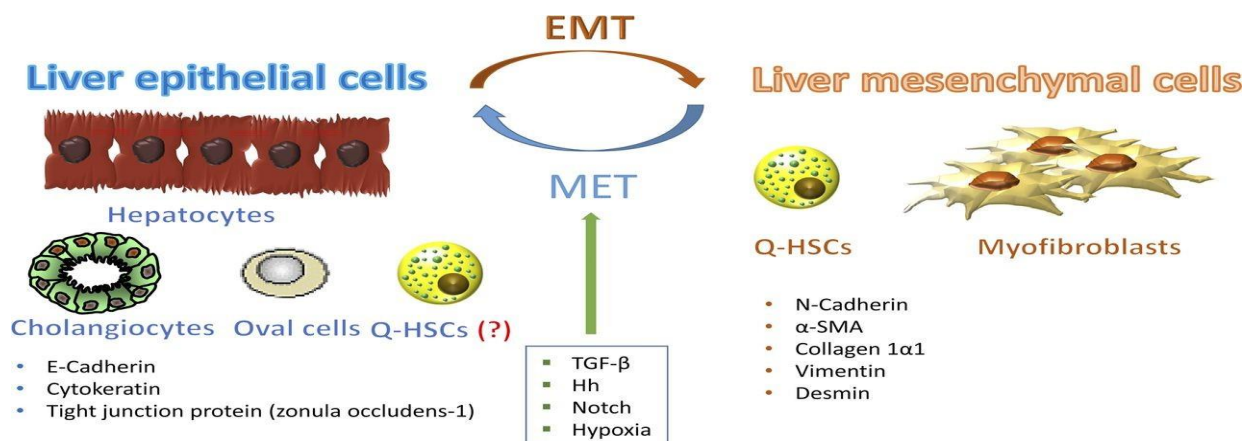


Figure 8: Summary of the EMT/MET processes in the hepatocytes, cholangiocytes, and HSCs (Xie & Diehl, 2013).

1.4.3 Different Fibrosis Models

Different fibrosis models are obtained with the help of the application of different chemicals and toxins such as TAA, CCl₄, DDC, and CDE to mice. In addition to these, some surgical methods help to develop fibrosis models such as BDL and PHx. All of these models display features of fibrosis mentioned previously. However, there are some major differences between these models in terms of the affected liver zone and disrupting mechanism of the liver (Delire et al., 2015).

1.4.3.1 TAA Induced Fibrosis Model

TAA which is the thioacetamide is a colorless, crystal organic compound. It leads to severe fibrosis in the liver by increasing doses and administration timeline. Generally, it distorts both the pericentral and periportal regions of the liver by the appearance of fibrotic septa through the portal-central and portal-portal axis. In the pericentral region, it induces oxidative stress by binding proteins and lipids and pericentral necrosis is formed. However, in the periportal area ductal hyperplasia and inflammation occur (Bao et al., 2021; Müller et al., 1988; Wallace et al., 2015). For the administration of TAA to mice, IP (intraperitoneal) injection or oral treatment is preferred with different doses and weeks. IP injection shows its fibrotic effects on the liver

quicker than oral administration. Therefore, IP injection is performed for almost 12 weeks and with a 150 mg/kg dose to promote severe fibrosis. On the other hand, oral administration is performed by the addition of TAA to the drinking water of animals. The occurrence of severe fibrosis takes 20-24 weeks with 300-600 mg/L by TAA (Wallace et al., 2015).

1.4.3.2 CCl₄ Induced Fibrosis Model

CCl₄ is another chemical to induce severe fibrosis in the liver. It is catalyzed by CP450 of hepatocytes resulting in highly radical metabolites. Due to these radicals, lipid and protein metabolisms are changed in hepatocytes and this situation leads to impairment in hepatocytes (Scholten et al., 2015; Weber et al., 2003). Generally, CCl₄ has negative effects on only pericentral hepatocytes by the development of centro-central septa compared to TAA. In addition to that, a short time after CCl₄ withdrawal, liver regeneration occurs quickly whereas liver regeneration takes much more time by TAA withdrawal (Delire et al., 2015). CCl₄ can be administrated to animals by different routes such as IP injection, gavage, and inhalation.

However, the most preferred technique is the IP injection twice a week for 6 weeks to obtain severe fibrosis (Scholten et al., 2015; Chang, 2005).

1.4.3.3 DDC Induced Fibrosis Model

DDC is the most common fibrotic agent which is administrated *ad libitum*. This agent is added to the diet of an animal because it is in powder. This diet causes the accumulation of protoporphyrin which is a precursor of heme. Extreme accumulation of this molecule damages the biliary system directly since the only way of the protoporphyrin removal from the liver is the biliary excretion (Clerbaux et al., 2018; Meerman et al., 1999). Therefore, fibrosis is observed mainly in the periportal region by indicators such as ductular reaction and porto-portal fibrotic septa (Delire et al., 2015). Generally, almost 3 weeks treatment of DDC diet to mice leads to severe fibrosis in the liver (Clerbaux et al., 2018).

1.4.3.4 CDE Induced Fibrosis Model

CDE is another *ad libitum* model for liver fibrosis as DDC. However, CDE consisted of both addition of ethionine into drinking water and a choline-deficient diet combination compared to DDC. Both choline absence and ethionine lead to hepatocyte impairment in functions. As seen in the DDC model, CDE affects the periportal region of the liver by DR, inflammation, and formation of fibrotic septa. Approximately 3 or 4 weeks are enough for the occurrence of severe fibrosis (Clerbaux et al., 2018; Lombardi et al., 1968).

1.4.3.5 Bile Duct Ligation (BDL) Induced Fibrosis Model

Bile duct ligation (BDL) is one of the common surgical methods to induce severe periportal fibrosis in the liver. BDL stimulates obstruction of the flow of bile acids which is called cholestasis. Thus, DR in cholangiocytes and liver progenitor cells, inflammation, and portal hypertension are observed mainly in that model (Takahashi & Fukusato, 2017; Geerts et al., 2008).

1.4.3.6 Partial Hepatectomy (PHx) Induced Fibrosis Model

Another common surgical method is PHx for liver fibrosis. In that model, almost 1/3 of the liver is taken from animals by surgical method. After almost 15 days of surgery, the liver maintains its whole mass completely thanks to regeneration. During the regeneration process of the liver to increase its liver mass, fibrosis is observed, and mainly hepatocytes and cholangiocytes are proliferating. However, this fibrosis is not zone-specific and it's distributed to the whole liver as the proliferation of hepatocytes and cholangiocytes (Liedtke et al., 2013).

1.4.4 Liver Regeneration

Regeneration is the capability of the tissue that can return to its original structure after injury without any remaining ruins of the wound. Regeneration capacity is dependent on fibrosis level, model, and administration timeline of toxic material. Generally, when fibrosis inducer chemical or toxic is eliminated, liver fibrosis is decreased gradually, and the regeneration process starts. During regeneration, opposite events to fibrosis are observed. Firstly, myofibroblasts, and activated HSCs are eliminated by apoptosis or senescence, so collagen level in ECM turns back into the basal level. Also, downregulation of TIMPS and upregulation of MMPs help to manage collagen levels. Generally, qHSCs level rises by vitamin A storage capability. As another process for regeneration, inflammation is decreased by the downregulation of cytokines, chemokines, and decreased population of immune cells in the regeneration area. Moreover, overactivity of signaling pathways is blocked and they return to their basal level so that proliferation of hepatocytes, cholangiocytes, and liver progenitor cells or differentiation between cholangiocytes and hepatocytes is not the case. Hypertension, hypoxia, angiogenesis, and capillarization are also not observed during regeneration by gaining LSECs characters of LSECs. Lipid and glucose metabolisms turn into homeostatic conditions. In addition to these, some fibrosis specific structures and cell types such as necrosis, fibrotic septa, ballooned hepatocytes disappear gradually. Briefly, liver turns into its basal and homeostatic conditions by regeneration (Cordero-Espinoza & Huch, 2018) (Kisseleva & Brenner, 2020).

1.5 The Primary Cilium

The primary cilium is a nonmotile organelle extending from the surface of the cell. It's one of the key factors for the cell cycle and differentiation steps of a developmental process. The primary cilium is a complex organelle and consists of mainly axoneme, basal body, transition zone, and lipid bilayer membrane (Pedersen et al., 2016). The axoneme is a microtubule-based structure. In motile cilia or flagella, the axoneme is formed by nine outer doublet microtubules (9+2) that cover a central microtubule pair, radial spokes, and dynein arms for motility. However, the axoneme of primary cilium lacks some of these structures and consists of 9+0 form (Pedersen et al., 2016; Silverman & Leroux, 2009; Fisch & Dupuis-Williams, 2011). The basal body is another important component of primary cilium for extension of it through the cell membrane. It is the differentiated version of the mother centriole which is a part of the centrosome (Kim & Dynlacht, 2013; Dawe et al., 2007). The transition zone is a functional region of the primary cilium compared to structural components, axoneme, and the basal body. It takes a role in controlling the selective passage of molecules into/out the cilium by functioning as a gate (Pedersen et al., 2016; Reiter et al., 2012; Jensen et al., 2015). In addition to that, in some organisms and mammalian cell types, primary cilium is surrounded by a ciliary pocket (CiPo) which is the cytoplasmic invagination to monitor the movement of vesicles (Pedersen et al., 2016; Benmerah, 2013).

The primary cilium is a specialized transport system called intraflagellar transport (IFT) for the delivery of protein and lipids between axoneme and transition zone. This transport system can process through two oppositely different directions, retrograde and anterograde by using different motor proteins. Retrograde transport is performed towards the basal body of cilium under the control of Dynein II motor protein. When lipids and proteins are carried from the basal body to the ciliary tip, Kinesin II motor protein is responsible for anterograde transport (Mansini et al., 2018; Lee & Gleeson, 2011).

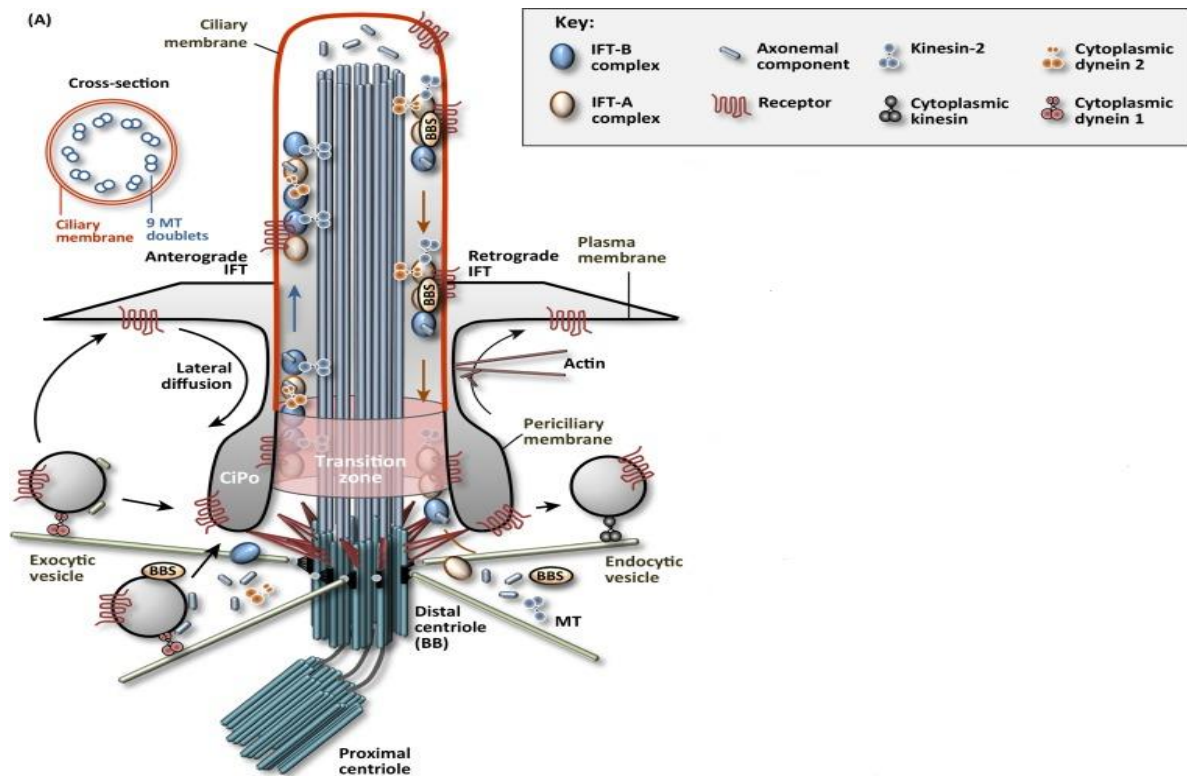


Figure 9: The primary cilium structure with its components (Pedersen et al., 2016).

The primary cilium is accepted as a cellular antenna of the cell by performing mechano, chemo, and osmo-sensory tasks. It takes roles in endocytosis and exocytosis of vesicles which can carry ligands, miRNAs, and other important factors for the signaling pathways (Mansini et al., 2018; Masyuk et al., 2010). Moreover, it regulates signaling pathways such as Hedgehog, Wnt, Notch, Tgf β , RTK, but its exact role in this regulation is not characterized well (Pedersen et al., 2016). On the membrane of the primary cilia, different GPCRs which are components of these pathways are located. For instance, the Gpr161 affecting factor of Hedgehog pathway is localized on the ciliary membrane when the pathway is inactive. It stimulates PKA by cAMP accumulation and this leads to degradation of the Gli transcription factor. When the pathway is on state by Hedgehog ligand binding, Gpr161 is carried from the ciliary membrane and Gpr175 enters cilia by preventing Gli cleavage (Pedersen et al., 2016; Goetz & Anderson, 2010; Mukhopadhyay & Rohatgi, 2014; Singh et al., 2015). In addition to that, primary cilium has effects on calcium metabolism by the existence of calcium channels, PKD1L1, PKD2L1. These channels control ciliary-cytoplasmic calcium concentration and modify Gli1 and Gli2 expression (Delling et al., 2013).

In the mouse liver, primary cilium is expressed only in cholangiocytes (Grzelak et al., 2014). However, 1.92% of hepatic stellate cells/myofibroblasts have primary cilium in addition to cholangiocytes in the human liver. Nevertheless, the exact function of cilia in these cell types

under homeostatic or disease conditions is not known (Grzelak et al., 2017).

Ciliopathy is the kind of disorder observed due to defects and distortions in the function, structure, organization of primary cilium (Waters & Beales, 2011). When ciliopathy happens specifically in the cholangiocytes, it is called cholangiociliopathy. It mainly affects the function of cilia in the cholangiocytes in the portal region of the liver. The most common cholangiociliopathies are polycystic liver disease (PCLD), autosomal dominant polycystic liver disease (ADPLD), and primary sclerosing cholangitis (PSC). Apart from liver, kidney is also an important organ affected by ciliopathy (Mansini et al., 2018; Masyuk et al., 2009).

1.6 Other Disease Models

Obesity and fasting/refeeding models are other disease states to change the function and structure of the liver. In this section, these models will be covered.

1.6.1 High Fat Diet and Western Diet Induced Obesity Models

Obesity models are mainly affecting lipid and glucose metabolism of the body and the main factors to induce non-alcoholic fatty liver disease (NAFLD) specifically in the liver (Steensels et al., 2020). High lipid accumulation in hepatocytes (steatosis), inflammation, and fibrosis occur in NAFLD (Steensels et al., 2020; Iwaisako et al., 2012). Also, the fundamental regulators of hepatic lipid deposition, PPAR α , PPAR γ , and PPAR δ , have different expression profiles during NAFLD. PPAR α and PPAR γ promote lipid accumulation and lipid biogenesis in the NAFLD liver, therefore their expressions are upregulated under NAFLD (Steensels et al., 2020; Montagner et al., 2016; Souza-Mello, 2015; Lee et al., 2018). However, the protective role to prevent extreme lipid storage belongs to PPAR δ , so its expression decreases in the NAFLD liver (Steensels et al., 2020; Kliewer et al., 1994). The increasing stages of NAFLD lead to non-alcoholic steatosis (NASH) and cirrhosis. As a final stage, hepatocellular carcinoma happens (Steensels et al., 2020; Benedict & Zhang, 2017).

The main diet-based models for obesity are performed by administration of high fat and western diet. These are very similar models to each other and the only difference is the percentage of fat level. A high fat diet includes 60% kcal of fat whereas a western diet contains 40% kcal of fat and 40% kcal of carbohydrate. Both models cause the phenotype which is observed in human steatosis in the mice in terms of lipid accumulation and insulin resistance. Nonetheless, these models are not enough to induce severe fibrosis models in the mouse liver. It barely promotes mild fibrosis (Steensels et al., 2020; Ito et al., 2007).

1.6.2 Fasting/Refeeding Models

Fasting/refeeding is another model affecting mainly lipid and glucose metabolism of the liver as observed in obesity models. During fasting, the liver starts acting like it's under steatosis. Therefore, lipid storage as lipid droplets in hepatocytes increases in order to provide energy for cellular functions. In these lipid droplets (LDs), mainly triacylglycerol, cholesteryl ester, and retinyl ester are accumulated. Apart from the liver, adipose tissue and small intestine are affected by fasting. Nevertheless, under refeeding conditions, liver initiates decreasing the number and size of lipid droplets, lipogenesis, and lipid deposition, so that it can maintain its homeostatic state (Kramer et al., 2018). Due to fasting and refeeding states, some transcriptional changes are observed in the liver. As previously mentioned PPAR α expression is upregulated in fasting due to its similar reaction to obesity, whereas this increase is reversed during refeeding. In addition to this, fasting induces increasing expression in Acot2 which is also related to lipid metabolism (Zhang et al., 2011).

2. OBJECTIVES AND AIMS

In this thesis, our main aims were to characterize the novel sub-population of Arl13b+ cholangiocytes under fibrosis and to show the response of primary cilium to fibrosis. It's known that the whole liver structure had a big alteration and different liver cell types showed different responses under fibrosis. Primary cilium was a significant structure that was expressed only in cholangiocytes. The response of the primary cilium against fibrosis was not understood, well. Thus, we generated different weeks of TAA induced fibrosis models. We performed various sets of IHC experiments and microscopy analyses. According to our observations, we hypothesized that partial EMT undergoing Arl13b+ cholangiocyte subpopulation arose upon fibrosis and it had effects on the progression of the fibrosis.

To evaluate our hypothesis, our main aims were to:

1. To show the responses of different cell types to fibrosis. With this goal, we could compare structural changes, proliferation, migration, and expansion of different cell types throughout the whole liver for different stages of TAA induced fibrosis models.
2. To confirm the specificity of the primary cilium in cholangiocytes under both homeostatic and fibrotic conditions. It was known that primary cilium is specific for cholangiocytes under homeostasis. However, its expression was not studied in liver diseases. It could be upregulated in other cell types upon fibrosis or cholangiocytes could lose their primary cilium. This goal will describe these possibilities.
3. To detect changes in primary cilium level in fibrosis. As mentioned, alteration of primary cilium due to fibrosis was not known, exactly. This aim will help us to show upregulation, downregulation, or stable expression of the primary cilium in a specific cell type, cholangiocyte.
4. To characterize partial EMT undergoing Arl13b+ cholangiocytes upon fibrosis. With this goal, we could identify a new subpopulation of Arl13b+ cholangiocytes observed in fibrosis, but not in homeostasis. In addition to that, we could compare the level and the location of this new subpopulation of cholangiocytes between different weeks of fibrosis models.
5. To identify niche components of partial EMT undergoing Arl13b+ cholangiocytes. This novel type of Arl13b+ cholangiocytes constituted cell masses around and between portal veins. Also, several cells of this subpopulation expanded through the central vein from the portal vein. With the help of this goal, we could describe these cell masses and

several cells around partial EMT undergoing cholangiocytes. These surrounding cells could be niche components of this novel Arl13b⁺ cholangiocyte subpopulation.

6. To discover novel markers to label partial EMT undergoing Arl13b⁺ cholangiocytes and its niche components. This aim could facilitate the characterization of partial EMT undergoing Arl13b⁺ BECs and other cell types surrounding them. Also, it could give clues about the contributions of niche components for the occurrence of partial EMT in Arl13b⁺ cholangiocytes.



3. MATERIALS AND METHODS

3.1 Generation of Mouse Models

All mouse models were generated at the Bilkent University Animal Facility (Ankara, Turkey) according to Ethical Committee requirements. TAA induced liver fibrosis model was developed by using 8-10 weeks old, female C57BL/6 strain of mice. For the experimental group, 24 mice (n=24) were used whereas 19 mice (n=19) constituted the control group. Thioacetamide, TAA, (Sigma Aldrich, USA, 163678) was administered to animals by addition of their autoclaved drinking water. For the first week of TAA administration, 150 mg/L dose was applied to drinking water. However, after the first week 300 mg/L TAA was administered into drinking water for 16, 20, and 24 weeks. Conversely, autoclaved regular drinking water was used for the control group.

High fat diet induced obesity models were generated from 8-12 weeks old, female-male, C57BL/6 strain mice. The experimental group of high fat diet induced obesity consisted of 37 mice whereas the control group was composed of 30 animals. High fat diet which had 60% kcal fat (Research Diets, NJ, USA, D12492) was administered to the experimental group *ad libitum* for 2,8,12,16,20,24 weeks. For the control group, a regular chow diet (Optima, Bolu, Turkey) was used *ad libitum*. Weights of both the control and experimental group were measured twice a month regularly.

Western diet induced obesity model was developed from 8-12 weeks old, female-male, C57BL/6 strain mice. The experimental group contained 17 mice (n=17) whereas the control group had 12 mice (n=12). A western diet including 40 kcal% fat and 20% protein (Research Diets, NJ, USA, D12079B) was applied to animals *ad libitum* for 24 weeks. The Control group was fed with a chow diet *ad libitum*. Weights of both groups were measured twice a month regularly.

The fasting/refeeding model was generated by using 8-12 weeks old, female-male, C57BL/6 strain mice. For the fasting part, mice were fasted for 24 hours and euthanized. For refeeding state, some groups of mice were refed for 3 hours after 24 hours fasting and euthanized. This group was called 3 hr refeeding. However, other mice known as 6 hr refeeding group were refed for 6 hours after 24 hours fasting and euthanized. Respectively, the numbers of the fasting, 3hr refeeding, 6 hr refeeding and control groups were 4,4,4, and 5. All groups were fed with a regular chow diet *ad libitum* and their weights were measured at the end of fasting and 3 hr and 6hr refeeding states.

3.2 Isolation of Liver and Tissue Embedding

The liver was collected from all disease and control models. Mice were fainted with isoflurane and euthanized. Midline laparotomy and heart retrograde perfusion were performed on each animal. During cardiac perfusion, blood was collected from the right ventricle of the heart. A small incision was made in the right atrium and ice cold 1x PBS was administrated through the left ventricle. From the same spot where PBS was administrated, 4% paraformaldehyde (PFA)/PBS (Fluka) or 10% formalin solution (Sigma Aldrich, MO, USA, HT501128) was applied. After the perfusion step, all parts (left, middle and right) of the liver were collected and fixed in 4%PFA on ice or 10% formalin solution at room temperature according to used fixative material during cardiac perfusion. If tissues were to be embedded in paraffin, their first step fixation took almost two hours, and their fixative material was refreshed. In the refreshed fixative, tissues were incubated overnight in 10% formalin or 4%PFA at room temperature or +4°, respectively. However, if tissues were to be embedded in OCT, their fixation took an hour. After fixation, tissues were washed with ice cold 1X PBS three times 5 minutes for each and incubated into 30% sucrose solution (Fisher Chemicals, NH, USA, 10634932) overnight at +4° without depending on their fixative material. Following overnight incubation, tissues which will be used in paraffin were washed with 1X PBS three times 5 minutes for each at room temperature. They were dehydrated with 30% EtOH/PBS/ddH₂O and 50% EtOH/ ddH₂O for an hour at room temperature, respectively. Tissues were put in cassettes and incubated into 70% EtOH/ddH₂O for upcoming tissue embedding into paraffin which will be performed by an automated tissue processor. After some overnight processing into tissues by tissue processor, liver tissues were embedded into paraffin (Sakura, Finetek, USA, Tissue Tek III Paraffin) horizontally. For the preparation of frozen tissues after overnight incubation of them into sucrose, they were horizontally embedded into molds (Sigma Aldrich, USA, E6032-1CS) containing OCT (Sakura Finetek, Tokyo, Japan, 4583) and incubated at -80° until they were totally frozen.

3.3 Immunohistochemistry in Paraffin and Frozen Samples

Paraffin embedded livers were prepared for immunohistochemistry by sectioning into 4µm thickness via microtome. These sections were incubated onto a 37°C stretching table (Meditex, Germany, OTS 40.3040) overnight. For the deparaffinization part, sections were incubated two times in xylol/xylene 7.5 minutes for each at RT. They were dehydrated into 100%, 96%, 80%, 70%, and 50% EtOH/ddH₂O two times 4 minutes for each dehydration at RT. After

dehydration, liver sections were washed three times with RT 1x PBS for 5 minutes. For the antigen retrieval, according to antibody working condition citrate working buffer (0.1 M citric acid anhydrous, 0.1 M tri-sodium citrate dihydrate, pH 6.0) or Tris-EDTA working buffer (10 mM Trizma base, 1 mM EDTA, pH 9.0) was heated into the microwave (Samsung, South Korea) until boiling. Antigen retrieval was performed in a steam cooker (Tefal, France) with citrate working buffer or Tris-EDTA working buffer at rice adjustment of the cooker for 35 minutes. Following antigen retrieval, tissues were cooled down at RT and washed RT 1x PBS two times, 5 minutes for each. For the blocking of the tissues, the blocking buffer was prepared with ddH₂O, 10X PBS, 0.3% TritonX100 detergent/PBS called stock solution, and donkey serum (Biowest, MO, USA). Tissues were incubated with this blocking solution for an hour at RT in aluminium covered wet chamber. After the blocking step, primary antibody solution was prepared with known dilutions of antibodies (Appendix I) in the blocking buffer. Tissues were incubated with primary antibody solution at +4° for overnight into an aluminium covered wet chamber. On the following day, tissues were washed with RT 1X PBS three times 5 minutes for each. They were incubated with secondary antibody solution prepared in blocking solution with known dilutions of secondary antibodies (Appendix I) at RT for an hour into an aluminium covered wet chamber. After secondary antibody administration, tissues were washed with 2 times RT 1X PBS and 2 times RT PBS-T 5 minutes for each. For the DAPI staining, DAPI solution (ThermoFisher Scientific, USA, 62248) was prepared into PBS-T and applied to these sections for 10 minutes incubation at RT into an aluminium covered wet chamber. Stained samples were washed with 2 times RT PBS-T and 2 times RT 1X PBS 5 minutes for each, respectively. As a final step, 2-3 drops of mounting medium (Abcam, Cambridge, UK) were applied to tissues and covered by a coverslip (Menzel-Glaser, VWR International). These samples were kept at +4°.

For the preparation of frozen samples to immunohistochemistry, they were sectioned into 5µm thickness by a cryostat. They were incubated on a 37° stretching table at RT for 30 minutes each, respectively. Slides were fixed with 4%PFA or 10% formalin solution for 10 minutes depending on the fixative material during the perfusion step. After fixation, they were washed three times with RT 1X PBS 5 minutes for each washing. Slides were blocked with blocking solution and administrated with primary antibody, secondary antibody, and DAPI solutions (Appendix I) as previously explained.

3.4 Image Collection From Confocal Microscopy

Images from IHC liver samples were collected with a Leica TCS SP8 DMI8 Confocal Microscope (Leica Biosystems, Wetzlar, Germany). Images and tile scans were taken in 63X magnification and 0.85 or 1.00 zoom. The thickness of Z-stacks was changeable between 3 microns and 10 microns depending on frozen or paraffin sections. Adjustments regarding the quality of images in the confocal microscope were format 1024/1024 or 2048/2048, line average 2 or 4, speed 400 according to tile scan images or regular image, respectively. Generally, in images portal vein, central vein, and parenchyma were concentrated according to zonation of stained marker whereas in tile scan images all structures were captured. Collected images were analyzed and processed via Fiji-Image J by adjusting RGB color, stack number, and projection type.

3.5 Image Analysis and Statistics

For the quantification, image analysis was carried out by ImageJ-Fiji. Normalization and different thresholding methods were applied to raw data images. Autofluorescence and unspecific signals were eliminated. After the image analysis, statistics were performed with GraphPad Prism software. Both TAA 24-week and control samples were subjected to normality and lognormality tests. According to normalization, unpaired, non-parametric (Mann Whitney test), or unpaired, parametric t-test was applied and p-value was obtained.

*=P<0.05 ; **=P<0.01 ; ***=P<0.001 ; ****=P<0.0001; ns=no significance.

4. RESULTS

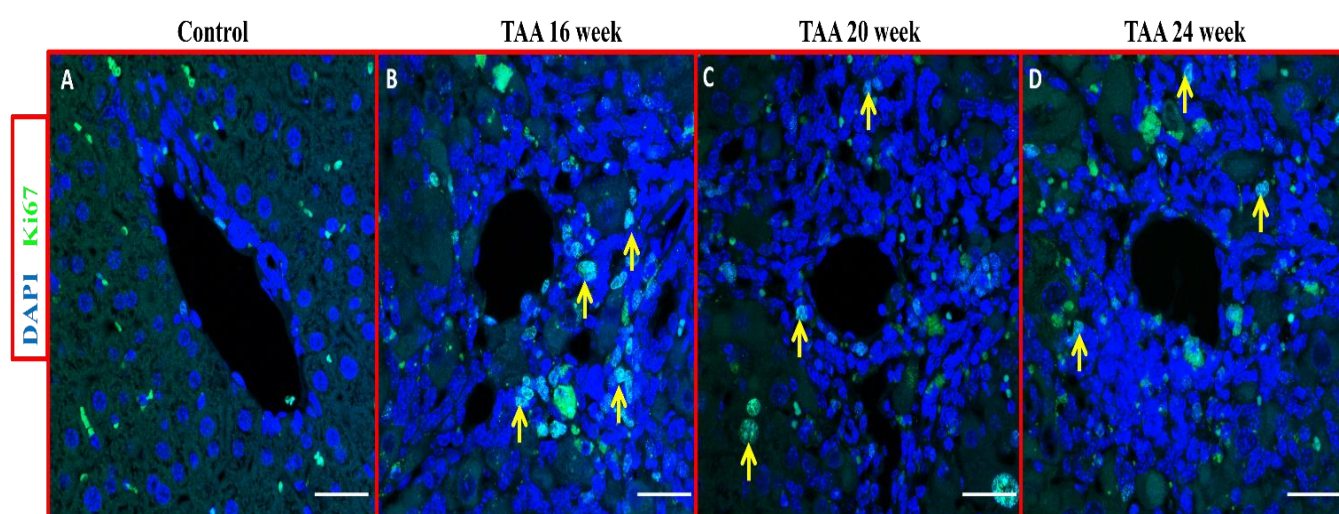
In this thesis, responses of different liver cell types to various metabolic and disease conditions were studied. In addition to that, phenotypic changes in these cell types under fibrosis were shown by comparing homeostasis. The main focus of this research was the characterization of partial EMT undergoing Arl13b⁺ cholangiocytes in TAA induced liver fibrosis model.

4.1 Generation of Fibrosis in the Liver

After administration of TAA to mice for 16, 20, and 24 weeks, some main indicators of fibrosis which were mentioned previously in section 1.4 of the introduction were checked with the help of immunohistochemistry to confirm development of the fibrotic state in the liver.

4.1.1 Proliferation Rate

To demonstrate proliferation of cells as an indicator of successful fibrosis model, Ki67 which is a proliferation marker was used in different weeks of TAA-induced fibrosis samples by fluorescent staining. All weeks of fibrotic samples contained proliferative cells around the portal vein compared to controls (Figure 1A-D). However, when TAA samples were compared to each other, TAA 16-week sample possessed the highest number of proliferative cells than TAA 20-week, and 24-week samples. We observed similar level proliferative cells in the TAA 20, and 24-week samples (Figure 1 B, C, D).



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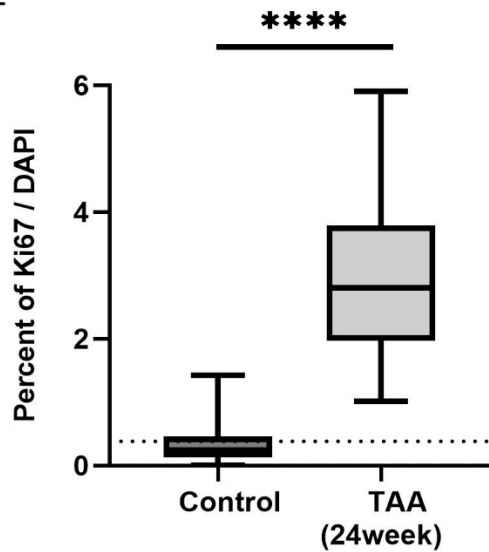


Figure 1: Ki67 (green) staining in control sample of liver (A)(n=8) and TAA induced fibrosis samples for different durations; 16 week (B)(n=2), 20 week (C)(n=2), and 24-week (D)(n=4). All images showed a portal vein of the liver. Yellow arrows indicated Ki67+ cells around the portal vein. DAPI was used for staining the nucleus. Scale bar represented 50µm. (E) Quantification of immunostaining experiment by ratio of specific signal to DAPI. Data presented as mean $\pm$ SEM, t-test, $P < 0.0001$

In order to compare structural changes throughout the fibrotic and homeostatic liver from extensive view, tile scan images were used. Due to the increasing proliferation level of cells under fibrosis, we observed that some cell types started to migrate from portal vein to another portal vein by following a clear path (Figure 2B). Interestingly, some cells were getting out of this path and initiated an expansion through the central vein. Ki67+ cells existed in the both migration area between the portal-portal axis and the expansion area to central vein. However, in the control liver we didn't notice any change in the proliferation or expansion of cells between veins (Figure 2A). We observed basal level Ki67 expression in the control liver.

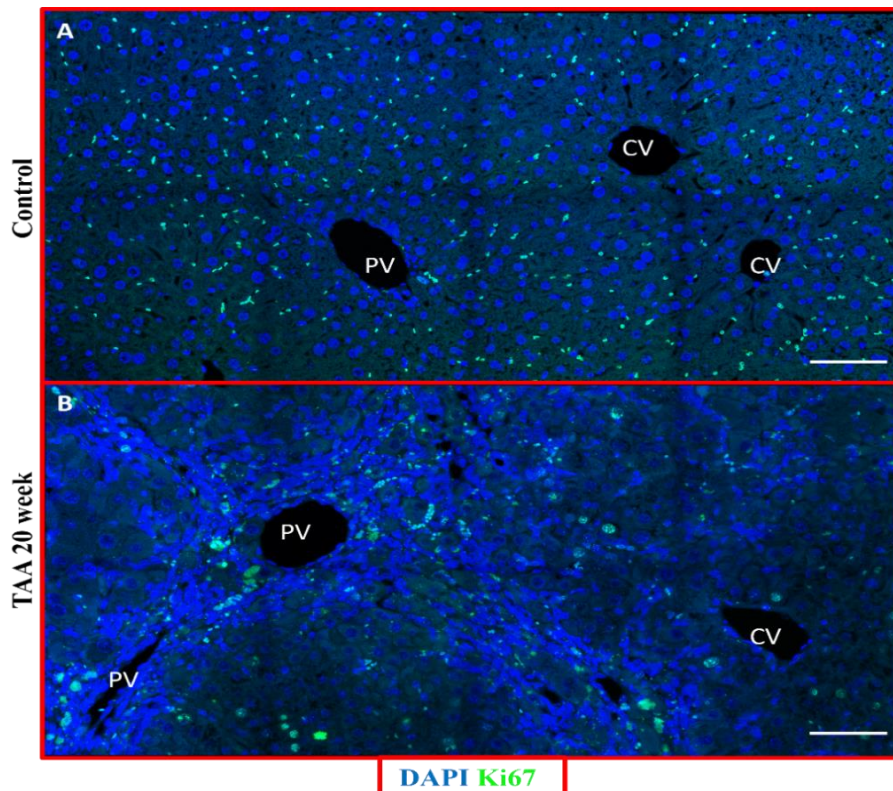
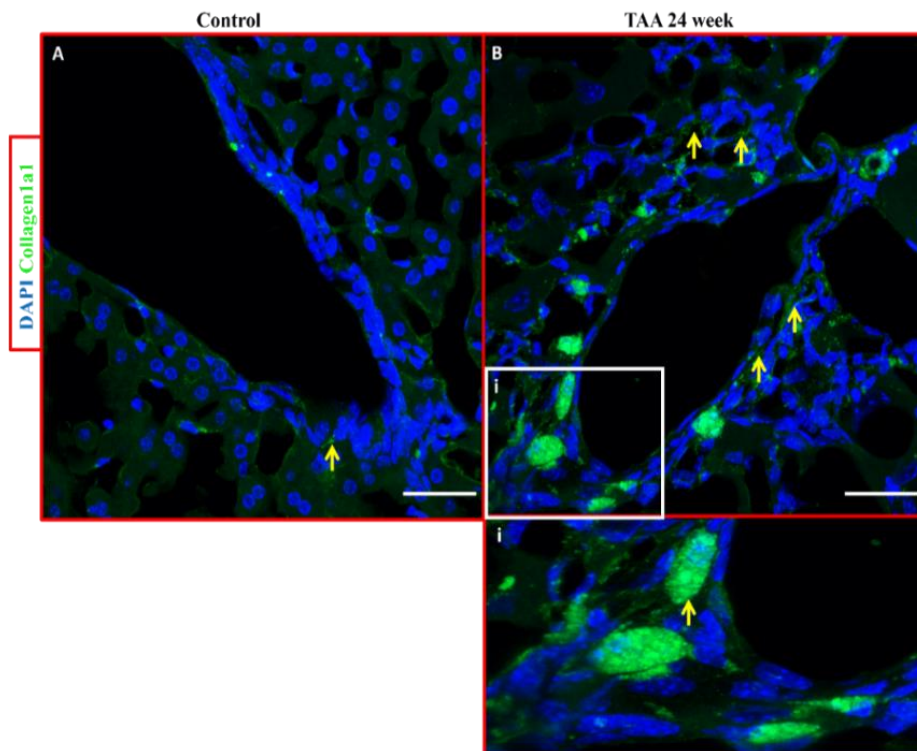


Figure 2: Immunohistochemistry against Ki67 (green) in control (A)(n=2) and TAA 20-week sample (B)(n=2). Both images showed portal and central veins of the liver as a tile scan. Between two portal veins, cells were generating a way to migrate (B). But some cells were expanded through portal to central axis. DAPI was used for staining the nucleus. Scale bar represented 200 μ m.

4.1.2 Collagen Accumulation and Retinoid Droplets

As explained in previous sections, another important sign for the generation of fibrosis was the collagen accumulation in the extracellular matrix. Thus, as a second proof for our model, we wanted to show collagen levels in the fibrotic liver compared to the homeostatic liver. For this purpose, we performed IHC staining against Collagen1 α 1 which is one of the most affected collagen types in the fibrotic liver. Collagen fibers around the portal vein were evidently high in the fibrotic liver compared to control samples (Figure 3A, B, C, t-test, $P=0.0024$). Apart from collagen fibers, we observed another signal (Figure 3i). This signal was very different from the collagen signal and was not observed in the control sample. When other unstained channels were operated under the microscope, we observed the same signal in other channels. Therefore, we concluded that this signal was coming from the autofluorescence of accumulated retinoid droplets in the fibrotic liver as another indicator of fibrosis.



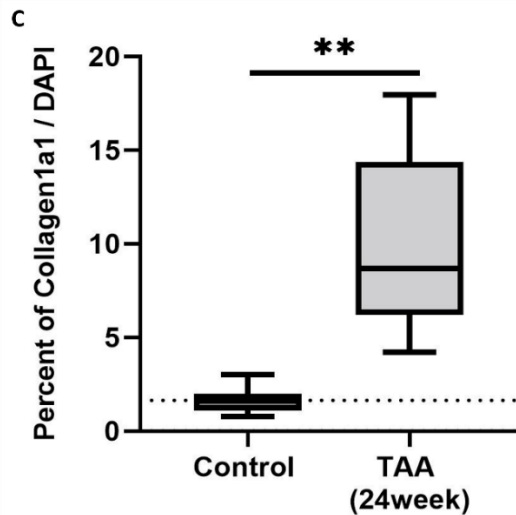


Figure 3: IHC staining against Collagen1 α 1 (green) in the control sample (A) (n=2) and TAA 24-week sample (B) (n=2). Both images belonged to portal veins of the liver. Yellow arrows in the A and B panels showed collagen fibers marked by Collagen1 α 1 whereas in the zoomed panel i, yellow arrow indicated autofluorescence of retinoid droplet. DAPI was used for staining the nucleus. Scale bar represented 50 μ m. (C) Quantification of immunostaining experiment by ratio of specific signal to DAPI. Data presented as mean $\pm$ SEM, t-test, P=0.0024.

4.1.3 Ballooned Hepatocytes

To confirm the generation of fibrosis, we aimed to analyze the appearance of giant hepatocytes. Because of changes in the lipid metabolism of the liver under fibrotic state, hepatocytes are getting bigger and called ballooned hepatocytes as mentioned previously. To detect hepatocytes and distinguish enormous ones, we performed IHC with one of the common hepatocyte markers, Hnf4 α . We observed that around the portal vein of TAA 24-week sample, several ballooned hepatocytes which were almost two times bigger than normal sized hepatocytes of control sample were present (Figure 4A, B). However, we still observed homeostatic hepatocytes in addition to ballooned hepatocytes in fibrotic sample as expected (Figure 4B). Moreover, these giant hepatocytes were not restricted around the portal zone of the fibrotic liver. Ballooned hepatocytes were distributed through all regions of the liver including periportal, pericentral, and midzone areas (Figure 4C).

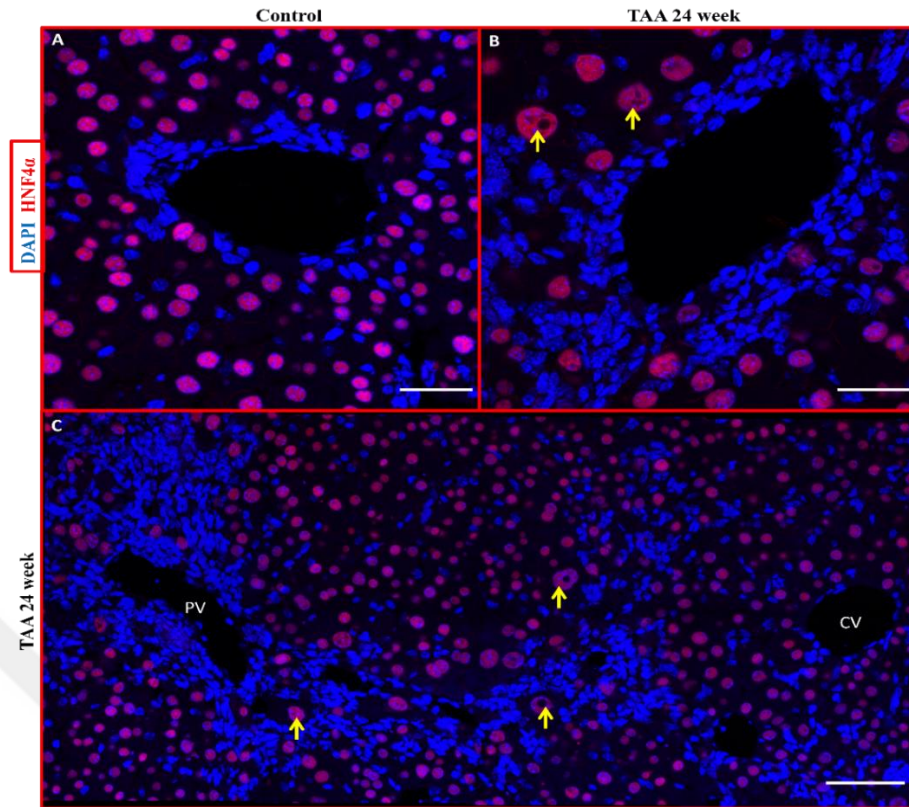


Figure 4: Hnf4 α (red) fluorescence staining in the control (A) (n=4) and TAA 24-week sample (B) (n=4). A and B panels showed the portal region of the liver tissues. C part was a tile scan image of TAA 24-week sample containing portal vein, central vein, and parenchyma. Yellow arrows demonstrated ballooned hepatocytes. DAPI was used for staining the nucleus. Scale bar represented 50 μ m (A and B), and 200 μ m (C). (PV: Portal Vein; CV: Central Vein)

4.2 Responses of the Liver Cell Types to Fibrosis

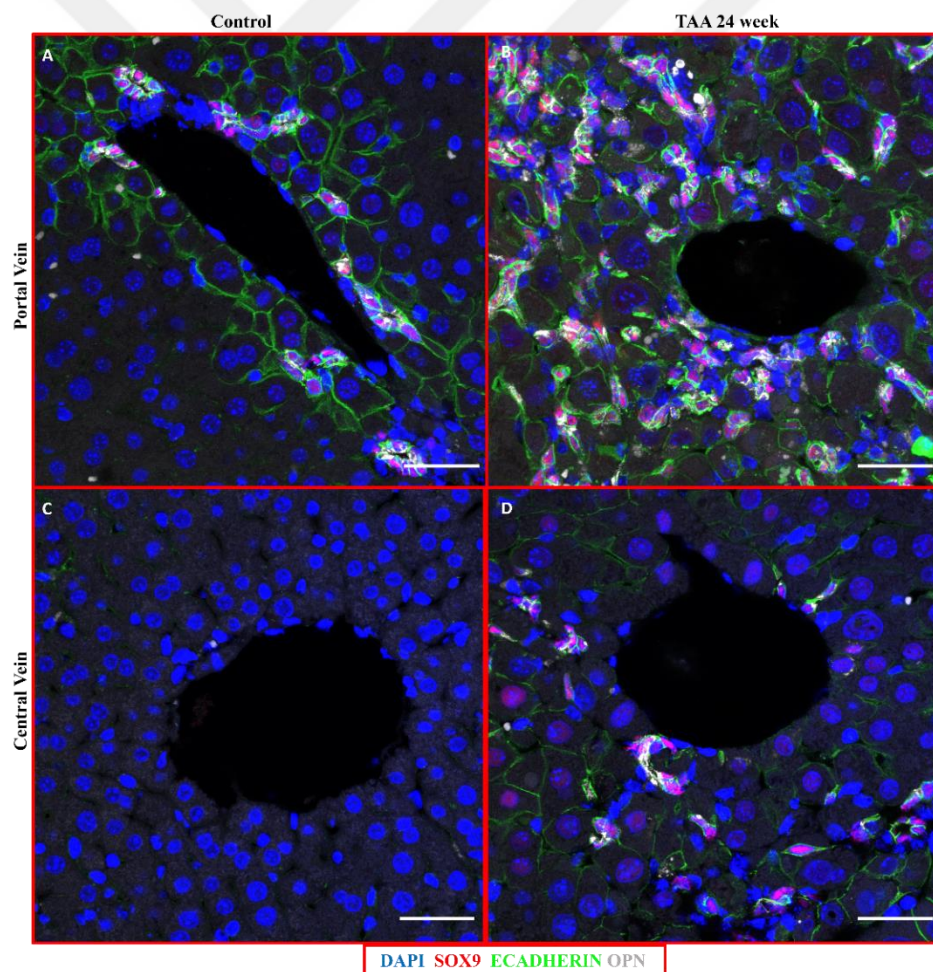
After confirmation of the development of fibrosis model with the help of proliferation level, collagen accumulation, and appearance of ballooned hepatocytes, we wanted to focus on responses and changes in the various liver cell types, cholangiocytes, hepatocytes, mesenchymal cells, and endothelial cells against fibrosis. For this purpose, we conducted different IHC stainings by diverse markers of different cell types and combinations of them.

4.2.1 Responses of Cholangiocytes to Fibrosis

Since our main focus was the Arl13b⁺ cholangiocytes, we started to analyze responses of the cholangiocytes to fibrosis. As a first step, we wanted to check the level of cholangiocytes under fibrosis compared to the control liver. We performed IHC staining by using well-known cholangiocyte markers, Opn and Sox9, and epithelial marker, E-cadherin. As mentioned in

section 1.3.2, cholangiocytes should locate only around the portal vein under homeostatic conditions. We observed cholangiocytes around portal veins of control and fibrosis samples (Figure 5A, B). Moreover, there was an extreme increment for cholangiocyte level in the portal vein of the fibrotic liver compared to controls (Figure 5E, F, t-test, $P < 0.0001$) Surprisingly we observed several cholangiocytes around the central vein under fibrosis unlike to central vein of the control sample (Figure 5C, D).

Also, we nicely showed an increase in the cholangiocyte number in both pericentral and periportal regions of the fibrotic liver with the help of tile scan images of this staining. Opn⁺, Sox9⁺, E-cadherin⁺ cholangiocytes were extremely increased around the portal vein and expanded through parenchyma and central vein due to fibrosis (Figure 6B). However cholangiocytes were detected only in the periportal zone, not the pericentral zone and parenchyma in the control liver (Figure 6A).



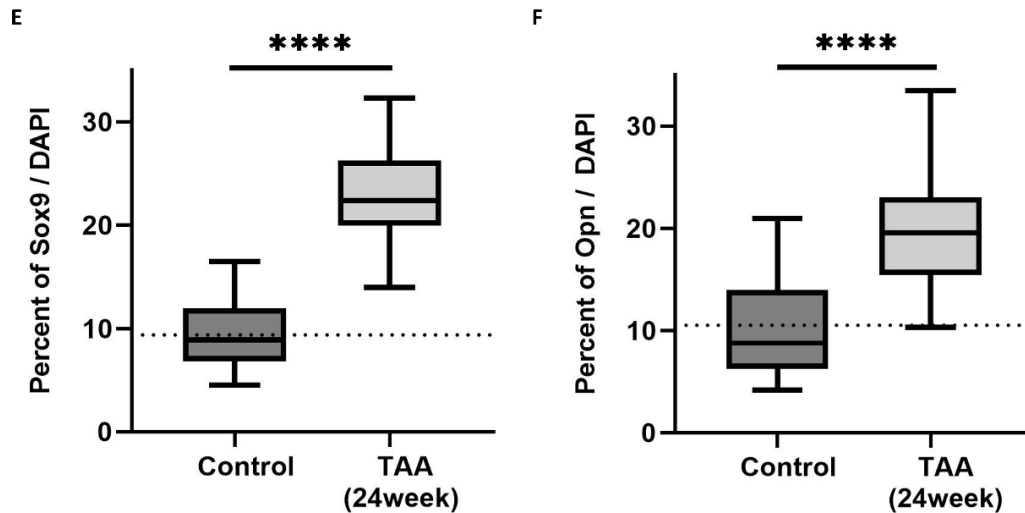


Figure 5: IHC staining by Sox9 (red), E-cadherin (green), and Opn (grey) for control (A, C) and TAA 24-week (B, D) samples. A and B panels showed portal veins whereas C and D panels demonstrated. Sox9 (n=4), Opn (n=6) and E-cadherin (n=3) were expressed periportal region of homeostatic liver. However, Sox9 (n=4), Opn (n=6) and E-cadherin (n=3) expressed cholangiocytes were cholangiocytes were detected in both central and portal veins of TAA 24-week sample. DAPI was used for staining the nucleus. Scale bar represented 50 μ m. (E, F) Quantification of immunostaining experiment by ratio of specific signal Sox9 and Opn to DAPI, respectively. Data presented as mean $\pm$ SEM, t-test, $P < 0.0001$.

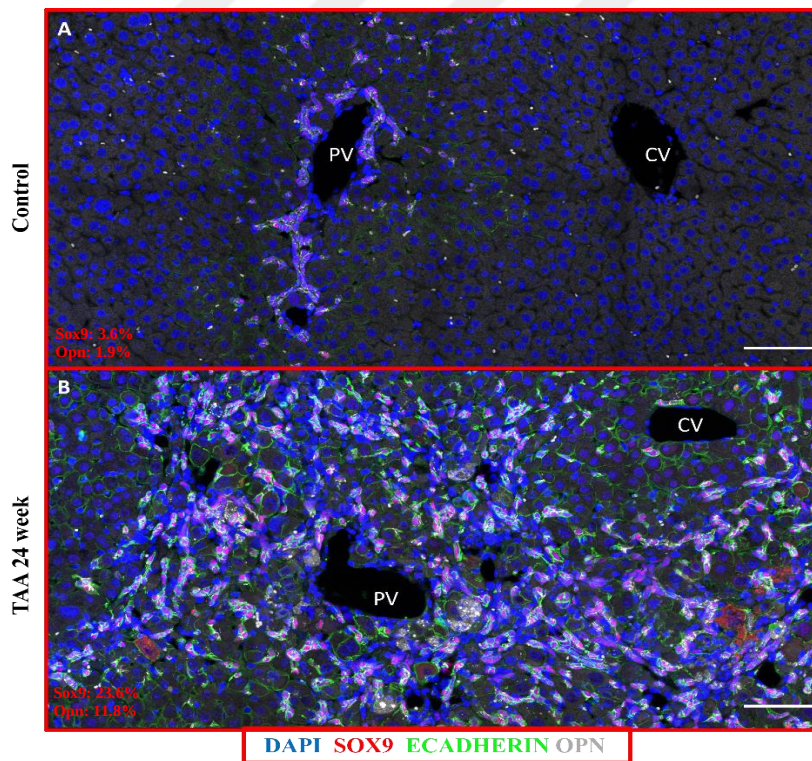


Figure 6: Tile scan images of combined Sox9, Opn, E-cadherin IHC staining for control (A) and TAA 24-week (B) samples. Tile scans indicated all zones of the liver, periportal zone, midzone and pericentral zone, from left to right. At the left bottom of images, percentages showed the ratio of the marker to DAPI for quantification. DAPI was used for staining of nucleus. Scale bar represented 200 μ m. (PV: Portal Vein; CV: Central Vein)

After demonstrating a sharp increase in the cholangiocyte level under fibrotic conditions, we were curious about whether these cholangiocytes were proliferating or not. We were wondering if these cholangiocytes were proliferating cells (Figure 1 and Figure 2B), how the proliferation rate of cholangiocytes changed among different weeks of TAA samples, 16, 20, and 24 week. In order to give answers to these questions, we conducted a co-staining experiment of Ki67 and Opn in the control and different stages of fibrosis samples. We didn't observe any overlap for Ki67 and Opn as expected (Figure 7A and Figure 1A). However, Ki67+ cholangiocytes were present in the portal veins of different weeks of TAA samples (Figure 7B, C, and D). Especially, TAA 16-week sample showed the highest number of Ki67 and Opn overlap compared to TAA 20-week and 24-week samples (Figure 7B, C, D, and Figure 1B). Furthermore, we saw that more Ki67+Opn- cells were present in both TAA 20-week and 24-week samples than TAA 16-week sample (Figure 7B, C, and D). This means that apart from cholangiocytes, other cell types were also proliferating in fibrosis.

Additionally, we observed autofluorescence of retinoid droplets in almost all stainings as an indicator of fibrosis without depending on a stained marker (Figure 7D, white arrow).

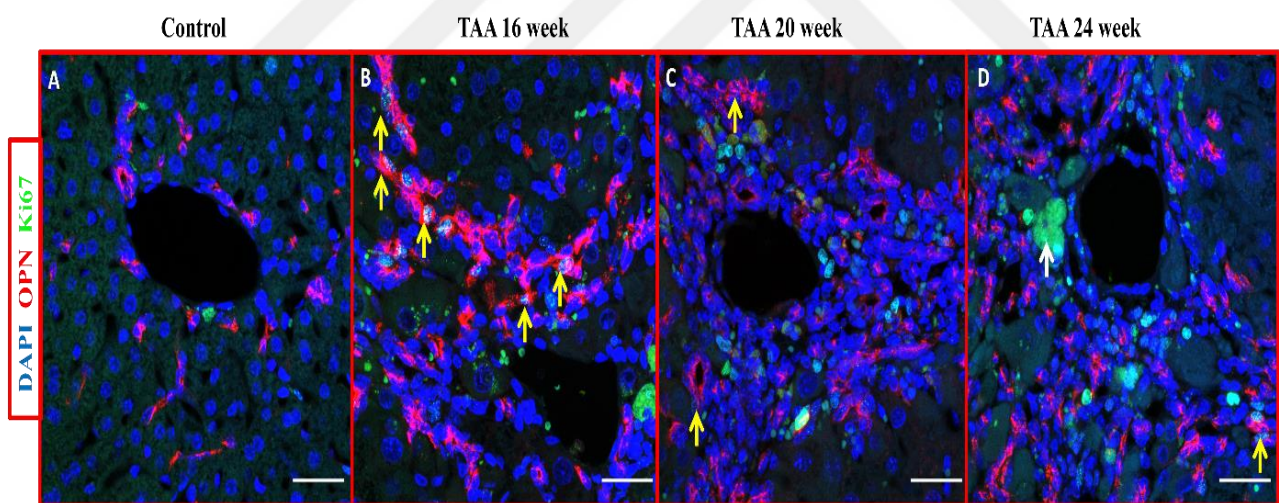


Figure 7: Fluorescence staining of Opn (red) and Ki67 (green) for control (A)(n=11, n=8), TAA 16-week (B)(n=3, n=2), TAA 20-week (C) (n=4, n=2), and TAA 24-week (D) (n=6, n=4) respectively. All images displayed portal veins. Yellow arrows indicated Opn and Ki67 co-expressed cells. White arrow in the D panel showed autofluorescence of the retinoid droplet. DAPI was used for staining the nucleus. Scale bar represented 50 μ m.

To check proliferative cholangiocytes from the wider view including various liver regions, tile scan images of Opn-Ki67 IHC experiments were useful. In TAA 16-week samples, fewer cholangiocytes which located around the portal vein and expanded through portal-portal and portal-central axis were present compared to other TAA samples (Figure 8B). However, in TAA 20-week and 24-week samples (Figure 8C and D) intense cell masses were generated around the portal vein and observed to be migrating through other portal veins by following a clear path. The main component of these cell masses were cholangiocytes (Figure 8C and D). Some cholangiocytes from these masses were escaping from their way and expanding through the central vein. Proliferative cholangiocytes of TAA 16-week sample existed around the portal vein (Figure 8i). Ki67+ cholangiocytes were detected in close proximity to the central vein and expansions to the central vein for TAA 20-week and 24-week samples, respectively in addition to proliferative cells around the portal vein (Figure 8 ii and iii). Nevertheless, in the control sample Ki67+ cholangiocytes or other proliferative cell types were absent as expected (Figure 8A).

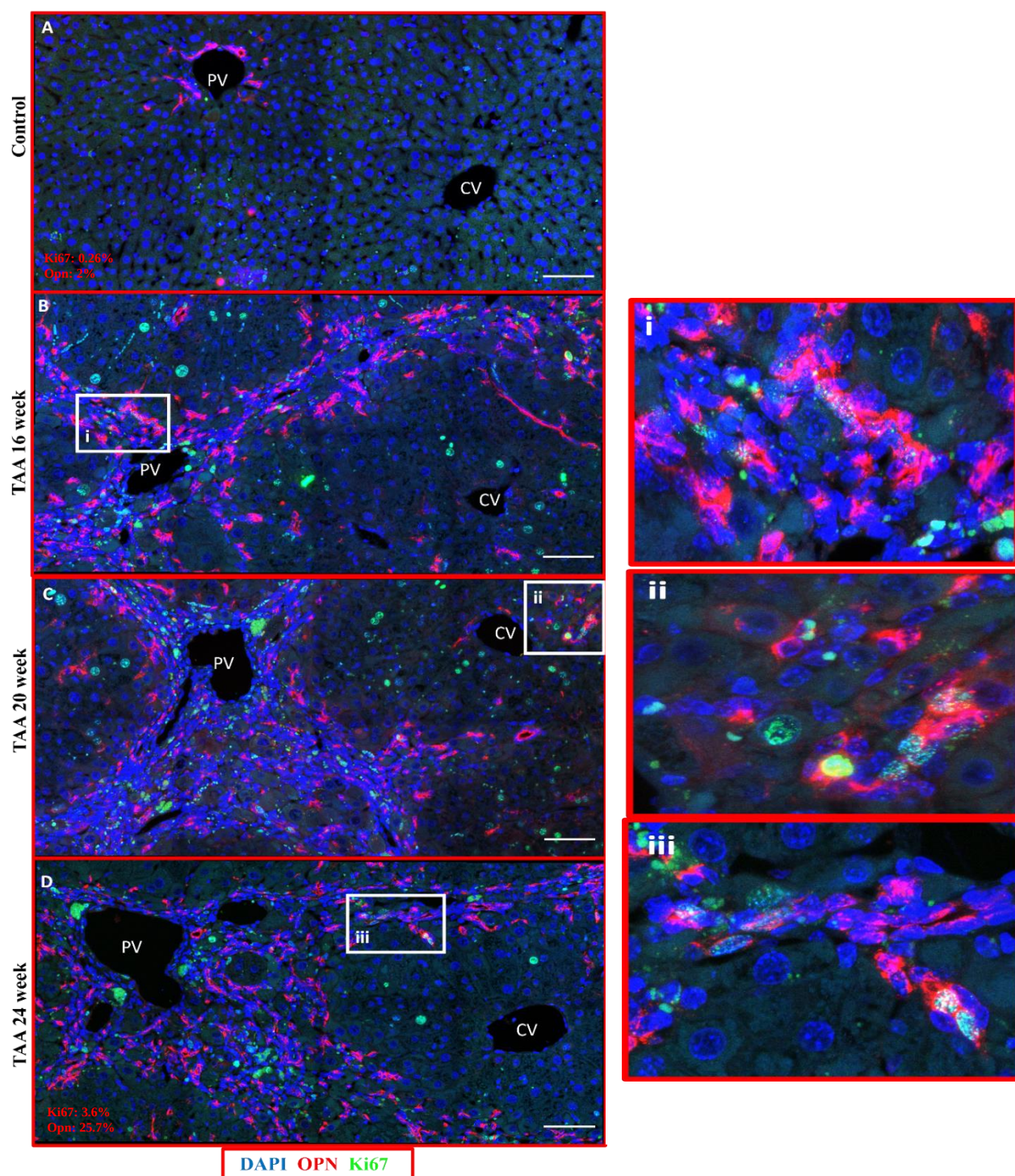


Figure 8: Tile scan images of Opn and Ki67 fluorescence staining for control (A), TAA 16-week (B), TAA 20-week (C), and TAA 24-week (D). All images showed pericentral, parenchyma, and pericentral zones from left to right. Zoomed panels i, ii, iii belonged to B, C, and D parts, respectively. For every different TAA sample, zoomed panel demonstrated Ki67-Opn overlaps clearly. At the left bottom of images, percentages showed the ratio of the marker to DAPI for quantification. DAPI was used for staining the nucleus. Scale bar represented 200 μ m. (PV: Portal Vein; CV: Central Vein)

After showing proliferative cholangiocytes with the help of Opn and Ki67 markers, we needed to demonstrate proliferation in the cholangiocytes by marking these cells with another marker, Arl13b, since the heart of the project was the Arl13b⁺ cholangiocytes. As mentioned in sections 1.3.2 and 1.5, every cholangiocyte has a primary cilium whose axoneme is marked by Arl13b. Thus, by conducting Arl13b and Ki67 fluorescence staining, we aimed to demonstrate the growth of Arl13b⁺ cholangiocytes for different weeks of TAA administration. Although we observed proliferative Opn⁺ cholangiocytes (Figure 7), unexpectedly we didn't detect any overlap for Ki67 and Arl13b for both control and TAA samples (Figure 9 A-D, i-iv). Therefore, we couldn't detect any proliferative Arl13b⁺ cholangiocytes in the fibrotic liver.

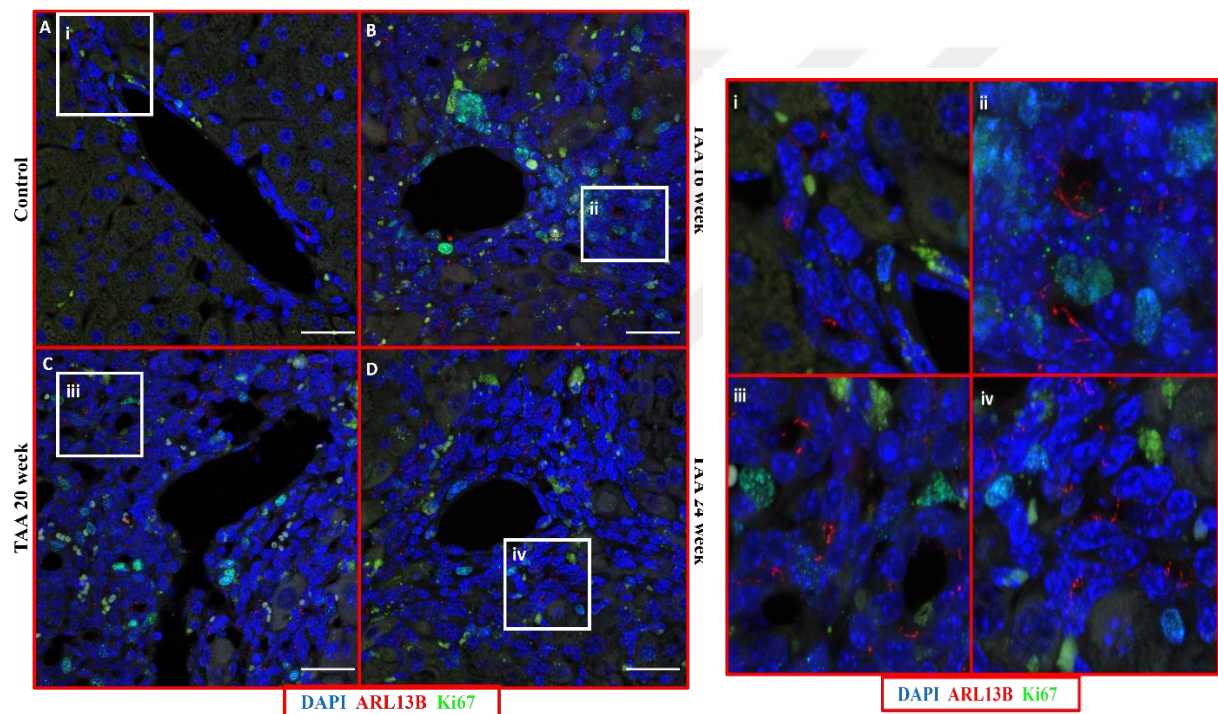


Figure 9: IHC against primary cilium marked by Arl13b (red) and proliferation showed by Ki67 (green). A, B, C, and D panels indicated portal veins of control and different TAA samples whereas i,ii,iii and iv showed zoomed areas from A, B, C, and D panels, in that order. There was no expression for Arl13b (n=19, n=4, n=3, n=11) and Ki67 (n=8, n=2, n=2, n=4) at the same cell in the control, TAA16-week, TAA 20-week, and TAA 24-week samples, respectively. DAPI was used for staining the nucleus. Scale bar represented 50µm.

4.2.1.1 Confirmation of the Primary Cilium in the Cholangiocytes

Following some confusing results regarding the presence of proliferative cholangiocytes but the absence of proliferative Arl13b⁺ cholangiocytes in the disease state, we decided to check

two important points. The first one was directly related to the specificity of Arl13b for primary cilium. For this purpose, we marked primary cilium in both fibrotic and homeostatic liver by Arl13b and another common marker PCNT which showed the exit point of primary cilium from the cell. In the IHC staining, we verified primary cilium in both control (Figure 10A) and TAA 24-week samples (Figure 10B). How primary cilium was protruding from the cell membrane and connecting to other cells was clear by a link between Arl13b and PCNT under steady and disease states (Figure 10i, ii).

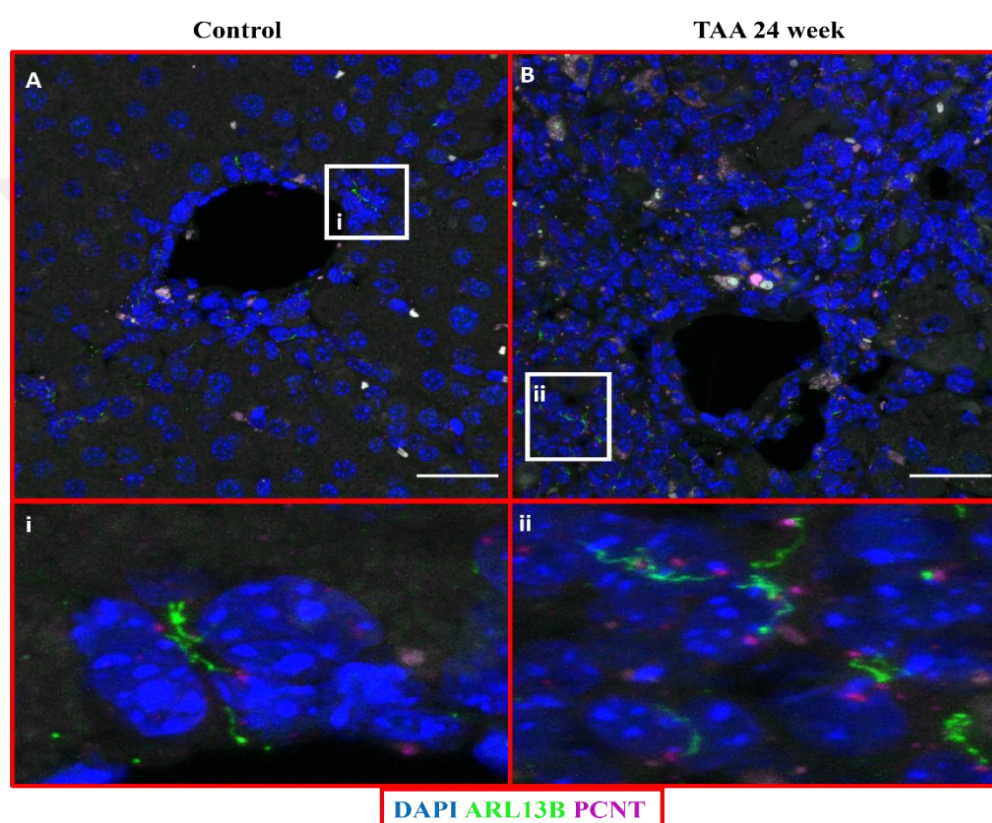
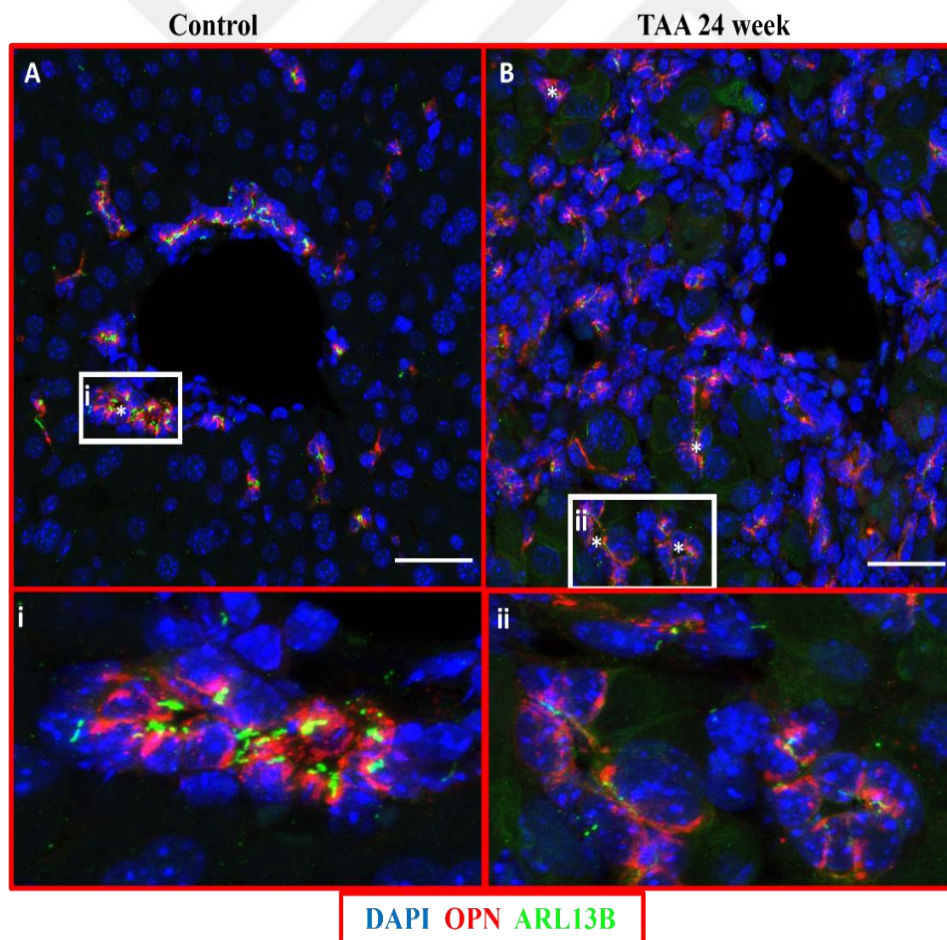


Figure 10: Primary cilium marked by Arl13b (green) (n=19,11) and PCNT (magenta) (n=2,2) in the control (A) and TAA 24-week (B) liver tissues by IHC. Zoomed panels (i and ii) showed a clear structure of primary cilium which was located around portal veins of control and fibrosis samples. DAPI was used for staining the nucleus. Scale bar represented 50 μ m.

As a second point, we focused on explaining whether primary cilium was specifically located in the cholangiocytes in both control and TAA samples and how primary cilium structure or expression level was altered during fibrosis. Identification of primary cilium response to fibrosis was one of the novel points of this study. Under the light of these questions, we performed Arl13b and Opn co-staining in the control and TAA 24-week samples. We observed almost one hundred percent overlap for Arl13b and Opn which means primary cilium was

specifically structured in the cholangiocytes of the liver under homeostatic (Figure 11A) and fibrotic (Figure 11B) conditions. Arl13b clearly marked primary cilium in the Opn+ cholangiocytes (Figure 11i and ii). When fibrosis and control samples were compared in terms of their primary cilium level, it was obvious that the number of primary cilia increased around the portal vein of the fibrotic liver compared to control. (Figure 11C, Mann Whitney test, $p < 0.0001$). Hence, we could easily conclude that cholangiocytes continued to keep their primary cilia despite of fibrosis.

As mentioned in section 1.3.2, cholangiocytes were located around the portal vein and the bile duct under homeostatic conditions. Control sample showed that Arl13b+ cholangiocytes were present surrounding the bile duct, supporting the literature (Figure 11i). When we checked the fibrotic liver, interestingly we observed an increase in the number of bile duct structures around the portal vein (Figure 11ii). These bile ducts were surrounded by lots of Arl13b+ cholangiocytes similar to the control sample.



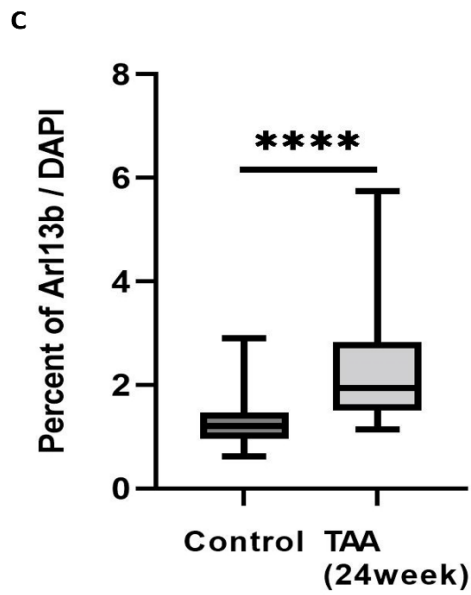


Figure 11: Validation of specific localization of primary cilium (marked by Arl13b, green) in the cholangiocytes (showed by Opn, red) by IHC. Both A and B panels were representations of portal veins of control and TAA 24-week samples, respectively. Zoomed panel i contained bile duct of control sample whereas bile ducts of fibrotic liver were shown in the zoomed panel ii. DAPI was used for staining the nucleus. Scale bar represented 50 μ m. (* : bile duct). (C) Quantification of immunostaining experiment by the ratio of specific signal to DAPI, respectively. Data presented as mean $\pm$ SEM, Mann-Whitney test, $P < 0.0001$

In addition to Opn-Arl13b co-staining, one more IHC experiment was designed by Sox9, Opn, and another well-known marker for primary cilium, α -acetylated tubulin, to confirm the presence of primary cilium in the cholangiocytes. We obtained the same results (Figure 11) with this staining (Figure 12). Sox9⁺ and Opn⁺ cholangiocytes were positive for α -acetylated tubulin around the portal vein (Figure 12A-D). Fibrotic sample (Figure 12B and D) could be distinguished from control sample (Figure 12A and C) with the help of an increase in the α -acetylated tubulin expression, so primary cilium level in the cholangiocytes.

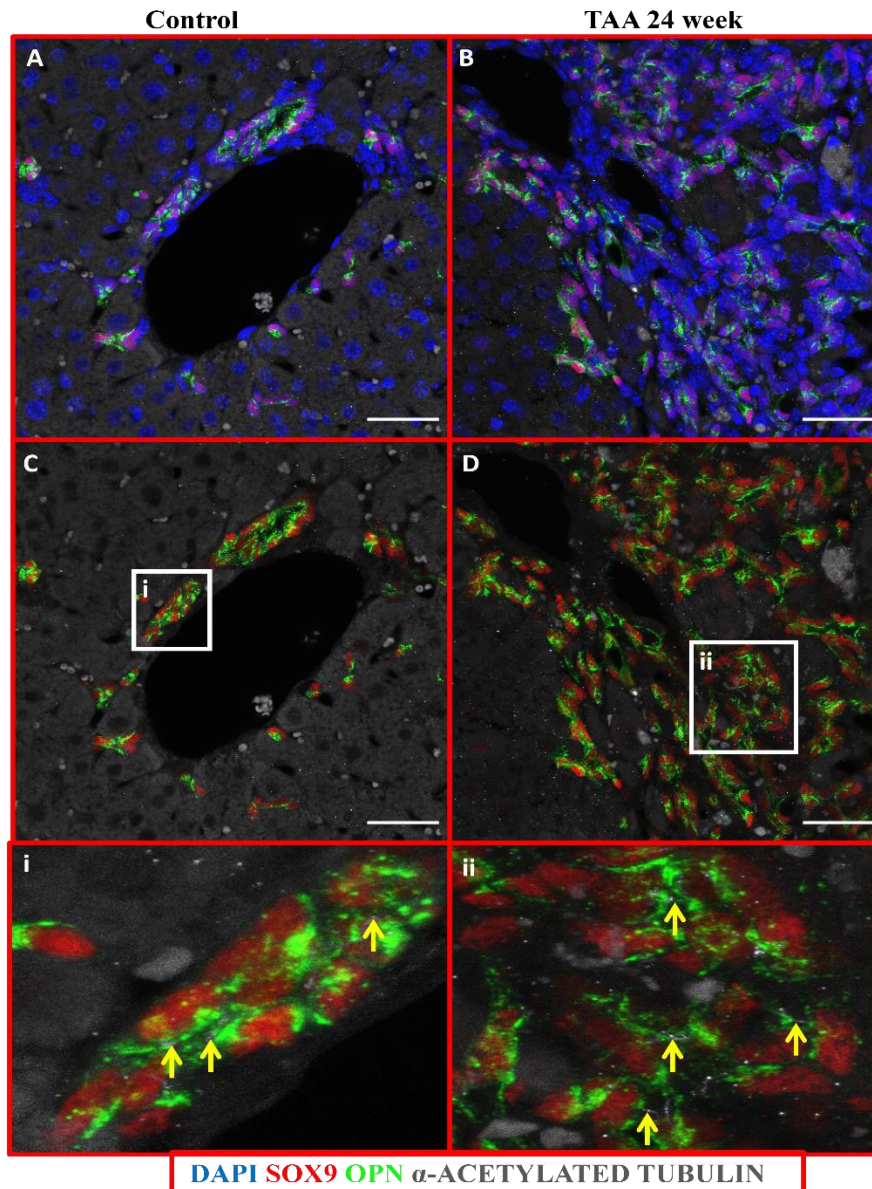


Figure 12: Sox9 (red)(n=4, n=4), Opn (green) (n=6,n= 6), and α -acetylated tubulin (grey) (n=2, n=2) staining for the portal regions of the control (A and C) and TAA 24-week (B and D) samples, respectively. Panel C and D showed Sox9, Opn and α -acetylated merged without DAPI. Panel i demonstrated zoomed version of a particular region in the C part whereas panel ii indicated a zoomed region in the panel D. Yellow arrows showed primary cilium. DAPI was used for staining of nucleus. Scale bar represented 50 μ m.

After observation of the increased level of primary cilium around the portal vein of fibrotic liver, we asked whether migratory cholangiocytes through portal-portal and portal-central axis expressed primary cilium or not (Figure 6). To exhibit this, tile scan images of Opn-Arl13b staining were used. Cholangiocytes generating a massive path between portal veins and cholangiocytes expanding separately through the central vein still contained primary cilium in the fibrotic state (Figure 13B). However, as observed in the previous figures no Arl13b+ cholangiocytes were observed in the pericentral region and midzone of the homeostatic liver (Figure 13A).

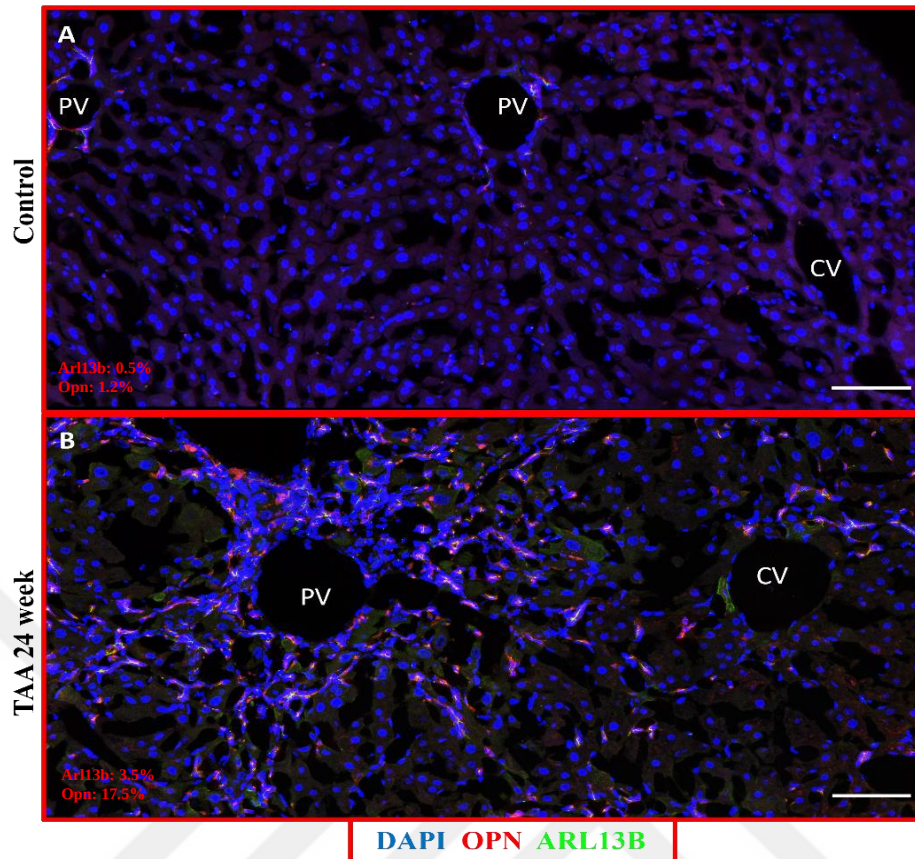


Figure 13: Tile scan images of Arl13b (green) and Opn (red) fluorescence staining for control (A) and TAA 24-week (B) samples. Both images included portal to central veins from left to right. At the left bottom of images, percentages showed the ratio of the marker to DAPI for quantification. DAPI was used for staining of nucleus. Scale bar represented 200μm. (PV: Portal Vein; CV: Central Vein)

To sum up, we could briefly conclude that primary cilium was a specific structure for cholangiocytes under both fibrotic and homeostatic conditions. Upon fibrosis, expanded and migratory cholangiocytes kept their primary cilium but while proliferating, they did not.

4.2.2 Responses of the Hepatocytes to Fibrosis

After investigation of cholangiocyte responses to fibrosis, we gave priority to check the reaction of hepatocytes under disease conditions. To achieve this goal, we performed Hnf4α and E-cadherin staining by using control and TAA 24-week animal samples. As we expected, we saw a high number of hepatocytes in both samples (Figure 14A and B). Cholangiocytes and other cell types were proliferating around the portal vein and these growing cells occupied the area nearby the portal vein in the fibrotic liver (Figure 14B). Thus, in TAA 24-week sample hepatocytes were a bit far away from the portal vein compared to their location in the control

samples (Figure 14A, B). As mentioned previously in section 4.1.3, we proved again the appearance of ballooned hepatocytes in the fibrotic liver with the help of Hnf4 α and E-cadherin staining (Figure 14B).

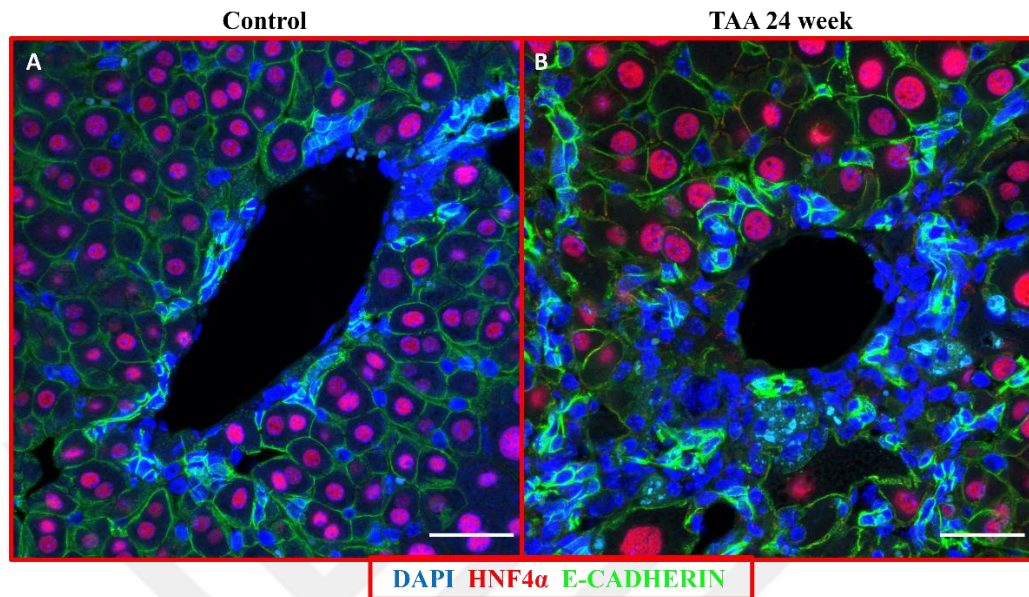


Figure 14: IHC staining for Hnf4 α (red) and E-cadherin (green) in the control (A) (n=4, n=3) and TAA 24-week sample (B) (n=4, n=3). Portal images of homeostatic and fibrotic livers were illustrated in both images. DAPI was used for staining the nucleus. Scale bar represented 50 μ m.

Hepatocytes are proper circle shaped cells and can be distinguished from other cell types with the help of this morphological form. Some hepatocytes were proliferating in all different week samples of fibrosis without showing any zonal preference (Figure 8B, C, D). However, during this growth, they didn't form a cell mass as observed in the proliferating cholangiocytes. They chose to proliferate individually.

4.2.2.1 Conversions Between HCs and BECs Under the Fibrosis

We mentioned in section 1.3.1 that due to severe fibrosis, hepatocytes cannot have enough capacity to regenerate the liver by proliferating from existing hepatocytes. Therefore, cholangiocytes differentiate into hepatocytes to maintain the hepatocyte level throughout the liver. Although we observed proliferation of both hepatocytes and cholangiocytes in TAA 16, 20, and 24-week samples, we wanted to assess the possibility of differentiation from cholangiocytes to hepatocytes or vice versa. In order to investigate this, we conducted IHC experiments against Hnf4 α and Opn. Since if there was a trans-differentiation between them, we expected to observe a bi-phenotypic, intermediate cell type which should express both

hepatocyte and cholangiocyte markers at the same time. According to the outcomes of our experiments, we observed that there was no overlap between Opn and Hnf4 α in zones of fibrotic liver (Figure 15B). As expected, we saw the same results for the control liver (Figure 15A).

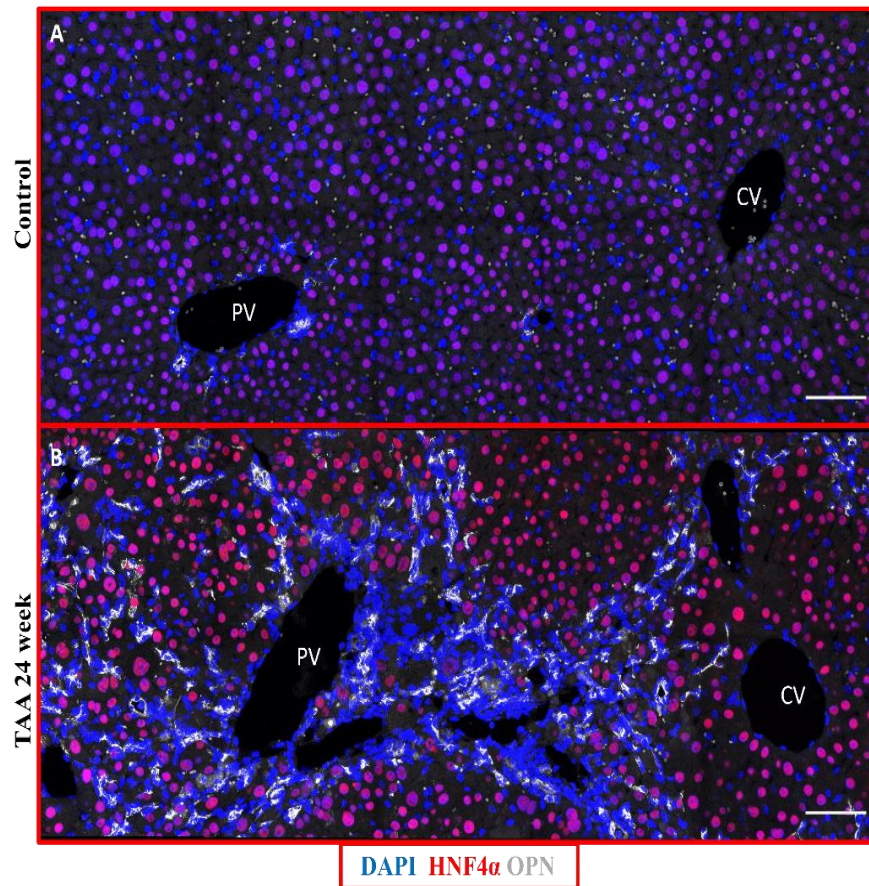


Figure 15: Tile scan images of fluorescence staining against Hnf4 α (red) and Opn (grey) in the control (A) and TAA 24-week (B) samples. Both tile scan images showed periportal, midzone and pericentral regions of the liver. DAPI was used for staining of nucleus. Scale bar represented 200 μ m. (PV: Portal Vein; CV: Central Vein)

We re-assessed this hypothesis regarding differentiation using Arl13b and Hnf4 α markers in IHC. Hnf4 α + hepatocytes didn't express primary cilia and Arl13b+ cholangiocytes didn't show any clue about the expression of hepatocyte marker under fibrosis (Figure 16B). Hence, we concluded that there was no differentiation from cholangiocytes to hepatocytes or from hepatocytes to cholangiocytes during fibrosis. Hepatocytes and cholangiocytes were increasing by proliferation from their respective resident cell types.

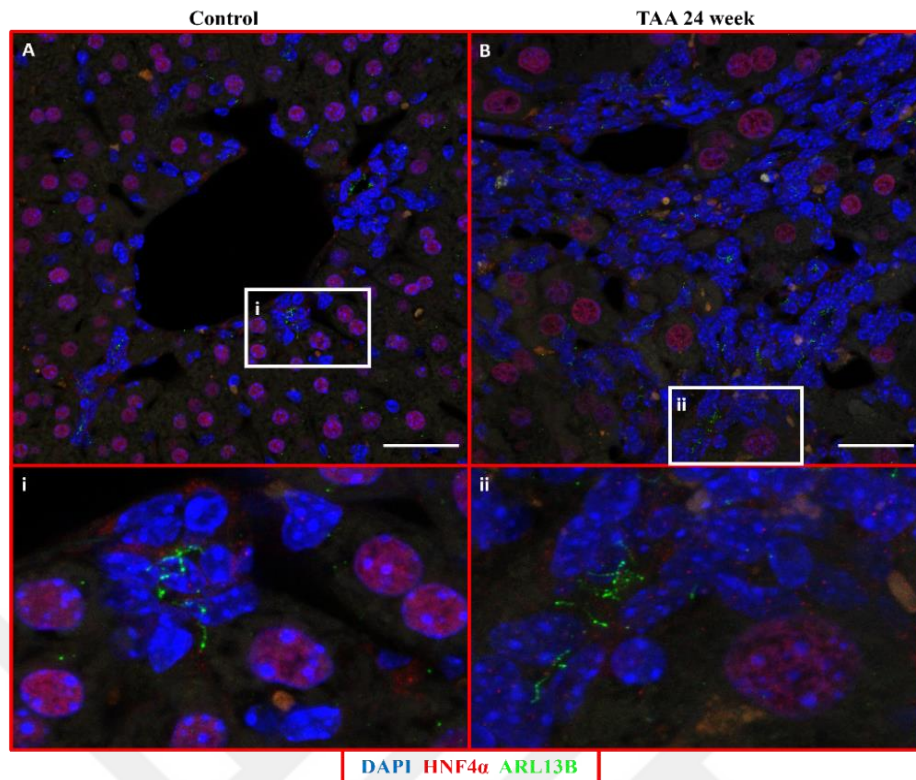


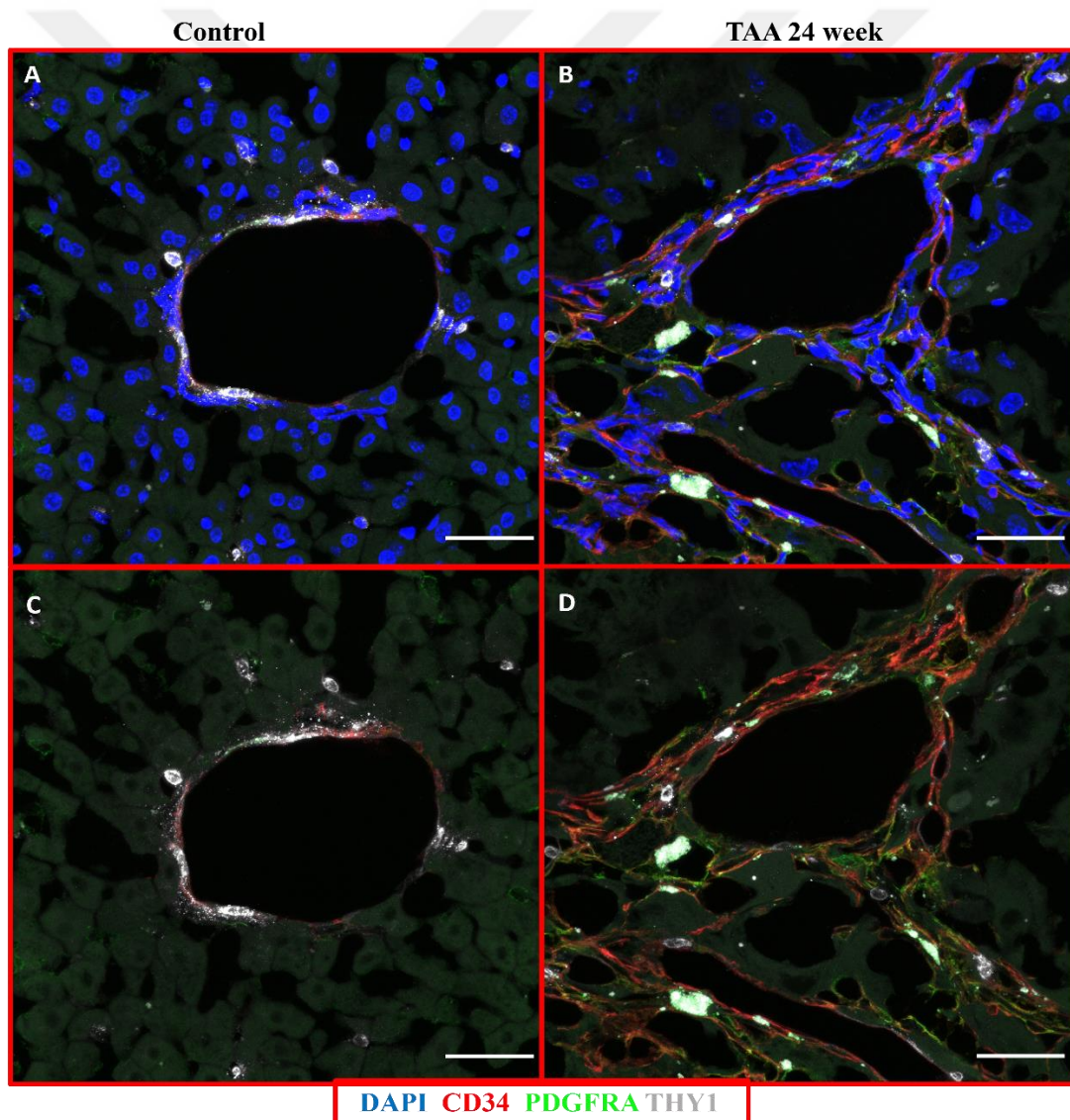
Figure 16: IHC experiment by using Hnf4 α (red) and Arl13b (green) in the control (A) (n=4, n=12) and experimental (B) (n=4, n=12) samples. Panel A and B indicated portal veins of the liver whereas panels i and ii showed zoomed regions in panels A and B, respectively. DAPI was used for staining the nucleus. Scale bar represented 50 μ m.

4.2.3 Responses of the Mesenchymal and Endothelial Cells to Fibrosis

In this part of the thesis, we examined the responses of mesenchymal cells such as portal fibroblasts, hepatic stellate cells, myofibroblasts and also, liver sinusoidal endothelial cells to fibrosis. Because there was no sharp marker to distinguish hepatic stellate cells and myofibroblasts from each other under fibrotic conditions, these cells were called as mesenchymal cells. We performed IHC staining by combining different mesenchymal markers. Thy1 was one of the common markers for portal fibroblasts whereas CD34 and Pdgfra were well-known mesenchymal markers for the liver. From this experiment, we obtained interesting results. First of all, in the portal vein of TAA 24-week sample we observed upregulation for CD34 and Pdgfra compared to control samples whereas Thy1 was downregulated in the fibrotic liver (Figure 17E, F, G, t-test, $p < 0.0001$, $p = 0.0011$, $p = 0.3403$, respectively). However, when we performed other Thy1 stainings in fibrotic and control samples, we observed increased expression of Thy1 in the fibrotic samples (data not shown). Hence, we could not conclude the

effects of fibrosis on Thy1 expression (Figure 17G).

As another point of this experiment, we observed that Pdgfra and CD34 almost one hundred percent colocalized in both fibrotic and control samples. However, the majority of Thy1 signals followed a complementary expression path to CD34-Pdgfra co-expression while a minority of Thy1⁺ cells expressed CD34 and Pdgfra under disease conditions. Hence, in TAA 24-week sample we saw low levels of overlap for these three mesenchymal markers. Nevertheless, in the control sample, Thy1 showed approximately one hundred percent overlap with CD34 and Pdgfra (Figure 17A). Also, we noticed that Thy1 high/low and CD34 high/low mesenchymal cell populations existed in the diseased sample (Figure 17B). This was another key clue for the heterogeneity of mesenchymal cells in the liver.



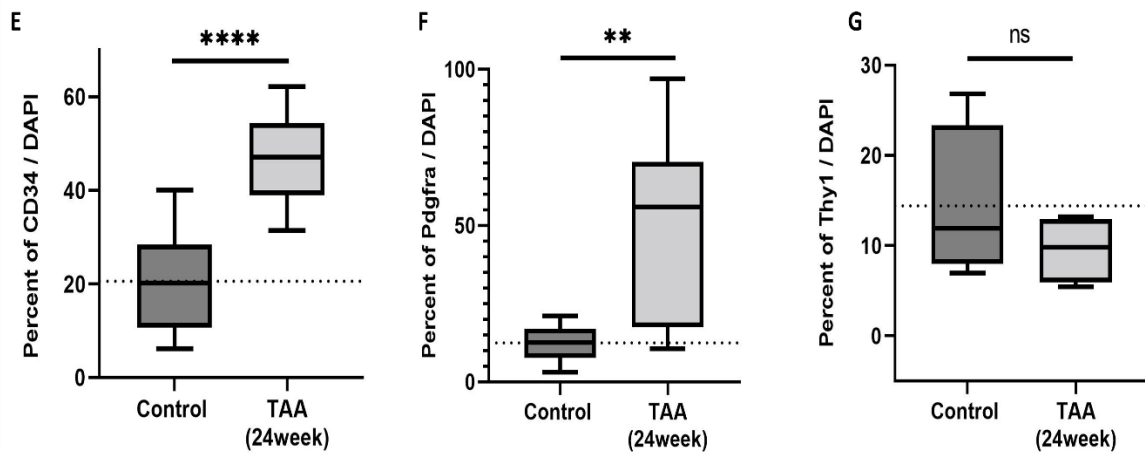


Figure 17: CD34 (red), Pdgra (green) and Thy1 (grey) fluorescence staining for control (A, C) (n=4, n=3, n=4) and TAA 24-week sample (B, D) (n=4, n=3, n=4). A and B parts showed DAPI merged version of CD34, Pdgra, and Thy1 staining whereas panels C and D indicated this staining without merging with DAPI to see every signal and overlap clearly. DAPI was used for staining the nucleus. Scale bar represented 50 μ m. (E, F,G) Quantification of immunostaining experiment by the ratio of specific signal CD34, Pdgra, and Thy1 to DAPI, respectively. Data presented as mean $\pm$ SEM, t-test, $P < 0.0001$, $P = 0.0011$ and $P = 0.3403$, respectively.

Tile scan images of CD34, Pdgra, and Thy1 fluorescence staining were analyzed so that we could compare the expression of these markers in the other zones of the liver under steady and disease states. Migratory cell masses which were formed between portal veins express CD34 and Pdgra similar to expanded cells through the central vein (Figure 18B). However, these migratory and expanded cells showed less Thy1 expression compared to CD34 and Pdgra in fibrosis. In addition to that, generally in these cell masses CD34 and Pdgra positive cells were not the ones in which Thy1 was expressed. Therefore, it was evident that there were not many cells which could express all these markers at the same time under fibrosis. Furthermore, Pdgra expression was distributed among all regions of the homeostatic liver whereas CD34 was observed mainly around the portal vein (Figure 18A). Thy1 was also localized mainly around the portal vein, but several Thy1+ cells which were negative for CD34 and Pdgra were present in close proximity to the central vein in controls. To sum up, we could say that CD34+ and Pdgra+ mesenchymal cells responded to fibrosis by increasing their populations without zonal preference whereas Thy1+ portal fibroblasts required more analysis. (Figure 17 and Figure 18).

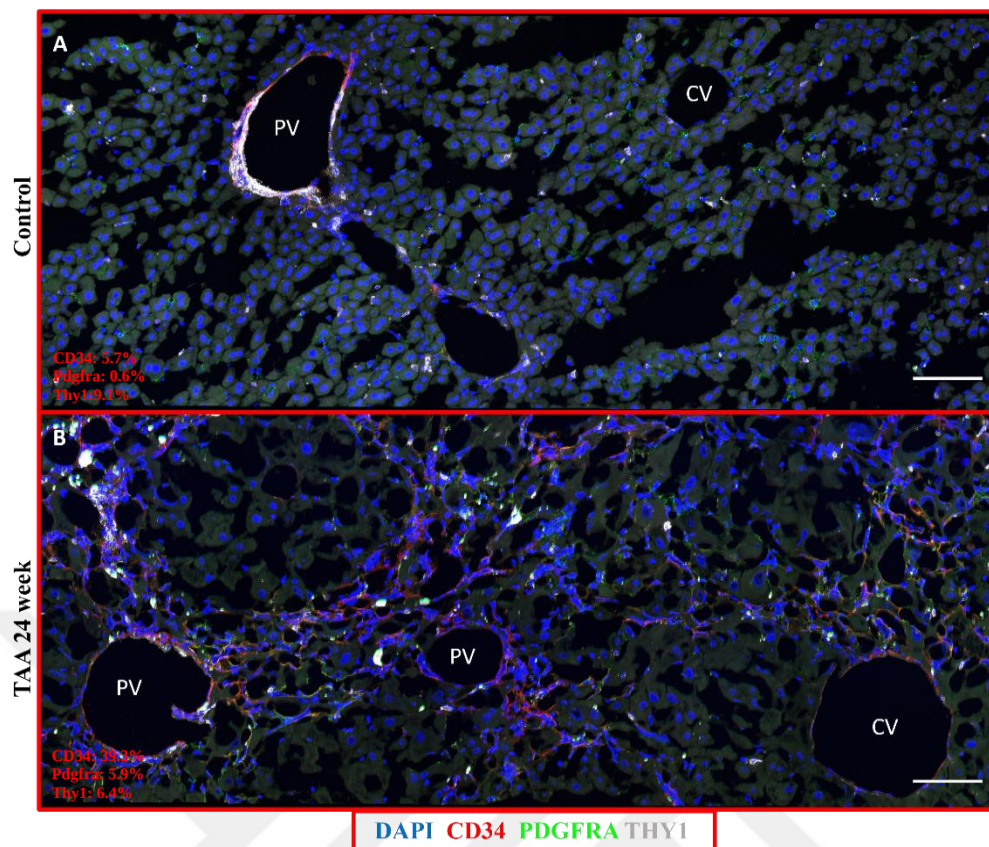


Figure 18: Tile scan images of CD34 (red), Pdgfra (green), and Thy1 (grey) IHC staining for control (A) and TAA 24-week (B) samples. Both tile scan images described expression profiles of these markers throughout all zones of the liver. At the left bottom of images, percentages showed the ratio of the marker to DAPI for quantification. DAPI was used for staining the nucleus. Scale bar represented 200 μ m. (PV: Portal Vein; CV: Central Vein)

In addition to the mesenchymal part, we aimed to monitor responses of the liver sinusoidal cell during fibrosis. For this goal, Lyve1 and Vimentin fluorescence staining was performed in control and TAA 24-week samples. Lyve1 was a typical LSEC marker whereas Vimentin was another significant marker for mesenchymal cells. We observed more Lyve1+ cells around the portal vein of fibrotic tissue than controls (Figure 19A, B). However, when we checked other portal veins of both samples (data not shown), Lyve1 expression was not stable. We saw lots of Lyve1+ cells around some portal veins of both samples whereas other portal regions didn't contain any Lyve1+ cells. Thus, we concluded that Lyve1 level for control and TAA 24-week samples were not comparable.

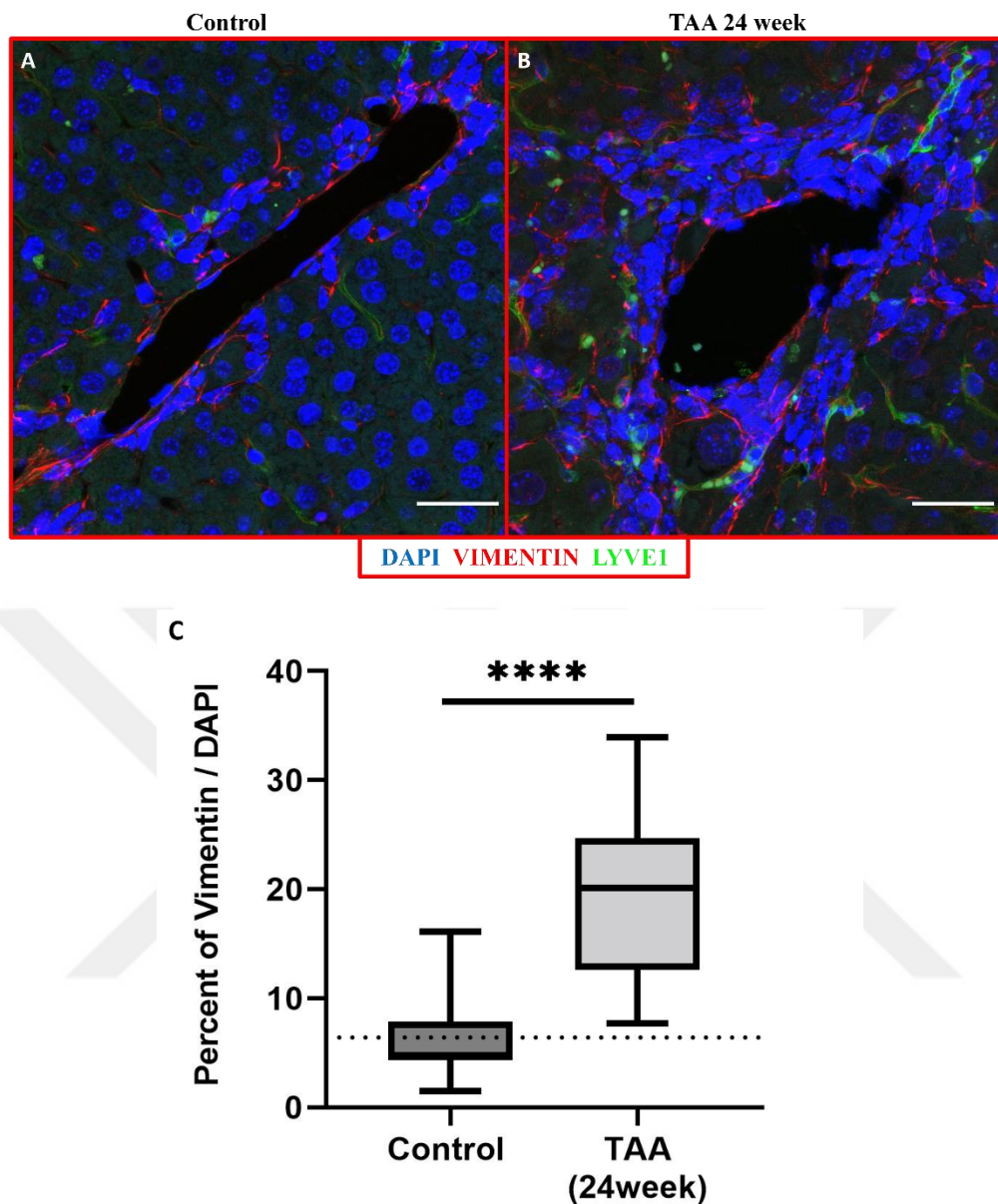


Figure 19: Control liver (A) and fibrotic liver (B) IHC staining showing Vimentin (red) (n=4, n=4) and Lyve1 (green) (n=2, n=2). Both A and B panels contained portal veins of the liver. DAPI was used for staining the nucleus. Scale bar represented 50 μm. (C) Quantification of immunostaining experiment by the ratio of specific signal to DAPI. Data presented as mean ± SEM, t-test, $P < 0.0001$.

Apart from the portal vein, we wanted to check the expression pattern of Lyve1 in the parenchyma and central vein. Lyve1 expression was visible in every zone of the fibrotic and normal liver tissues (Figure 20A and B). Unfortunately, the unstable expression of Lyve1 was still valid for other regions of the liver. Therefore, the expression of Lyve1 was not homogenous throughout the liver. Nevertheless, we could easily comment that migratory cells along portal to portal and portal to central axis were negative for Lyve1 (Figure 20B). This means that

LSECs were not the constituents of the expanded cell masses/several cells between portal veins and portal-central veins, respectively.

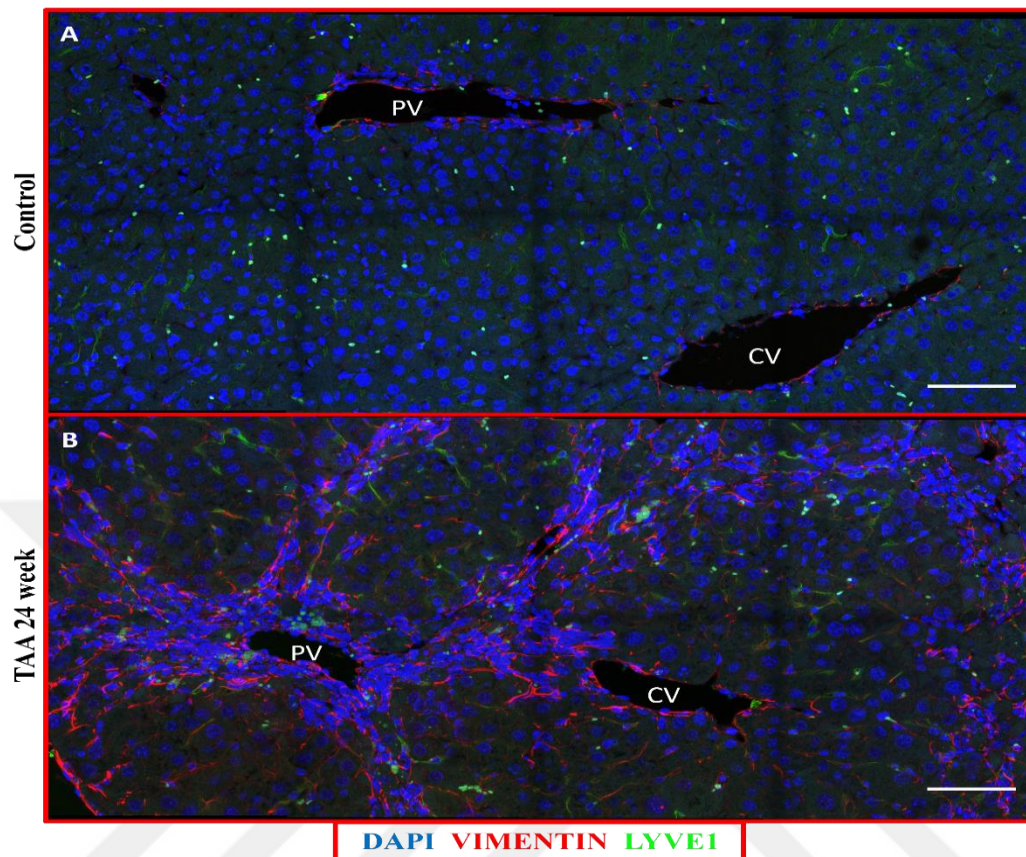


Figure 20: Tile scan images of IHC staining conducted for Vimentin (red) and Lyve1 (green) markers in the control (A) and TAA 24-week (B) samples. All images showed portal vein, parenchyma, and central vein of the liver from left to right. DAPI was used for staining the nucleus. Scale bar represented 200µm. (PV: Portal Vein; CV: Central Vein)

Regarding Vimentin expression, we observed very similar expression profiles with CD34 and Pdgfra (Figures 19B and 20B). Vimentin level was evidently higher in fibrotic liver than control (Figure 19C, t-test, $P < 0.001$). Additionally, a majority of expanded cells through the portal and central veins was positive for Vimentin as observed in CD34 and Pdgfra. (Figure 20B). In tile scan images of the control sample, we saw the basal level of Vimentin around both central and portal veins (Figure 20A). Therefore, we decided that Vimentin⁺ mesenchymal cells increased in all regions of the fibrotic liver as observed in CD34⁺ and Pdgfra⁺ mesenchymal cells.

4.3 Epithelial to Mesenchymal Transition in Cholangiocytes During the Fibrosis

In previous figures, we mentioned that due to fibrosis, massive cell densities were formed

around the portal vein and these cells were migrating through portal vein and central veins as masses and individuals, respectively. We showed that the major constituent of these cell masses was Arl13b⁺ BECs (Figures 8D and 13B). Moreover, we saw significant Vimentin, CD34, Pdgfra expression, and minimal Thy1 expression in these cell masses and individuals. After these results, we questioned regarding whether Arl13b⁺ cholangiocytes in cell masses started to express these mesenchymal markers or Arl13b⁺ cholangiocytes were surrounded with mesenchymal cells as other components of these cell masses. If fibrosis promotes the expression of the mesenchymal markers in Arl13b⁺ cholangiocytes, this could be explained by epithelial to mesenchymal transition in the cholangiocytes. However, if Arl13b⁺ cholangiocytes and mesenchymal cells constitute the migratory cells in paths, mesenchymal cells could have a contribution for the movement of Arl13b⁺ cholangiocytes which are non-motile epithelial cells. At that point, we suggested two different hypotheses about migratory cell masses in the fibrotic liver. To evaluate these hypotheses, we performed sets of IHC stainings against epithelial, mesenchymal, and primary cilium markers. We began with CD34, Opn, and Arl13b staining by focusing on the area around the portal vein. Arl13b and Opn were co-expressed in the cholangiocytes which were negative for CD34 (Figure 21A, C, and i). Neighboring cells expressing CD34 surrounded these Arl13b⁺ cholangiocytes under homeostatic conditions. However, upon fibrosis, we observed two different cholangiocyte sub-populations around the portal vein. (Figure 21B, D). Arl13b⁺ cholangiocytes were very similar to control cholangiocytes in terms of their expression profile (Figure 21ii). Interestingly, we observed Arl13b⁺ Opn⁺ CD34⁺ cholangiocytes around the portal vein of fibrotic liver (Figure 21iii). In addition to that, CD34⁺, Opn⁻, Arl13b⁻ mesenchymal cells were another component of these cell masses around the portal vein (Figure 21B, D). In the light of these results, we proved both our hypotheses that some sub-populations of Arl13b⁺ cholangiocytes were undergoing partial EMT. They formed big cell masses with homeostatic cholangiocytes and mesenchymal cells. Characterization of these cell masses was another significant novelty of this thesis. Nevertheless, we needed to re-assess the accuracy of these hypotheses with other mesenchymal markers. Therefore, we repeated the same IHC experiments with Vimentin instead of CD34. We observed the same cholangiocyte subpopulations (Figure 21 ii and iii) and also, Vimentin⁺ mesenchymal cells (Figure 22B, D, ii, iii) under fibrosis. Still, we observed a homeostatic cholangiocyte subpopulation that didn't express Vimentin (Figure 22 ii) and another cholangiocyte subpopulation which upregulated Vimentin expression by undergoing partial EMT in fibrosis (Figure 22 iii).

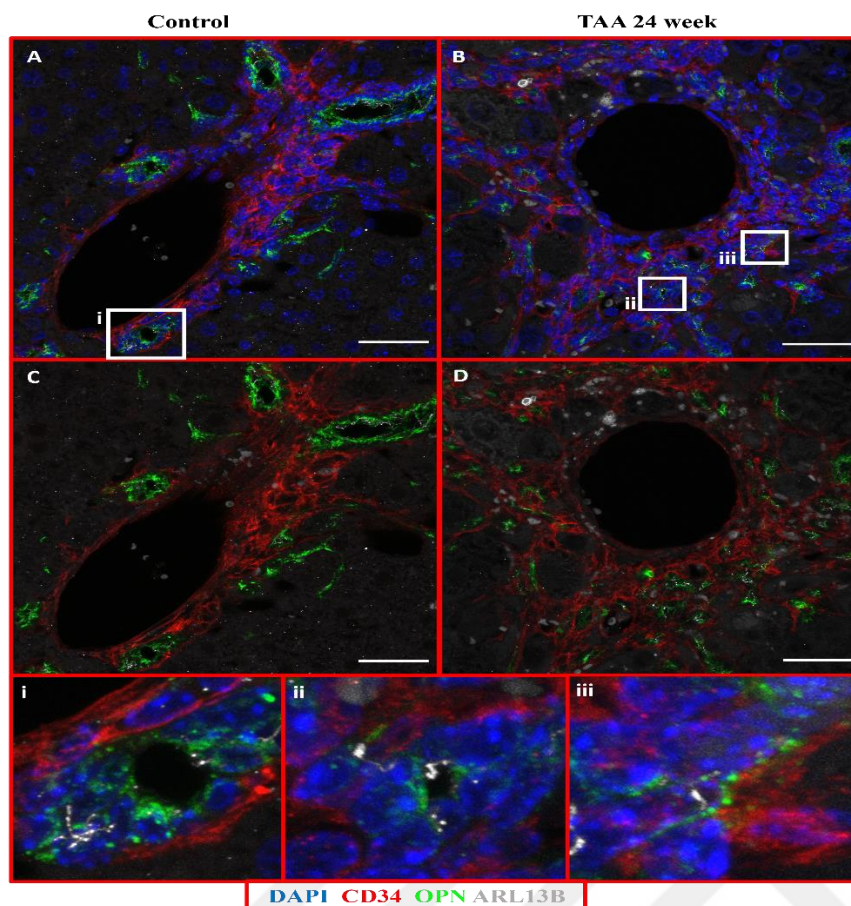


Figure 21: IHC staining for CD34 (red), Opn (green) and Arl13b (grey) in the portal veins of control (A, C) and TAA 24-week (B, D) samples. Panel C and D showed CD34, Opn and Arl13b merged without DAPI. Panel i demonstrated zoomed region in the A part whereas panel ii and iii indicated other zoomed regions in the panel B. DAPI was used for staining of nucleus. Scale bar represented 50µm.

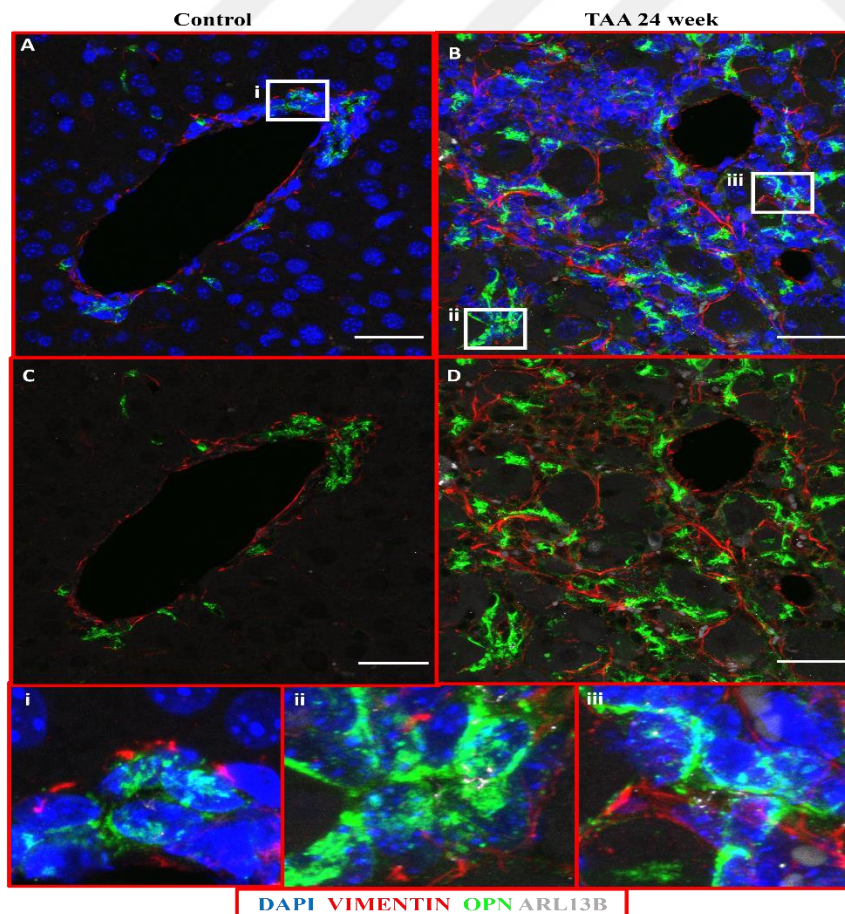


Figure 22: Fluorescence staining of Vimentin (red), Opn (green) and Arl13b (grey) in the portal regions of homeostatic (A, C) and TAA 24-week fibrosis (B, D) samples. Panel C and D showed CD34, Opn and Arl13b merged without DAPI. Panel i was an example of zoomed region in the A part whereas panel ii and iii showed other zoomed regions in the panel B. DAPI was used for staining of nucleus. Scale bar represented 50µm.

After CD34 and Vimentin staining, we focused on another mesenchymal marker, Pdgfra to prove a partial EMT in cholangiocytes. For this purpose, we performed Arl13b and Pdgfra fluorescence staining in control and TAA 24-week samples (Figure 23) and identified the same cholangiocyte subpopulations and Pdgfra+ mesenchymal cells around the portal vein (Figures 21 and 22).

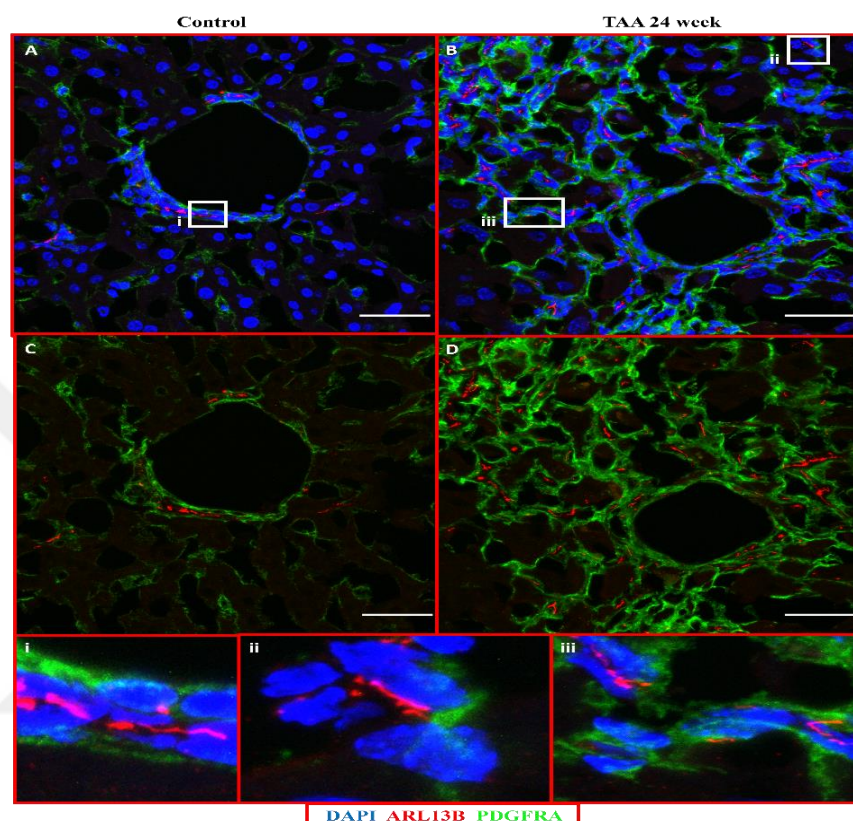


Figure 23: Upon homeostatic (A, C) and fibrotic (B, D) conditions, Arl13b (red) and Pdgfra (green) staining by indicating portal veins. Panel C and D showed Pdgfra and Arl13b merged without dapi. Panel i showed zoomed region in the A part whereas panel ii (homeostatic BEC) and iii (partial EMT undergoing BEC) clarified other zoomed regions in panel B. DAPI was used for staining of the nucleus. Scale bar represented 50 μ m.

Following analysis of cholangiocyte subpopulations and mesenchymal cells around the portal vein, we wanted to check the presence or absence of these populations in the migratory cell masses through the portal vein and expanded individual cells through the central vein. For this purpose, we checked tile scan images of E-cadherin-Vimentin-Opn, Arl13b-Pdgfra and CD34-Opn stainings. In the migratory cell densities and individual ones, we observed homeostatic cholangiocytes (shown in blue, yellow circles, and purple circles), partial EMT undergoing cholangiocytes (shown in black, white circles, and green circles), and mesenchymal cells (shown in orange, pink circles, and grey circles) under fibrotic conditions (Figures 24B, 25B and 26B). Partial EMT undergoing cholangiocytes obtained CD34, Vimentin, Pdgfra

expressions in addition to Arl13b and Opn expressions whereas homeostatic cholangiocytes could express Opn, Arl13b.

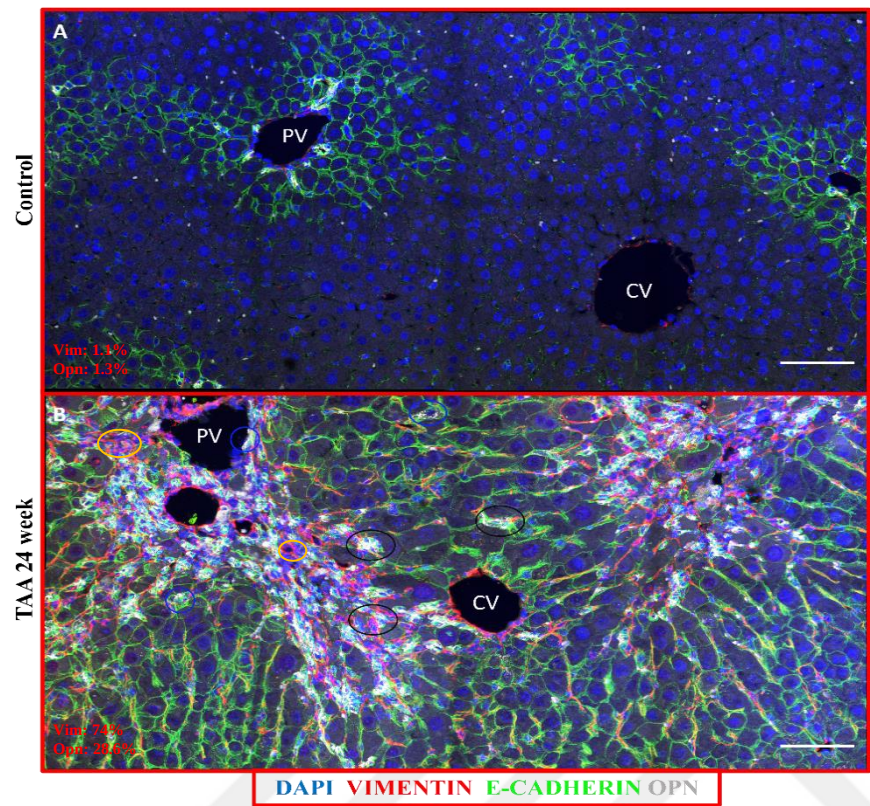


Figure 24: Tile scan images of Vimentin (red), E-cadherin (green) and Opn (grey) in control (A) and fibrotic (B) samples. Both images contained all three regions of the liver. Black circles were examples of partial EMT undergoing BECs, blue ones showed normal BECs and orange circles were for mesenchymal cells in B panel. At the

left bottom of images, percentages showed the ratio of the marker to DAPI for quantification. DAPI was used for staining of nucleus. Scale bar represented 200um.

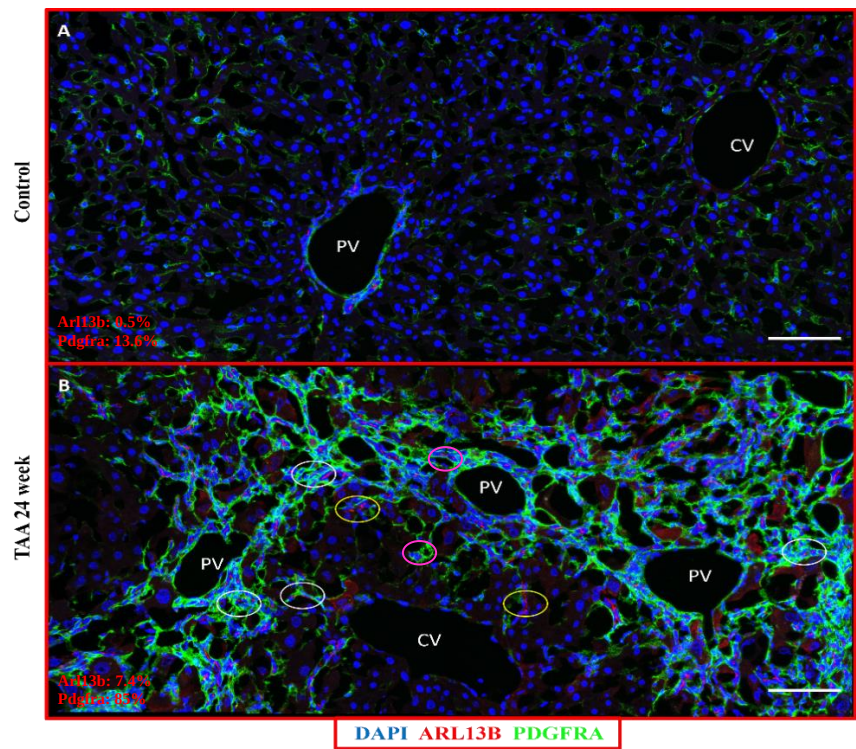


Figure 25: Tile scan images of Arl13b (red), and Pdgfra (green) in control (A) and fibrotic (B) samples. Both images displayed all zones of the liver. White circles were examples of partial EMT undergoing BECs, yellow ones showed normal BECs and pink ones demonstrated mesenchymal cells in B panel. At the left bottom

of images, percentages showed the ratio of the marker to DAPI for quantification. DAPI is used for staining of nucleus. Scale bar represented 2000 μ m. Portal vein; CV: Central vein

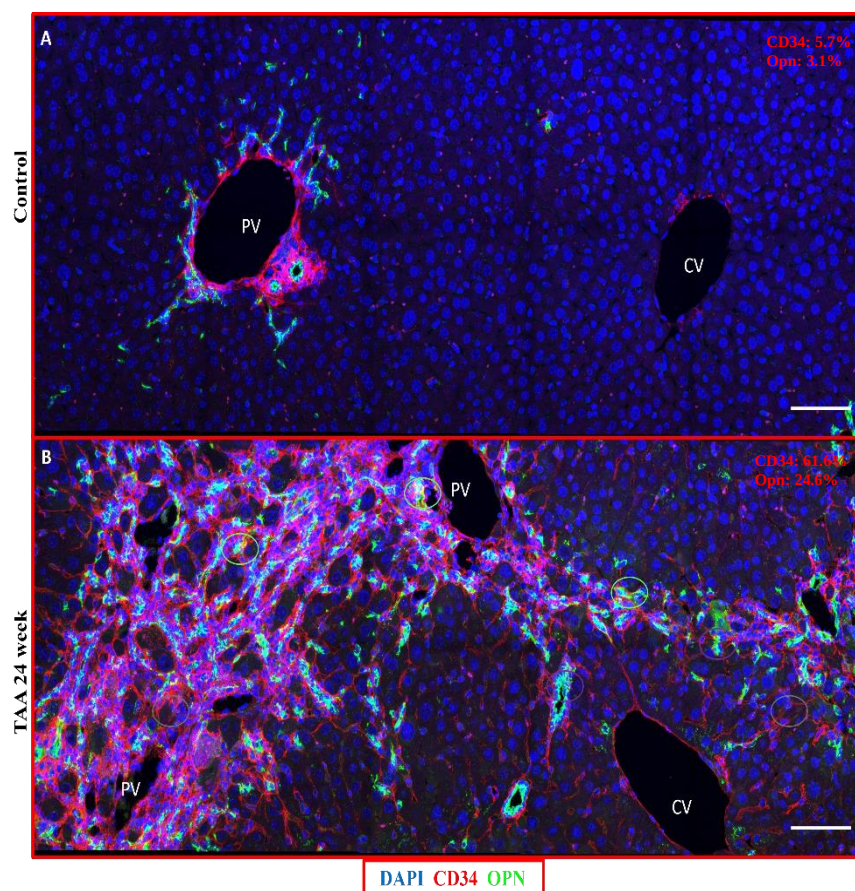


Figure 26: Tile scan images of CD34 (red), and Opn (green) in control (A) and TAA 24-week (B) samples. Both images displayed all zones of the liver. Green circles showed partial EMT undergoing BECs, purple ones showed homeostatic BECs and grey ones demonstrated mesenchymal cells in B panel. At the right top of images, percentages showed the ratio of the marker to DAPI for quantification. DAPI was

used for staining of nucleus. Scale bar represented 200 μ m. Portal vein; CV: Central vein

Previously, we observed cell masses with different densities expanding along portal-portal and portal-central axis in various weeks of TAA (Figure 8). Arl13b⁺ cholangiocytes were the main components of these cell masses. We showed in the last figures that partial EMT undergoing Arl13b⁺ cholangiocytes and mesenchymal cells were present in these cell masses upon TAA 24-week fibrosis. At that point, we were curious about whether partial EMT was happening in Arl13b⁺ cholangiocytes during TAA 16 and 20-week samples. As a first step, we preferred to check the periportal zone of different TAA samples by staining with E-cadherin, Vimentin, and Opn. Similar to TAA 24-week sample, we observed mesenchymal cells, partial EMT undergoing Arl13b⁺ cholangiocytes, and homeostatic Arl13b⁺ cholangiocytes around portal veins of TAA 16 and 20-week samples (Figure 27B, C, and D). However, there were fewer partial EMT undergoing Arl13b⁺ cholangiocytes in TAA 16-week samples compared to TAA 20 and 24-week samples.

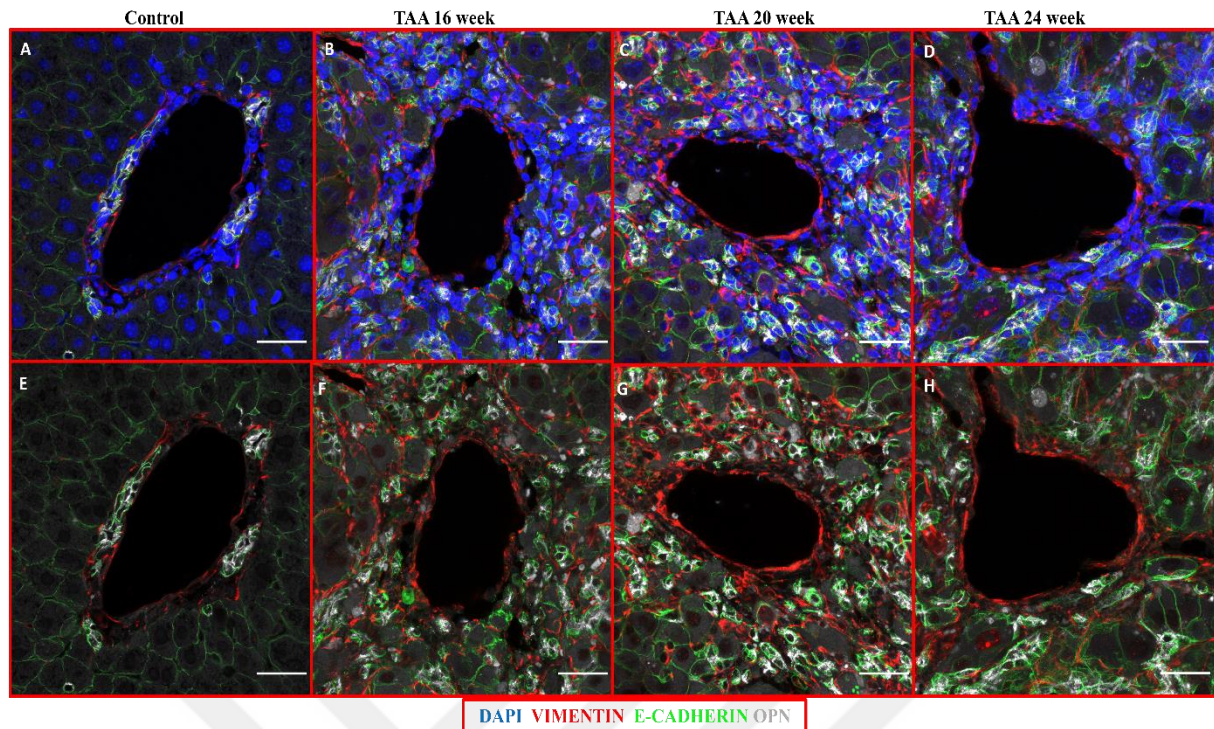


Figure 27: IHC staining performed with Vimentin (red), E-cadherin (green) and Opn (grey) markers in the control (A, E) (n=7,5,11), TAA 16-week (B, F) (n=1,1,3), TAA 20-week (C, G) (n=2,2,4), and TAA 24-week (D, H) (n=4,2,6) samples, respectively. All images showed the portal region of the indicated sample. Panel E, F, G, and H were corresponding Vimentin, E-cadherin, and Opn signals merged without DAPI. DAPI was used for staining the nucleus. Scale bar represented 50 μ m.

Secondly, we aimed to check the possibility of partial EMT in Arl13b⁺ cholangiocytes which were migrating between veins of TAA 16 and 20-week samples. For this purpose, we benefited from tile scan images of E-cadherin, Opn, and Vimentin IHC staining. As a consequence, we showed that in TAA 16 and 20-week samples some sub-populations of migratory Arl13b⁺ cholangiocytes along the central and portal vein were undergoing partial EMT similar to TAA 24-week sample (Figure 28B, C, and D). However, as expected, partial EMT undergoing Arl13b⁺ cholangiocyte level in these migratory cells was lower in TAA 16-week sample compared to other fibrosis samples. The highest level of partial EMT undergoing Arl13b⁺ cholangiocytes in expanded cell densities and individuals belonged to TAA 24-week sample (Figure 28D). Also, we observed mesenchymal cells in migratory cell masses apart from homeostatic and partial EMT undergoing Arl13b⁺ cholangiocytes in every TAA sample (Figure 28B, C, D).

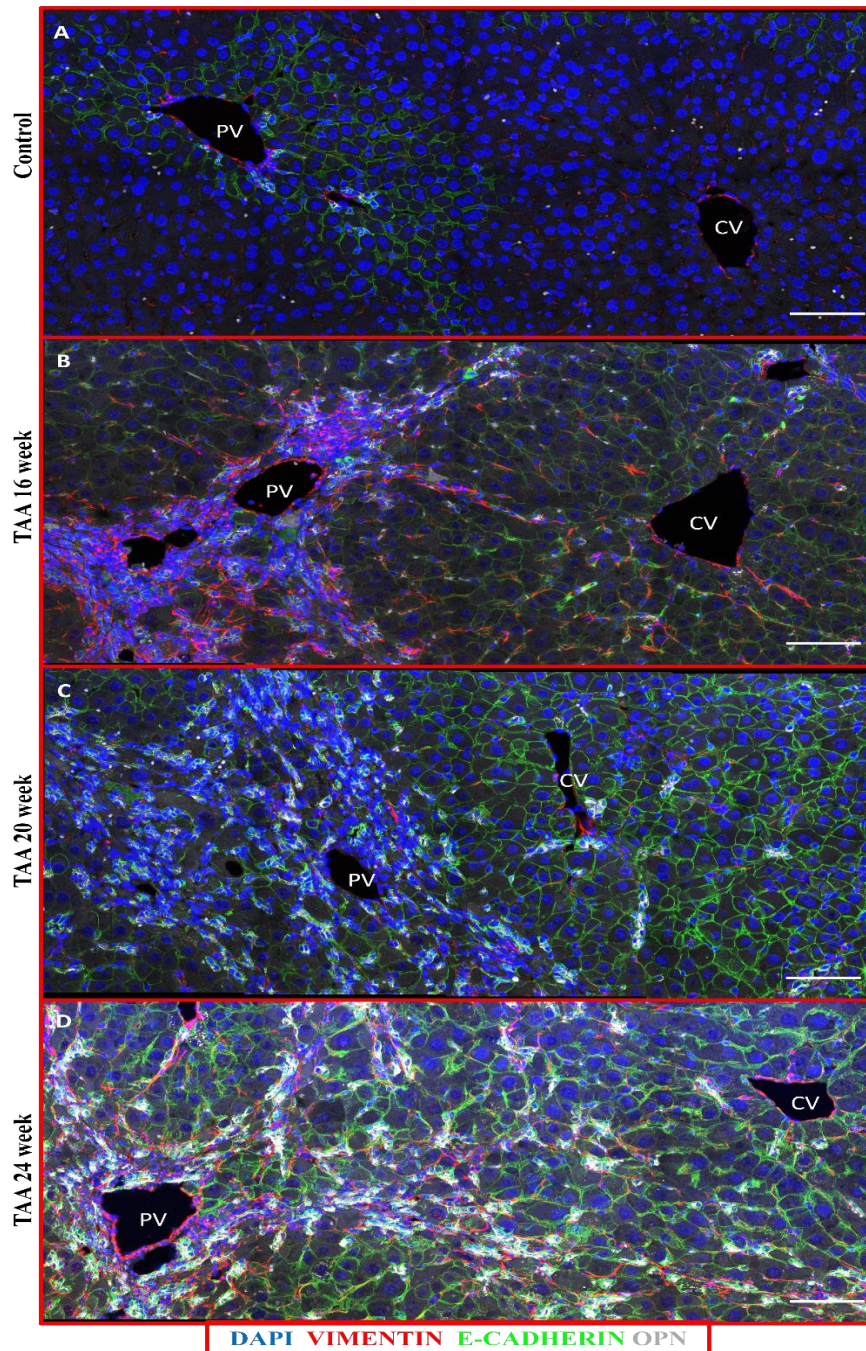


Figure 28: Tile scan images of Vimentin (red), E-cadherin (green) and Opn (grey) IHC experiment for the control (A), TAA 16-week (B), TAA 20-week (C) and TAA 25-week (D) samples. Portal vein, central vein and parenchyma regions were found in all tile scan images. DAPI was used for staining of the nucleus. Scale bar represented 200 μ m. PV: Portal vein; CV: Central vein

As mentioned previously, Thy1 expression in the expanded cell masses and individuals along the portal-portal and portal-central axis was very low (Figures 17 and 18). Despite of this observation, we wanted to re-assess whether partial EMT undergoing cholangiocytes showed expression for Thy1 as observed in other mesenchymal markers, CD34, Vimentin, and Pdgfra. We explained that there was a very low amount of expression for Thy1 in the Arl13b+ cholangiocytes around the portal vein under fibrosis (Figure 29B and D). However, some Thy1+ cells were located surrounding Arl13b+ cholangiocytes and constituted cell masses in addition to other mesenchymal cells.

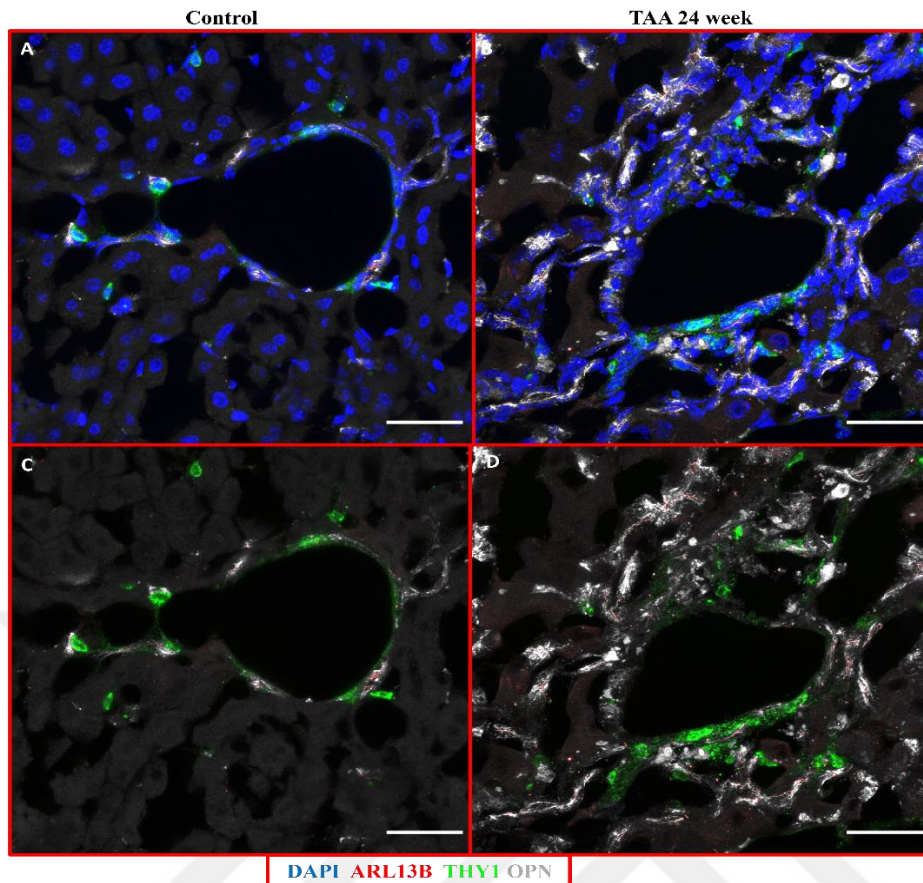


Figure 29: Arl13b (red), Thy1 (green), and Opn (grey) fluorescence staining in control (A, C) and TAA 24-week (B, D) samples. Both images belonged to portal veins of the liver tissue. Panel C and D were corresponding Arl13b, Thy1, and Opn signals merged without DAPI. DAPI was used for staining the nucleus. Scale bar represented 50 μ m.

When we checked the expression of Thy1 in expanded Arl13b⁺ cholangiocytes with the help of tile scan images of Thy1, Opn, and Arl13b staining (Figure 30B), we observed similar results (Figure 29B). Also, we saw a lower amount of Thy1⁺ cells around expanded Arl13b⁺ cholangiocytes compared to Thy1⁺ cells around the portal vein (Figure 30B). Hence, we concluded in this section that Thy1 could not be accepted as an EMT marker for partial EMT undergoing cholangiocytes. Possibly, Thy1⁺ portal fibroblasts could be another contributor of niche components for partial EMT undergoing cholangiocytes in the cell masses similar to other mesenchymal cells (Figure 17B and 29B).

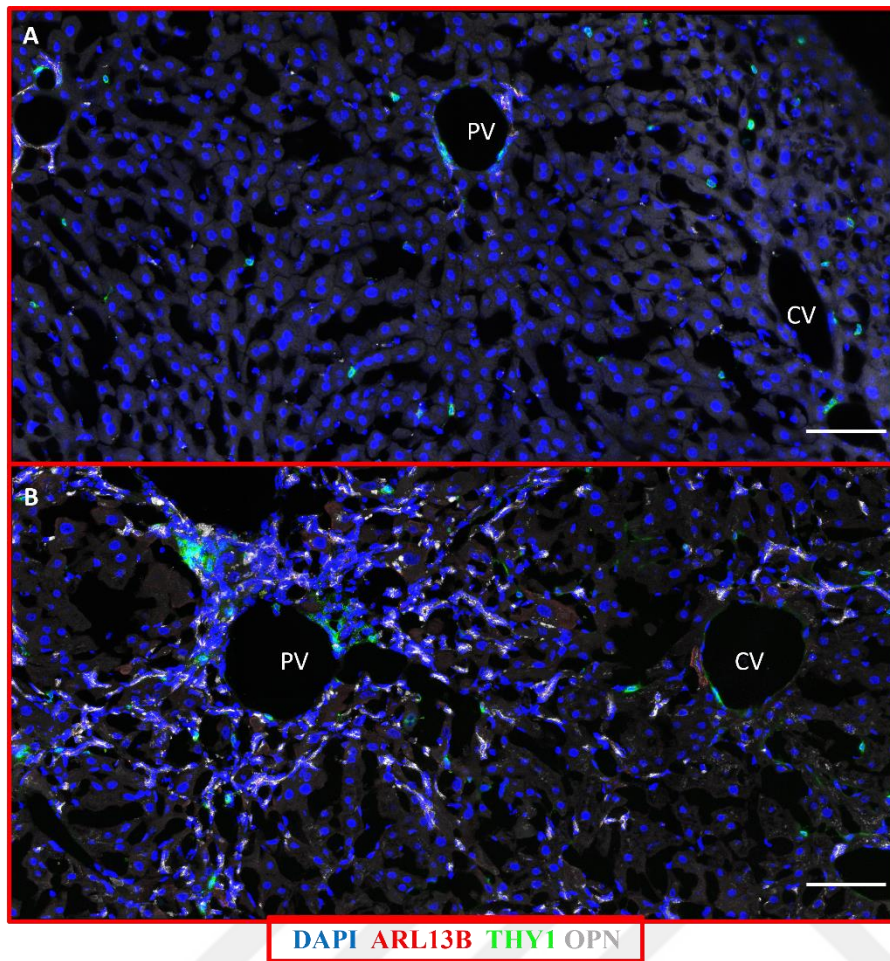


Figure 30: Tile scan images of Arl13b (red), Thy1 (green) and Opn (grey) staining in homeostatic (A) and 24-week duration of fibrotic (B) liver tissues. Both images indicated periportal, midzone and pericentral areas of the liver. DAPI was used for staining of nucleus. Scale bar represented 200 μ m. PV: Portal vein; CV: Central vein

4.3.1 Novel Marker for the Partial EMT Undergoing Arl13b+ BECs

In the previous section, we nicely showed constituents of crowded cell masses (mesenchymal cells, homeostatic Arl13b+ cholangiocytes, and partial EMT undergoing Arl13b+ cholangiocytes, a new sub-population of Arl13b+ cholangiocytes,) upon TAA induced fibrosis. To clarify this new sub-population of partial EMT undergoing Arl13b+ cholangiocytes, we used conventional mesenchymal markers Vimentin, CD34, and Pdgfra. However, we wanted to demonstrate partial EMT in Arl13b+ cholangiocytes via novel markers in addition to common ones. We knew that Igfbp7 was a downstream target of Tgf β signaling, so it could have an effect on EMT processes (Zhong et al., 2019). Thus, we wanted to show whether Igfbp7 was detected in partial EMT undergoing cholangiocytes or its niche components. We started to investigate the expression of Igfbp7 in Arl13b+ cholangiocytes by Igfbp7-Arl13b IHC staining in the homeostatic and diseased liver. It was clear that there was no overlap for Igfbp7 and Arl13b in the homeostatic liver (Figure 31, i). However, upon TAA induced fibrosis Arl13b+ cholangiocytes began to express Igfbp7 (Figure 31, ii). At the same time, Igfbp7 level extremely

increased in TAA 24-week sample compared to control sample (Figure 31A and B).

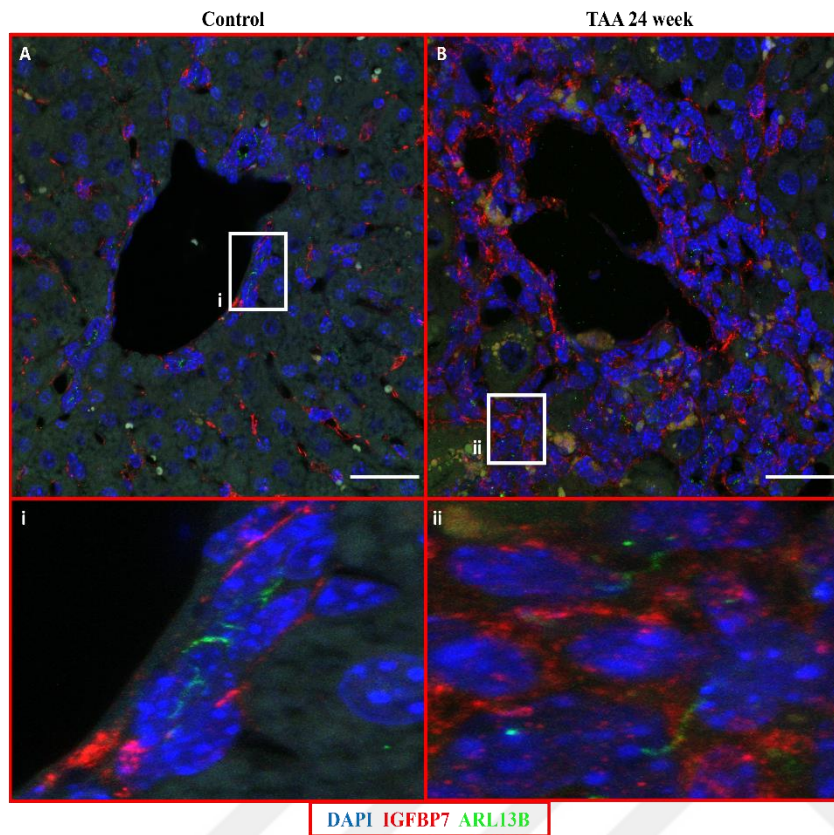


Figure 31: IHC staining against Igfbp7 (red) (n=3, n=3) and Arl13b (green) (n=12, n=12) for control (A,i) and TAA 24 week (B, ii) samples. A and B panels showed portal veins of liver samples whereas zoomed panels indicated specific areas determined in Panel A and B, respectively. DAPI was used for staining of nucleus. Scale bar represented 50µm.

At that point, we decided to check the Igfbp7 expression in cholangiocytes with another BEC marker Opn and the epithelial marker, E-cadherin. We observed a very low number of Opn+, E-cadherin+, Igfbp7+ cholangiocytes were present in control liver (Figure 32A, C), while fibrotic sample included a higher number of these cells (Figure 32B, D). Additionally, overlaps in both samples were obvious without DAPI (Figures 32C and D). These Opn, E-cadherin, and Igfbp7 expressed cells were very similar to partial EMT undergoing cells around the portal vein in terms of morphology (Figure 32B, D).

As another comment for this experiment, apart from cholangiocytes in which Igfbp7 was expressed we observed another cell type expressing only Igfbp7, not the other two markers in fibrosis (Figure 32B, D).

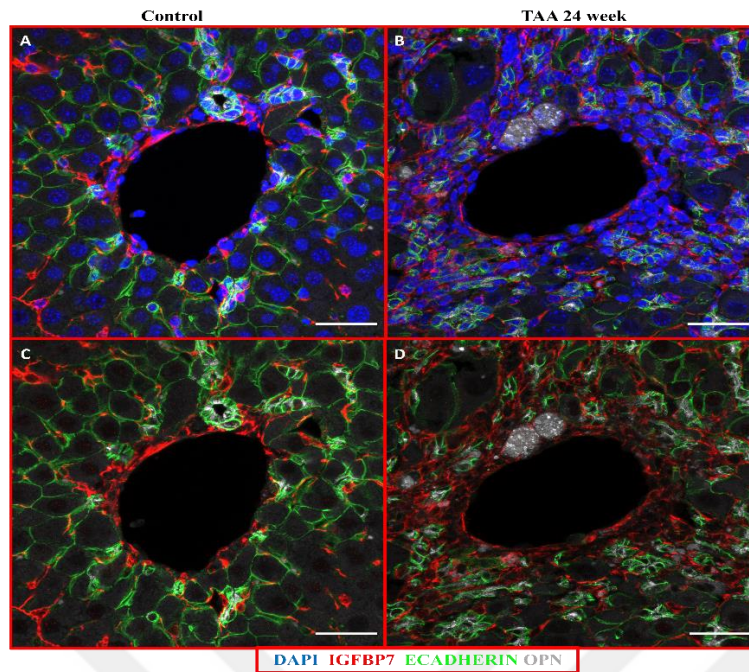


Figure 32: In the control (A, C) and TAA 24-week (B, D) samples, IHC analysis for Igfbp7 (red), E-cadherin (green) and Opn (grey). All images were showing portal veins. C and D panels were versions of A and B panels without merging with DAPI. DAPI was used for staining of nucleus. Scale bar represented 50µm.

When we analyzed tile scan images of this staining, we could remark several points. Firstly, under both homeostatic and fibrotic conditions Igfbp7 expression was distributed throughout the whole liver, but expression level significantly increased in the fibrotic sample (Figure 33A and B). Secondly, giant cell masses (including both homeostatic and partial EMT undergoing BECs) around the portal vein and individual cells expanding through other veins contained Opn+E-cadherin+ Igfbp7+ cholangiocytes in fibrosis but control samples did not. Hence in this part, we claimed that Igfbp7 could be accepted as a new EMT marker for partial EMT undergoing cholangiocytes, but to be sure, further analysis should be carried out.

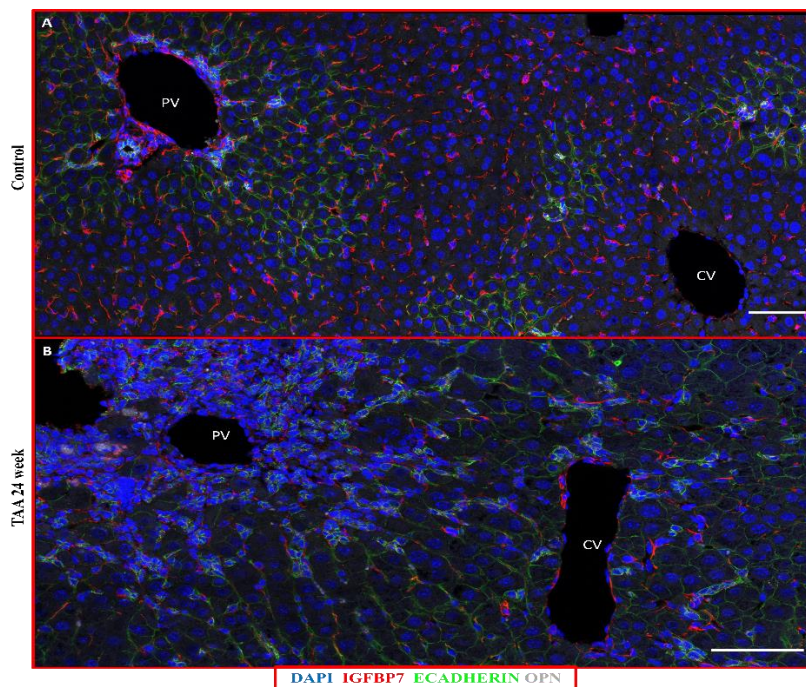


Figure 33: Tile scan images of Igfbp7 (red), E-cadherin (green) and Opn (grey) staining for control (A) and fibrotic (B) models. All zones of the liver were present in both figures. DAPI was used for staining of nucleus. Scale bar represented 200µm. PV: Portal vein; CV: Central vein

4.3.2 Novel Marker for Niche Components of the Partial EMT Undergoing BECs

After we decided that Igfbp7 could be a strong candidate to label partial EMT undergoing Arl13b⁺ cholangiocytes, we chose another marker which was called Fabp4. This marker was a kind of fatty acid binding protein (Furuhashi et al., 2014) which could be affected by fibrosis owing to changes in lipid metabolism as mentioned in section 1.4. Thus, we thought that maybe this marker could be another new marker for partial EMT undergoing cholangiocytes as Igfbp7. We performed the same sets of experiments which were designed for Igfbp7. However, we obtained completely opposite results compared to Igfbp7 stainings. According to Arl13b and Fabp4 staining, we didn't observe any co-expression for these markers in both fibrotic and steady state samples (Figure 34A, B). Additionally, some Fabp4⁺ cells were in very close proximity to some Arl13b⁺ cells in both samples. However, it was evident that the Fabp4 levels were highly increased in fibrotic tissue compared to controls.

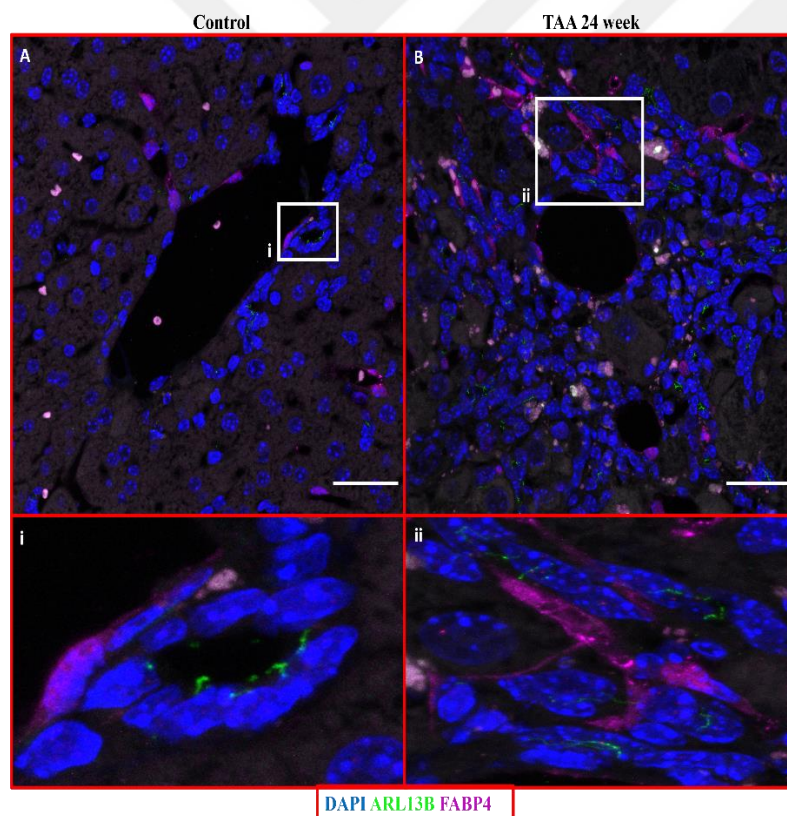


Figure 34: Fluorescence staining for Arl13b (green) (n=12, n=12) and Fabp4 (magenta) (n=3, n=3) in the control (A) and TAA 24-week (B) samples. Both A and B figures belonged to portal veins of models. Panels i and ii demonstrated zoomed versions of determined areas in A and B parts. DAPI was used for staining of nucleus. Scale bar represented 50µm.

Despite of our observations regarding the absence of Fabp4 in Arl13b⁺ cells, we wanted to check Fabp4 expression in cholangiocytes by using E-cadherin, and Opn. As expected, there were no Opn⁺ and E-cadherin⁺ cholangiocytes which were able to express Fabp4 around the

portal vein. (Figure 35B and D). Similar to our observations (Figure 34B and D), some Fabp4⁺ cells were surrounding Opn⁺ and E-cadherin⁺ cholangiocytes (Figure 35B and D).

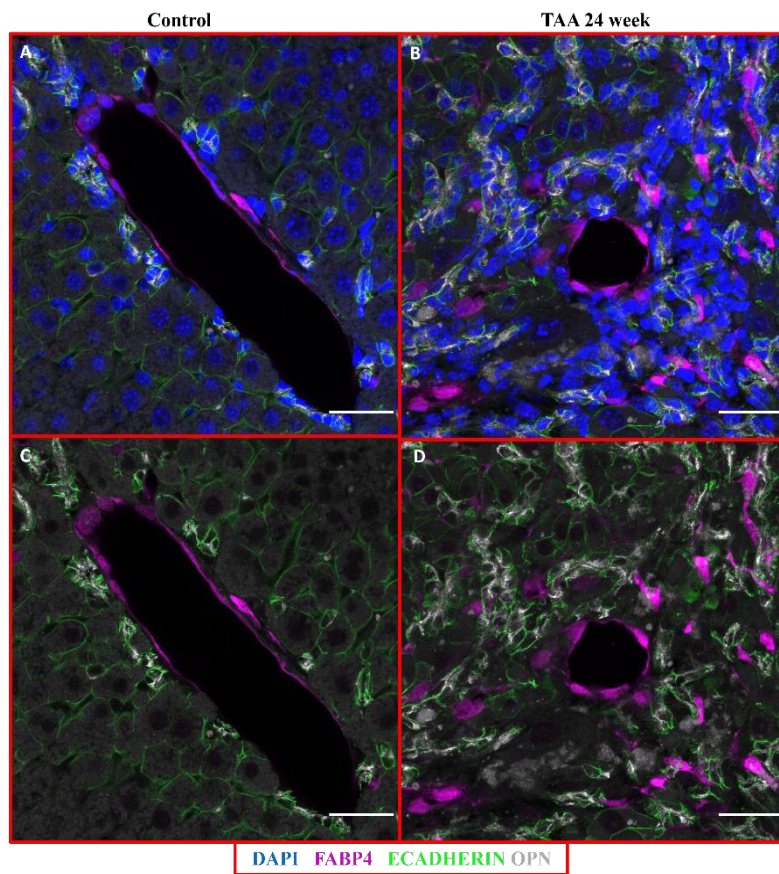


Figure 35: IHC staining against for Fabp4 (magenta), E-cadherin (green) and Opn (grey) for homeostatic (A, C) and fibrotic (B, D) samples. All images represented portal veins of the liver. C and D panels were the same images with A and B parts without DAPI, respectively. DAPI was used for staining of nucleus. Scale bar represented 50 μ m.

To detect the expression profile of Fabp4 in all zones of the liver, we benefited from tile scan images of E-cadherin, Opn, and Fabp4 staining. Fabp4 expression was visible around central and portal veins, not in parenchyma for the control sample (Figure 36A). Moreover, similar to our previous observations there was no overlap for Fabp4 and cholangiocytes under homeostatic conditions. However, Fabp4 expression showed an upregulation for both periportal and pericentral regions by fibrotic conditions (Figure 36B). Still, there was no Fabp4 expression in cholangiocytes of cell masses in the periportal region and also, migratory area through the portal or central veins. Nonetheless, some cholangiocytes in these big and small cell densities along the portal-portal axis and portal-central axis were encircled with some Fabp4⁺ cells. Hence, we could conclude that Fabp4⁺ cells possessed a possibility of being one of the niche components of partial EMT undergoing cholangiocytes. However, further experiments are needed to make sharp comments.

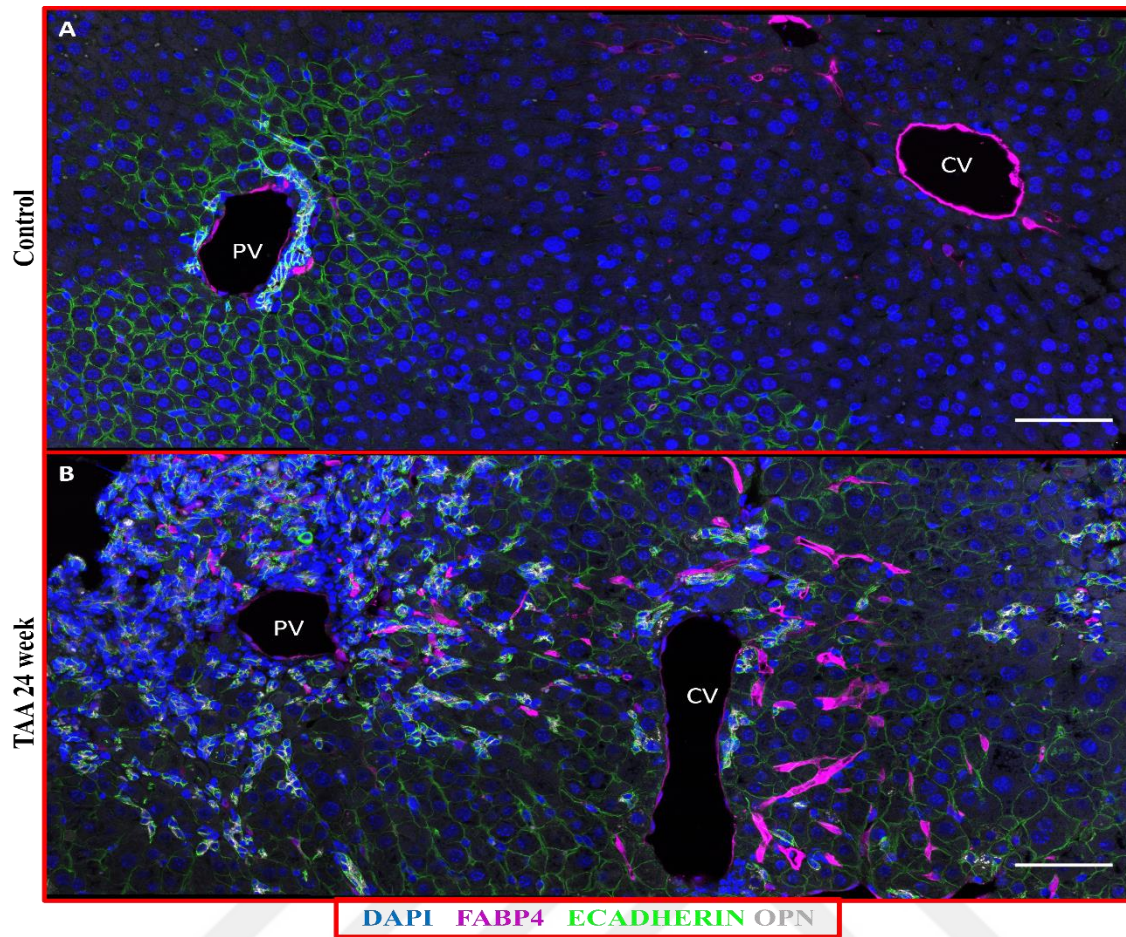


Figure 36: Tile scan images of IHC analysis performed by Fabp4 (magenta), E-cadherin (green), and Opn (grey) for control (A) and fibrotic (B) liver. Images included periportal, midzone, and pericentral regions of the liver from left to right. DAPI was used for staining the nucleus. Scale bar represented 200µm. PV: Portal vein; CV: Central vein

4.3.3 Vesicle Secretion in the Fibrotic Liver

As mentioned in previous sections, it was known that different cell types such as cholangiocytes communicate with each other by secreting vesicles under fibrotic conditions. The primary cilium took several roles in endocytosis and exocytosis of vesicles. At that point, we speculated that vesicles could be secreted from or taken by cholangiocytes via the primary cilium. For this purpose, we performed IHC staining against Opn, Vimentin, and CD63 which is a common marker to detect vesicles. We showed that CD63 was expressed in both homeostatic and fibrotic livers (Figure 37A and B). However, we observed that there was no overlap for Opn-CD63, and Opn-Vimentin-CD63 markers in both control and fibrosis models. This means that both homeostatic and partial EMT undergoing cholangiocytes did not secrete vesicles in our TAA induced fibrosis model. Thus, we could not comment on the role of the primary cilium in vesicle

secretion. However, we observed a co-expression for Vimentin- CD63 and only CD63+ cells in both samples.

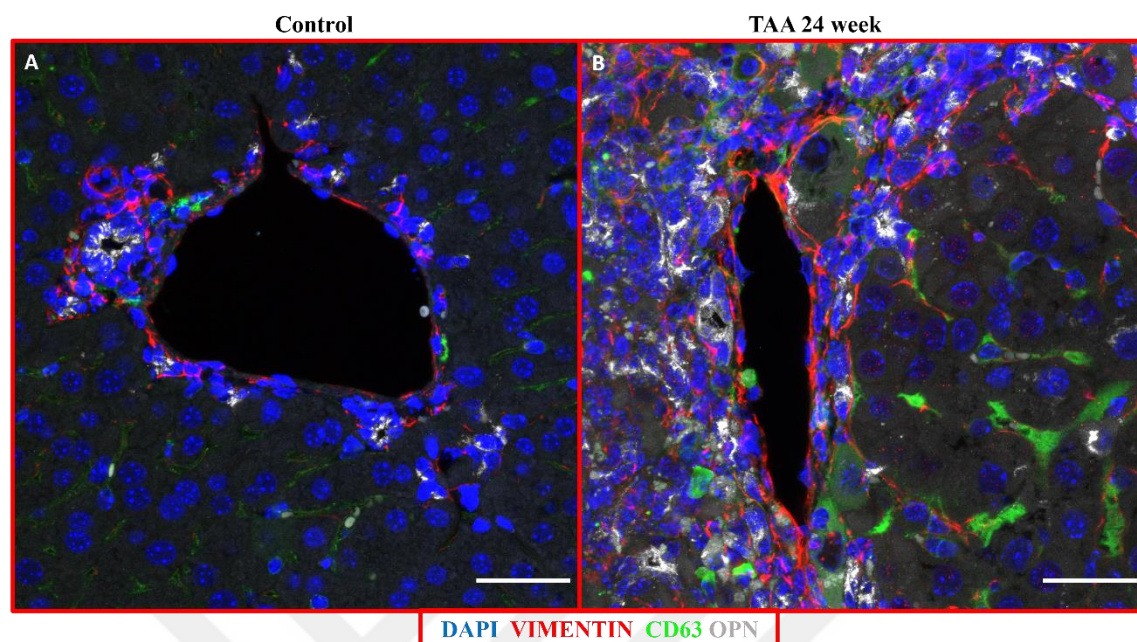


Figure 37: IHC analysis performed by Vimentin (red) (n=4, n=4), CD63 (green) (n=2, n=2) and Opn (grey) (n=6, n=6) for control animal (A) and TAA 24 week animal (B). Both images showed portal veins of the tissue. DAPI was used for staining the nucleus. Scale bar represented 50µm

After our study regarding CD63 expression around portal veins of liver samples, we focused on other regions of the liver. However, the problem which was observed in Lyve1 expression (Figure 20) was observed for CD63 signaling. In some regions of both fibrotic and homeostatic liver samples, we saw a high level of CD63 expression whereas, at some parts, there was no noteworthy CD63 signal (data not shown). Hence, we could not compare a change in CD63 expression level between control and fibrosis animals. However, we obtained several results from tile scan images of CD63, Vimentin, and Opn staining. Firstly, both homeostatic and partial EMT undergoing cholangiocytes around the portal vein, in cell masses between portal veins, and in several cells extending central vein were not expressing CD63 (Figure 38B). Secondly, CD63+ cells were not the components of giant cell masses between portal veins of the fibrotic liver. As a third point, the signaling style of CD63 was very similar to the signaling style of Lyve1 (Figure 20). When Lyve1 and CD63 expressions in big cell masses through the portal-portal axis were checked (Figure 20B and Figure 38B), it was clear that no expression for these markers was found in these cells. Thus, Lyve1+ cells could express CD63; so vesicle secreting cells could be LSECs but more analyses were required for further explanations.

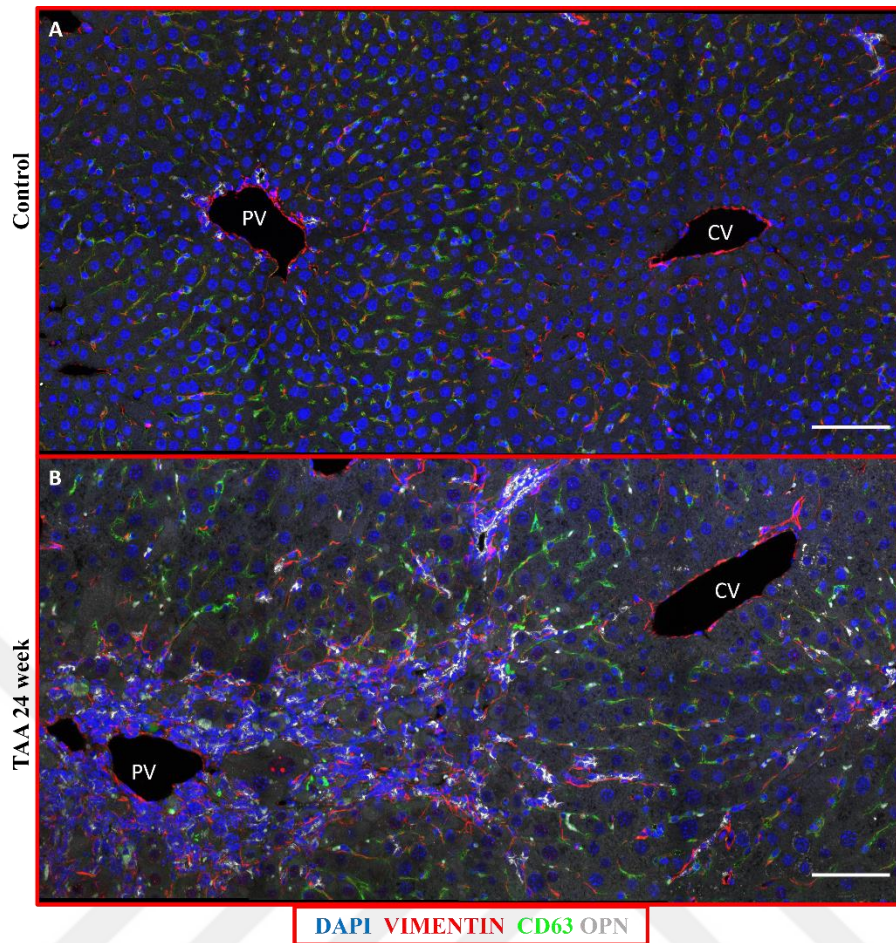


Figure 38: Tile scan images of Vimentin (red), CD63 (green), and Opn (grey) in control (A) and fibrotic (B) samples. Both images demonstrated portal vein, parenchyma, and central vein of the tissue from left to right. DAPI was used for staining the nucleus. Scale bar represented 200 μ m. PV: Portal vein; CV: Central vein

4.4 EMT in the Cholangiocytes by Other Disease Models

Apart from TAA induced fibrosis models, we generated High Fat Diet induced and Western Diet induced obesity models and fasting-refeeding models in mice. We wanted to identify whether a subpopulation of Arl13b⁺ cholangiocytes was undergoing partial EMT in these different disease models. We started our study with HFD 24-week model by IHC against CD34 and Arl13b. In this experiment, we didn't observe any overlap for these two markers in our both control and HFD 24-week samples (Appendix II). CD34⁺ cells were surrounding Arl13b⁺ cholangiocytes under both control and diseased state. To compare the general liver structures between control and HFD 24-week samples, tile scan images of Opn staining were checked. According to the results, both tissues were very similar to each other (Appendix III). There was no proliferation, migration, or expansion between or through different veins. At the same time,

Opn⁺ cholangiocytes were located around only portal veins of both samples and their level was the same under both conditions. Therefore, we concluded that there was no partial EMT undergoing Arl13b⁺ cholangiocytes in HFD 24-week samples.

After HFD induced obesity model, we continued with Western Diet induced obesity model to show whether partial EMT undergoing Arl13b⁺ cholangiocytes were present or not. As expected, we didn't observe any change for Arl13b⁺ cholangiocytes in terms of proliferation around the portal vein upon WD 24-week by Arl13b, Opn, and Thy1 staining (Appendix IV). Portal veins of control and WD 24-week samples were very similar to each other. Hence, we commented that there was no alteration for Arl13b⁺ cholangiocytes between control and WD 24-week samples, so there should be no partial EMT undergoing Arl13b⁺ cholangiocytes in WD 24-week samples.

Last but not least, we checked the appearance of partial EMT undergoing Arl13b⁺ cholangiocytes under different metabolic states such as homeostasis, fasting, 3 hours refeeding, and 6 hours refeeding. For this purpose, we performed Arl13b, Opn, and CD34 fluorescent staining. However, we could not detect any partial EMT undergoing Arl13b⁺ cholangiocytes in any of these metabolic conditions (Appendix V). CD34⁺ cells were surrounding Arl13b⁺ cholangiocytes in all samples. In addition to that, we did not observe any distortion or change in fasting, 3hr refeeding, and 6hr refeeding samples compared to control samples.

5. DISCUSSION

In this master's thesis study, one of our fundamental aims was to show responses of different liver cell types, hepatocytes, cholangiocytes, mesenchymal cells, and endothelial cells to liver fibrosis. For this purpose, we generated TAA induced fibrosis model in different weeks such as 16, 20, and 24 weeks, to compare reactions of various cell types against different fibrotic states. As mentioned in section 1.4.3, there were various chemicals and toxins to develop fibrosis in the mouse. However, we preferred to generate our fibrosis model by administration of TAA due to several reasons. The first motive behind this selection was that the focused cell type among all liver cell types was the Arl13b⁺ cholangiocytes which were located at the periportal zone. Therefore, to observe the effects of fibrosis on these cells we had to damage mainly the periportal area of the liver. Hence, CCl₄ which led to harm in the pericentral area was eliminated. Apart from CCl₄; DDC, CDE, and TAA were the possible chemicals causing distortion around the portal area. Although DDC and CDE showed their fibrotic effects on the liver faster (generation of fibrosis took 5-7 days) compared to TAA model, their administration style carried much more risks compared to TAA. They were in powder and given to animals by adding to their food *ad libitum*. However, there was a possibility that they wouldn't eat up their food due to the presence of the chemical. On the other hand, TAA was administrated to animal by dissolving it in their drinking water. Compared to feeding, drinking water was more essential, so the generation of the fibrosis model via TAA was a more guaranteed method.

We collected various samples from different weeks of TAA promoted fibrosis model. Unfortunately, there were no observable and physiological alterations between fibrotic and homeostatic animals unlike the weight gain observed in obese animals. Nevertheless, we clearly observed that the fibrotic liver contained a rough, external layer and some nodules compared to the control liver during collecting liver tissues (data not shown). Despite this observation, we needed to prove the generation of fibrosis in a microscopic state. As clarified previously, the main indicators of fibrosis were collagen accumulation and increasing cell proliferation. With the help of Ki67 fluorescence staining in 16, 20, and 24 weeks of TAA samples, we observed increasing proliferation around mainly portal veins of all samples with a significant p-value. But in control samples, there were no noteworthy Ki67 expressed cells. This means that we succeeded in developing fibrosis fundamentally around the portal vein. At the same time, we compared proliferation level in TAA 16, 20, and 24-week samples. TAA 16-week samples showed the highest number of proliferative cells around the portal vein compared to other samples. This was very logical because the progression of fibrosis (increase in fibrosis score)

could be provided by keeping TAA administration through weeks (Salguero Palacios et al., 2008). Among our samples, we should observe the weakest fibrosis in TAA 16-week due to the lowest fibrosis score. The proliferation of cells happened under fibrotic conditions in order to regenerate the liver (to reverse fibrosis) by developing liver cell populations. At that point, the highest possibility for liver regeneration by the proliferation of cells belonged to TAA 16-week samples compared to further samples. However, TAA 20-week, and TAA 24-week samples exhibited more progressive fibrosis. Hence, observation of less proliferative cells at portal veins of these samples could be understandable.

After analysis of proliferation level, we checked changes in liver structure due to fibrosis. We observed packed cell masses around portal veins of diseased liver. Moreover, these cell masses drew a path for themselves along the portal-portal axis and moved in that path. Some cells from this path were expanding through the central vein as either individual or several cells. Thus, we could say that a much more complicated and distorted liver structure appeared upon fibrosis compared to a properly structured control liver.

As another key point for fibrosis, we conducted an experiment to show collagen level in fibrotic and control livers. We observed similar results with Katsumata et al., 2017 regarding significantly increasing collagen level by fibrosis. Surprisingly, in addition to collagen fibers, we observed bigger and fully circle shaped signals which were observable in every channel of the microscope. We thought that they were coming from autofluorescence of some structures specific for fibrosis since they were not visible in the homeostatic liver. When we investigated this situation in literature, we found that some metabolic/catabolic and structural proteins in the liver have endogenous fluorophores under different cellular and diseased states. Especially, structural proteins for instance collagen, ECM matrix components, elastin, and other fibrous proteins generate autofluorescence due to endogenous fluorophores excited by suitable wavelengths during fibrosis (Croce et al., 2018). In another study, they showed that a type of hypertrophied hepatic stellate cells is arising under fibrosis. These cells are capable of storing retinoids (retinyl-ester). In the control liver, quiescent hepatic stellate cells accumulate retinoid at the basal level which don't exceed the threshold for autofluorescence. However, although activated HSCs lose their ability to deposit retinoid by fibrosis, this specific type of HSCs increases ability for storage of retinoid. This retinoid overload in these cells causes autofluorescence (Hoffmann et al., 2020). Our observed signal apart from collagen fibers was very similar to retinoid droplets shown in that study. Thus, we decided that these signals belonged to the retinoid autofluorescence which could be used as another marker for the generation of liver fibrosis.

For the final proof for the generation of TAA induced fibrosis model in the liver, we wanted to show the appearance of ballooned hepatocytes. In hepatocytes, several alterations in lipid metabolism were observed under fibrosis. Hepatocytes started to store various lipid molecules and increased their size. This type of hepatocytes could be easily observed in fibrotic tissue thanks to their extremely big shapes under the microscope. With the help of this aspect of ballooned hepatocytes, we observed them as distributed throughout the whole liver without any zonal preference under fibrotic conditions.

After being sure that our TAA promoted fibrosis model was accomplished, we began to analyze responses of different cell types against this model. The heart of this thesis was Arl13b⁺ cholangiocytes because responses or changes in primary cilia to fibrosis were not shown, before. Hence, we started with this cell type. For this purpose, we used Opn, Sox9, and E-cadherin, well-known cholangiocyte markers and epithelial cell marker, respectively. Under fibrotic conditions, we showed that cholangiocytes demonstrated a dramatic increase around the portal vein. Our statistics results supported our observation. While cholangiocytes were absent around the central vein at homeostasis, we observed that upon fibrosis they arose at the pericentral zone. The main reason behind this was the expanded cells from the portal vein to the central vein. As previously mentioned, due to fibrosis enormous cell masses around portal vein were formed and they were migrating and expanding through portal veins and central veins. With this experiment, we proved that the main component of these cell masses was the cholangiocytes. They should proliferate by fibrosis and migrate through other zones. By combining proliferative marker and cholangiocyte marker, we wanted to check the proliferative state of cholangiocytes in different weeks of TAA model. As expected, around the portal vein highest number of proliferative cholangiocytes belonged to TAA 16-week samples. On the other hand, we saw a bunch of proliferative cholangiocytes as migratory cell masses and single cells through the portal vein and central vein in TAA 20-week and 24-week samples compared to TAA 16-week samples. The reason behind the difference in location of proliferative BECs in different weeks of fibrosis was directly related to the fibrosis stage. As described earlier, TAA 16-week model should be the least severe fibrosis model among others. Since we observed fewer cell masses (including cholangiocytes) around the portal vein and fewer migratory cells (including cholangiocytes) through different zones in TAA 16-week model. At that point, the cells have started to proliferate around the portal vein and will increase their density around other zones by upcoming weeks. However, fibrosis stages increased in TAA 20-week and 24-week samples. BECs already proliferated around the portal vein and currently, they are migrating through other zones to increase their populations as big cell masses and individuals.

Thus, we observed much more cell densities through the portal-portal axis and more cells around central veins in TAA 20-week and TAA 24-week samples. This was another significant indicator that TAA induced fibrosis model was affecting mainly portal veins. Since under less severe fibrosis (TAA 16-week), this model showed its effect around mainly portal veins. However, when fibrosis stages were progressing with continuous weeks, it started to influence the pericentral zone in addition to the periportal area.

Apart from the proliferation of BECs in the portal vein and in migratory cell masses, we observed the proliferation of other cell types for all TAA samples. For example, we observed that hepatocytes were proliferating by fibrosis. They could be distinguished from other cell types without any labeling technique thanks to their specific shape and morphology. Moreover, other cell types in cell masses between portal veins showed expression of proliferation marker in different stages of fibrosis.

As another additional and technical point, it is known that catching proliferative cells via IHC is extremely difficult. We might have missed some proliferative cells in all TAA models, but with different sets of experiments related to the proliferation of cells (including BECs) results were similar to our previous results.

We were sure that cholangiocytes were proliferating at periportal zones and migrating through other zones. We aimed to show this for Arl13b⁺ cholangiocytes. As mentioned formerly, every cholangiocyte has a primary cilium. Hence, Arl13b⁺ cholangiocytes should show proliferation under fibrosis as Opn, Sox9, and E-cadherin⁺ cholangiocytes because these should be the same cholangiocytes with primary cilium containing ones. However, we obtained contradictory results with our previous results. There were no proliferative Arl13b⁺ BECs for none of the TAA samples. At that point, we thought several speculations. One possibility could be that maybe during proliferation BECs lost their primary cilium, so we didn't observe any overlap for Arl13b and Ki67 in proliferating cholangiocytes. Another possibility could be explained for this situation as maybe all cholangiocytes were absent for primary cilium due to fibrotic distortion in cells. The further hypothesis could be related to the non-specificity of a primary cilium for cholangiocytes. Although all studies for mice in the literature show that the only cell type containing a primary cilium in the liver is cholangiocytes, maybe another cell type started to express primary cilium under fibrotic conditions. We checked these possibilities from starting to the last one. First of all, we should be certain whether Arl13b signals possessed specifically primary cilium. With another marker for primary cilium, PCNT, we made sure that Arl13b expression was observed in specifically primary cilium structure. Furthermore, we verified with different primary cilium and cholangiocyte markers that primary cilium was

formed specifically in cholangiocytes under both homeostatic and fibrotic states. Hence, we could say that fibrotic cholangiocytes didn't lose their primary cilium. Even, cholangiocytes which were migrating through the portal and central veins kept their primary cilium. Hence, the total primary cilium level increased as a response to the fibrosis which was a novelty for literature. Thanks to these results, we explained previous contradictory outcomes by our first hypothesis. Most probably, during proliferation due to cytokinesis (loss of some structures and changes in microtubules and centrioles), primary cilium was lost temporarily since primary cilium structure is directly related to microtubules and centrioles. However, this hypothesis should be further checked.

About the increasing level of Arl13b under fibrotic conditions, our statistics analysis confirmed our IHC experiments. However, outliers of fibrotic Arl13b were very wide, so data was not normalized. The reason behind this situation could be related to a too small size of Arl13b and the heterogeneity in the size of portal veins. Some portal veins were huge and surrounded by numerous Arl13b+ cholangiocytes whereas some of them were smaller and less Arl13b+ cholangiocytes were located. Effect of fibrosis on Arl13b level with heterogeneity in vein size could cause these outliers in Arl13b expression level.

As mentioned previously, cholangiocytes surrounded the portal vein and bile duct under homeostasis. During the investigation of reaction Arl13b+ cholangiocyte to fibrotic conditions, we observed that around portal vein there was an increment for bile duct structures which were positive for primary cilium containing cholangiocytes. This could be explained by the evolutionary concept of injured organs for the gastrointestinal tract (small intestine, colon, liver). It was known that when these tissues are injured, they have a tendency to turn into the embryologic state in terms of some ways. The ductal plate is a primitive version of a bile duct. According to a study, it was claimed that the liver responds to fibrosis by ductular reaction in which the generation of new ductal plates (bile ducts) occurs around the portal vein (Desmet, 2011). The reason behind our observation regarding an increase in bile duct level might be related to this approach. But more studies were required to prove these results.

After cholangiocytes, we continued to analyze responses of liver cell types to fibrosis with hepatocytes. We observed that hepatocyte level was high in every zone under fibrosis. However, they were not the components of formed, big cell masses around the portal vein and between portal veins. It seemed that hepatocytes were proliferating, and also migrating individually. Thus, under fibrotic conditions hepatocytes were located a bit distant from the portal vein compared to their locations in control samples. As previously mentioned, ballooned hepatocytes were also another response of the hepatocytes to fibrosis.

In literature, there was a study (Deng et al., 2018) mentioning that in TAA 12-week samples, they observed proliferation of both cholangiocytes and hepatocytes from their existing counterparts. However, when TAA 24-week samples are checked, they saw that cholangiocytes differentiate into hepatocytes. Since there are not enough hepatocytes that can proliferate and increase their population to regenerate the liver. Although we observed proliferation of both cholangiocytes and hepatocytes in our TAA model, we should investigate the possibility of this differentiation of hepatocytes into cholangiocytes or vice versa. During differentiation of hepatocytes into cholangiocytes, Deng et al. clarified a new, bi-phenotypic cell type that can express both cholangiocyte and hepatocyte markers at the same time. We used this strategy in our study and with different combinations of BEC and HC markers, we didn't observe any overlap for these markers. Therefore, we concluded that there was no differentiation from hepatocytes to cholangiocytes or vice versa for our TAA induced fibrosis model. The reason behind these contradictory results between two studies could be related to the dose of TAA. Deng et al., 2018 used 600 mg/L TAA to induce fibrosis in mice whereas we administered 300 mg/L TAA to the drinking water of the animals. This causes a significant difference in the severity of the fibrosis models. Since they generated a more intense injury model, differentiation between different cell types could be possible compared to our less severe fibrosis model.

Responses of hepatic stellate cells, myofibroblasts, portal fibroblasts, and endothelial cells to fibrosis were one of the hot topics of liver injury area. Especially, conversion of quiescent HSCs to activated HSCs/myofibroblasts and contribution of this differentiation to fibrosis were studied a lot. Unfortunately, there was no clear marker to distinguish activated HSCs from myofibroblasts in the literature, so we called mesenchymal cells to both cell types. However, to characterize portal fibroblasts from HSCs and myofibroblasts we used Thy1. As expected, we observed a significant increase in mesenchymal cell population in all zones of the liver under fibrotic conditions. This made sense because as quiescent HSCs, portal fibroblasts differentiated into myofibroblasts upon fibrosis. By increasing myofibroblast level via contributions of HSCs and PFs, collagen accumulation occurred. Thy1⁺ portal fibroblasts were gradually decreasing upon fibrosis in some experiments, but we observed increasing Thy1 expression in fibrotic liver by several IHC experiments. Thus, we could not conclude changes in expression of Thy1 due to fibrosis. Our statistics about Thy1 expression showed non-significant results by supporting our contradictory Thy1 expression in stainings. This could be related to Thy1 antibody or heterogeneity of portal fibroblasts in the liver.

In addition to these results about mesenchymal cells, we detected CD34^{high}/Thy1^{low} and

Cd34^{low}/Thy1^{high} populations around the portal vein of the fibrotic liver. More analyses were necessary for the characterization of these populations, but according to their physiological and morphological features CD34^{high}/Thy1^{low} populations could represent HSCs/MFs whereas CD34^{low}/Thy1^{low} populations could be examples of PFs. Because HSCs looked like star-like shaped whereas, portal fibroblasts were much more spindle-shaped, long cells.

Regarding responses of the LSECs against fibrosis, we couldn't obtain convincing results for comparison of LSEC levels between control and fibrotic samples. Since LSECs were expressed highly some parts of both fibrotic and homeostatic liver whereas some parts didn't contain any LSECs. This problem could arise from the LSEC antibody, Lyve1 or it could be related to the heterogeneity of LSECs. However, we clearly observed that LSECs were not located in giant cell masses around the portal vein and between different portal veins in the fibrotic liver. As known, LSECs were important for carrying oxygen and nutrients from blood vessels to liver cell types. Also, hypoxia was observed during fibrosis and facilitated the progression of a disease state as mentioned earlier. Thus, we thought that hypoxia could be occurring in these big cell masses due to the absence of LSECs around them. This situation could contribute to increasing fibrosis stages.

As mentioned previously, the mesenchymal cell population showed an increase upon fibrosis. We identified the expression of mesenchymal markers in the cell masses around the portal vein, in the path containing migratory cell densities between portal veins, and in the expanded several cells through the central vein. We knew that Arl13b⁺ cholangiocytes were the main component of these cell masses. At that point, we focused on two hypotheses. The first hypothesis could be that Arl13b⁺ cholangiocytes started to express mesenchymal markers by EMT due to fibrosis. Another hypothesis could be that another component of these cell masses was the mesenchymal cell population taking a role of niche components of migratory Arl13b⁺ cholangiocytes. Also, both hypotheses could be possible at the same time. According to sets of IHC experiments with mesenchymal and cholangiocyte markers, we clearly observed that not all, but some populations of Arl13b⁺ BECs were undergoing partial EMT. This term was called as "partial" EMT because Arl13b⁺ cholangiocytes expressed both mesenchymal (Vimentin, Pdgfra, and CD34; but not Thy1) and cholangiocyte/epithelial markers simultaneously. These partial EMT undergoing cholangiocytes were seen in cell masses around the portal vein, in the path including migratory cell densities between portal veins, and in the expanded, several cells along the portal-central axis with homeostatic Arl13b⁺ cholangiocytes and mesenchymal cells. Identification of these cell masses was another key and novel point of this thesis. When we thought about the EMT concept in cancer, our partial EMT idea was very logical. Cancer

originates from a specific tissue. However, to spread through whole body cancer cells need to move and migrate other organs which was called as metastasis. For this purpose, EMT happens in cancer cells so that they can gain mesenchymal features temporarily and migrate toward other organs (Fares et al., 2020). In our model, Arl13b⁺ cholangiocytes were epithelial cells that were not able to move. However, via partial EMT they obtained mesenchymal characteristics by maintaining their epithelial identity and migrated through other regions of the liver. In addition to that, if TAA administration lasts for 52-56 weeks, hepatocellular carcinoma is occurring (Wallace et al., 2015). Maybe in HCC, all Arl13b⁺ cholangiocytes would be undergoing full EMT to occupy all zones of the liver and also other organs. Since our model was less severe than HCC, perhaps we observed partial EMT in some populations of Arl13b⁺ cholangiocytes. However, we needed more analysis to verify our results by comparing both cancerous and fibrotic liver. With the same logic, it was very rational that TAA 16-week samples showed the lowest number of partial EMT undergoing cholangiocytes compared to TAA 20-week and 24-week samples due to less severity of fibrosis in TAA 16-week. As expected, the highest number of partial EMT undergoing Arl13b⁺ cholangiocytes was observed in TAA 24-week samples, the most severe fibrosis among our models.

In the light of these hypotheses, we thought that partial EMT undergoing cholangiocytes could not be the cells who started fibrosis. Because the main contributor to the fibrosis was the collagen accumulation by myofibroblasts. Therefore, HSCs should be the cells initiating fibrosis by their differentiation to myofibroblasts. After fibrosis began, hepatocytes and cholangiocytes were growing. However, at the threshold point hepatocytes and cholangiocytes could not proliferate due to insufficient level of existing HCs and BECs by developing fibrosis. Most probably at that point, cholangiocytes started to contribute fibrosis by partial EMT according to our hypothesis. Thanks to partial EMT, some sub-population of Arl13b⁺ cholangiocytes were able to migrate through other veins. Thus, we supposed that Arl13b⁺ cholangiocytes could not proliferate and migrate simultaneously. They should proliferate initially, and then migrate by undergoing partial EMT. The observation regarding the presence of the highest number of partial EMT undergoing Arl13b⁺ cells in TAA 24-week samples was another confirmation for our hypothesis.

For undergoing partial EMT, a sub-population of Arl13b⁺ cholangiocytes required a niche. CD34, Pdgfra, and Vimentin positive mesenchymal cells might be components of this niche by secreting factors to the subpopulation of Arl13b⁺ cholangiocytes. Level of the Thy1⁺ portal fibroblasts that surrounded partial EMT undergoing Arl13b⁺ cholangiocytes were less than the other mesenchymal cell populations. However, there was still a possibility that maybe these

Thy1+ PFs could be another niche component of partial EMT undergoing Arl13b+ BECs under fibrosis.

EMT concept in cholangiocytes upon fibrosis was a significant and complicated area in liver biology. There were lots of contradictory studies regarding this subject in both mouse and human samples. We nicely showed the appearance of partial EMT undergoing sub-population of Arl13b+ cholangiocytes by TAA induced fibrosis. Our study confirmed one of the main papers which supported EMT in cholangiocytes by fibrosis. According to Omenetti et al. EMT in cholangiocytes is facilitated by myofibroblastic hepatic stellate cells. Under fibrosis, these cells are secreting Hh ligands which regulate EMT in cholangiocytes (Omenetti et al., 2018). This study could work in our partial EMT model because Arl13b+ cholangiocytes were undergoing partial EMT. It was known that the primary cilium takes roles in Hedgehog signaling. As a hypothesis, maybe cholangiocytes containing a primary cilium took hedgehog signals from mesenchymal cells in their niche environment and primary cilium was a responsible structure for the occurrence of partial EMT. In addition to this study, we verified other known studies, Rygiel et al., 2007 and Xiao et al., 2014. These studies showed EMT in cholangiocytes upon fibrosis and Tgf β regulation in EMT. Tgf β related hypothesis by these studies could be valid for our model because of the same reason seen in Hedgehog signaling. Primary cilium is also significant for Tgf β signaling, however the exact role of the primary cilium in that pathway is not known. Likely, the primary cilium of cholangiocytes contributed to the occurrence of partial EMT by regulation of Tgf β signaling.

While our study was supporting many research in literature, it contradicted several studies. Scholten et al., 2010 denied the EMT in cholangiocytes upon fibrosis. They clarified in their study that there is no overlap for mesenchymal and cholangiocytes markers under fibrotic state. They used a genetically labeled mouse in which cholangiocytes are genetically labeled by YFP tagged K19 and they generated different fibrosis models such as BDL and CCl₄ (Scholten et al., 2010). In another study, Chu et al., 2011, they labeled all epithelial cells with Alfp genetically. With DDC and BDL models, they showed that no expression of mesenchymal markers is found in these labeled epithelial cells.

There were several reasons leading to these conflicting results between EMT supporting and against studies. First of all, EMT against studies performed genetic labeling to observe K19+ and Alfp+ cholangiocytes with the Cre-LoxP system. However, their Cre-LoxP system might be not 100% successful, they possessed a possibility to miss some K19+ or Alfp+ cholangiocytes which could undergo partial EMT. In addition to that, to label cholangiocytes fluorescently we used Opn, Sox9, and E-cadherin markers whereas they marked cholangiocytes

with K19 and Alfp. These populations of cholangiocytes maybe different from each other and their labeled cholangiocytes might not be undergoing partial EMT under the fibrosis.

As a new marker to characterize partial EMT undergoing Arl13b⁺ cholangiocytes, Igfbp7 whose effect on the EMT process was known as one of the candidates. According to our study, Igfbp7 was expressed in Arl13b⁺, Opn⁺ and E-cadherin⁺ cholangiocytes located around the portal vein, in cell masses between portal veins, and in several cells through central vein upon fibrosis. However, these cholangiocytes were negative for Igfbp7 in control samples; cells surrounding cholangiocytes were expressing Igfbp7. Therefore, we commented that Igfbp7 could be a novel marker to identify partial EMT undergoing Arl13b⁺ cholangiocytes. We couldn't perform co-staining of Igfbp7 with mesenchymal markers (CD34, Vimentin, or Pdgfra) and cholangiocyte marker to observe direct overlaps of Igfbp7 with partial EMT undergoing Arl13b⁺ cholangiocytes because working conditions and sources of antibodies were not applicable with each other. Therefore, we wanted to check Igfbp7 expression in cholangiocytes in both control and fibrosis samples. Since homeostatic cholangiocytes in control did not express Igfb7, we thought that homeostatic cholangiocytes still didn't have an expression for Igfbp7 under fibrosis. Therefore, partial EMT undergoing cholangiocytes should express Igfbp7. However, further analyses were required to be sure.

Fabp4 was a protein related to lipid metabolism. We hypothesized that maybe Fabp4 could be another novel marker for partial EMT undergoing cholangiocytes or a novel niche component of them similar to mesenchymal cells and Thy1⁺ PFs. We observed that, unlike Igfbp7, Arl13⁺, Opn⁺, E-cadherin⁺ cholangiocytes were not expressing Fabp4 under both homeostatic and fibrotic conditions. However, Fabp4 expressing cells were detected in cell masses around portal veins and several cells around central veins. These Fabp4⁺ cells seemed in close proximity to partial EMT undergoing cholangiocytes. Thus, we concluded that Fabp4⁺ cells could be a niche component of partial EMT undergoing cholangiocytes. We were not able to perform Fabp4 fluorescence staining with mesenchymal and cholangiocyte markers due to the same reasons occurring in Igfbp7. Nevertheless, further explanations were necessary about Fabp4⁺ cells being a niche component of partial EMT undergoing cholangiocytes.

Secretion of the vesicles was one of the most significant communication methods between cell types under different conditions. As mentioned in previous sections, almost all liver cell types communicated with each other by releasing vesicles upon fibrosis. Cholangiocytes were one of the cell types secreting vesicles under fibrosis. We hypothesized that maybe the primary cilium of cholangiocytes took a role in endocytosis or exocytosis of vesicles. To assess this hypothesis, we used a well-known vesicle marker, CD63. Unfortunately, we didn't observe any expression

of CD63 in cholangiocytes. Therefore, we could not make any comments about the role of the primary cilium in vesicle secretion. Moreover, we could not compare CD63 levels between control and fibrotic samples due to the same problem which was observed in Lyve1 staining. In addition to that, we detected some cells expressing only CD63. However, these CD63 expressing cells were not observed in massive cells densities in the fibrotic liver. The reason behind this situation could be related that cells in these masses were too close to each other and contacted physically. Thus, they could communicate with each other physically touching instead of secreting vesicles.

As another point of the CD63 staining experiment, we noticed that the signaling style of CD63 was very specific and similar to the Lyve1 expression style. We thought that maybe Lyve1+ cells could express CD63 to communicate with other cells. However, both Lyve1 and CD63 expressions were observed in control and fibrotic samples. This means that this communication between LSECs and other cells might not be specific for fibrotic conditions. Nevertheless, solving the relationship between Lyve1+ cells and CD63 expression were required more analysis.

When we checked other disease models such as high fat induced and western diet induced obesity models, we didn't observe any partial EMT undergoing Arl13b+ cholangiocytes. In addition to that, there was no structural change, proliferation, migration, and expansion of cells throughout the whole liver of both models. This result was expected. Since as mentioned in section 1.6.1, these obesity models are representations for steatosis. They cause mild fibrosis in the liver. Possibly, we didn't generate severe fibrosis by obesity models as much as TAA induced fibrosis model. Therefore, we could not observe any change in protein level by diet models. However, there was a possibility that mild fibrosis might show its effect on mRNA level. To be sure about that, more analyses related to RNA studies were required in these models.

As final models of this thesis, we focused on different metabolic conditions which were mainly affecting lipid and glucose metabolisms of the liver. These metabolic conditions were fasting, 3hr refeeding, and 6hr refeeding in addition to homeostasis. We thought that due to fast metabolic changes throughout the liver by these models, disrupted lipid metabolism caused fibrosis. According to our studies with these models, we did not detect any partial EMT undergoing Arl13b+ cholangiocytes and also any clue for generation of fibrosis. Most probably, the liver could fit these fast metabolic changes by adjusting its lipid and glucose metabolisms without the formation of any damage.

6. CONCLUSION AND FUTURE DIRECTIONS

In this thesis, we showed that noteworthy structural changes happened in the liver under fibrotic conditions. Almost all liver cell types gave responses such as proliferation to different stages of fibrosis. We observed that cells started to constitute giant masses around the portal vein in TAA induced fibrosis model. These cells were migrating between different portal veins as big masses, but several cells from these densities were expanding through the central vein. The formation of these cell masses, their migration between portal veins, and expansion through the portal-central axis were dependent on the severity of fibrosis. We identified a new subpopulation of Arl13b⁺ cholangiocytes which were the main focus of this thesis in these cell masses. This new sub-population was undergoing partial EMT by expressing both mesenchymal and epithelial markers at the same time. In addition to this new subtype, we observed homeostatic Arl13b⁺ cholangiocytes, mesenchymal cells, and portal fibroblasts (as a minor level) in these cell masses. Furthermore, we showed that primary cilium was structured specifically in cholangiocytes and its level extremely increases under fibrotic conditions compared to control samples as another goal of this thesis. Last but not least, we found two novel markers, Igfbp7 and Fabp4, as candidates to label partial EMT undergoing cholangiocytes and to be a niche component of partial EMT undergoing cholangiocytes, respectively.

For future perspectives, the first main goal is to mimic *in vivo* results *in vitro*. For this purpose, liver organoids from cholangiocytes can be generated. Organoids can be cocultured with mesenchymal cells under the homeostasis and also stimulation of Hh, Tgfb β to observe whether organoids from cholangiocytes show partial EMT or not. With the help of this method, the effect of mesenchymal cells on the occurrence of partial EMT in BECs can be identified *in vitro*.

The second goal is to characterize partial EMT undergoing Arl13b⁺ cholangiocytes and homeostatic Arl13b⁺ cholangiocytes in terms of a transcriptional profile under fibrotic conditions for the future. A sharp and novel marker can be detected to distinguish these two cholangiocyte types apart from mesenchymal markers with the help of transcriptional analysis. Spatial transcriptomics can be logical to compare both types of cholangiocytes. Partial EMT undergoing Arl13b⁺ cholangiocytes should contribute to fibrosis, but not regeneration. Due to fibrosis and partial EMT, they can return to their embryologic state by losing their regenerative capacity. Markers which will be obtained from the transcriptional analysis can be helpful to prove this. According to results from expression profile analysis, partial EMT types and partial EMT undergoing Arl13b⁺ cholangiocytes between fibrotic liver and embryonic liver can be

compared.

Overall, our novel findings with this thesis were the response of the primary cilium to fibrosis, the identification of the new subtype of Arl13b⁺ cholangiocytes (partial EMT undergoing) upon fibrosis, and the characterization of components of big cell masses which was observed around portal veins of the fibrotic liver.



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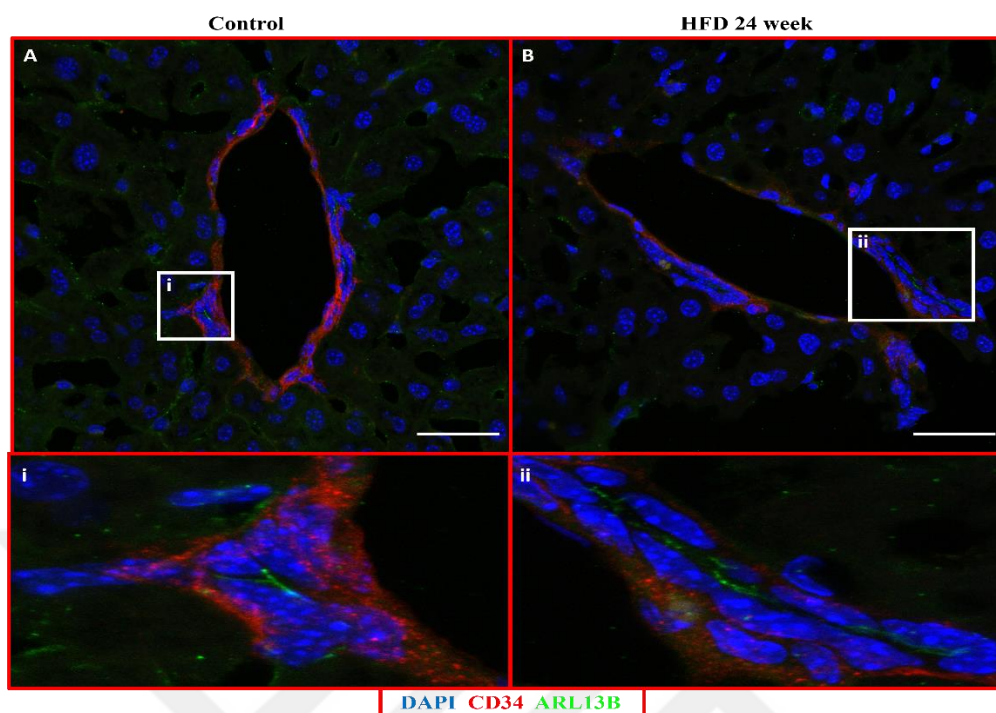
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Appendix I: List of primary, and secondary antibodies which were used in IHC experiments.

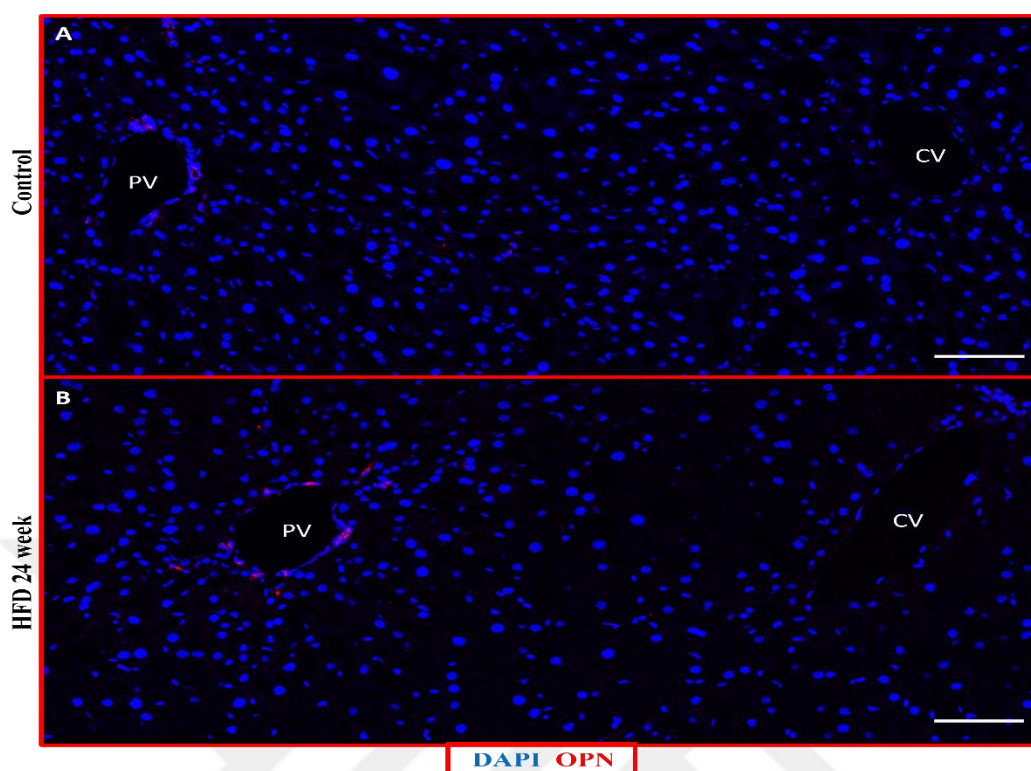
Antibody	Host Species	Clonality	Supplier Firm	Catalogue Number	Dilution in Technique
anti-Arl13b [N295B/66]	Mouse	Monoclonal	Abcam	ab136648	1/100
anti-Arl13b	Rabbit	Polyclonal	ProteinTech	17711-1-AP	1/100
anti-alpha acetylated tubulin	Mouse	Polyclonal	Sigma Aldrich	T7451	1/100
anti-CD34 [EP373Y]	Rabbit	Monoclonal	Abcam	ab81289	1/150
anti-Vimentin	Rabbit	Polyclonal	Invitrogen	PA5-27231	1/100
anti-Pdgfra	Goat	Polyclonal	R&D Systems	AF1062	1/50
anti-CD90/Thy1 [IBL-6/23]	Rat	Monoclonal	Abcam	ab3105	1/100
anti-Sox9	Rabbit	Polyclonal	Millipore	ab5535	1/100
anti-Osteopontin/OPN	Goat	Polyclonal	R&D Systems	AF808	1/60
anti-E-cadherin	Mouse	Monoclonal	BD Biosciences	610182	1/100
anti-Hnf4a (C11F12)	Rabbit	Monoclonal	Cell Signaling	3113	1/100
anti-PCNT	Rabbit	Polyclonal	Abcam	ab4448	1/100
anti-Ki67 [SP6]	Rabbit	Monoclonal	Abcam	ab16667	1/100
anti-Fabp4	Rabbit	Polyclonal	Abcam	ab13979	1/200
anti-Igfbp7	Rabbit	Polyclonal	Abcam	ab74169	1/200
anti-Lyve1 (ALY7)	Rat	Monoclonal	ThermoFisher	14-0443-82	1/25
anti-collagen I	Rabbit	Polyclonal	Abcam	ab34710	1/100
anti-Cd63	Mouse	Monoclonal	Santa Cruz	sc-5275	1/50
Cy2 Conjugated AffiniPure Donkey anti-Rat IgG (H+L)	Donkey	Polyclonal	Jackson Immuno Research	712-225-153	1/400
Cy5 Conjugated AffiniPure Donkey anti-Rat IgG (H+L)	Donkey	Polyclonal	Jackson Immuno Research	712-175-153	1/400
Cy3 Conjugated AffiniPure Donkey anti-Rat IgG (H+L)	Donkey	Polyclonal	Jackson Immuno Research	712-165-153	1/400
Cy2 Conjugated AffiniPure Donkey anti-Goat IgG (H+L)	Donkey	Polyclonal	Jackson Immuno Research	705-225-147	1/400
Cy2 Conjugated AffiniPure Donkey anti-Mouse IgG (H+L)	Donkey	Polyclonal	Jackson Immuno Research	715-225-151	1/400
Cy3 Conjugated AffiniPure Donkey anti-Mouse IgG (H+L)	Donkey	Polyclonal	Jackson Immuno Research	715-165-151	1/400
Cy3 Conjugated AffiniPure Donkey anti-Rabbit IgG (H+L)	Donkey	Polyclonal	Jackson Immuno Research	711-165-152	1/400
Cy5 Conjugated AffiniPure Donkey anti-Rabbit IgG (H+L)	Donkey	Polyclonal	Jackson Immuno Research	711-175-152	1/400
Cy5 Conjugated AffiniPure Donkey anti-Goat IgG (H+L)	Donkey	Polyclonal	Jackson Immuno Research	705-175-147	1/400

Appendix II: IHC experiment for control and HFD 24-week samples of liver.



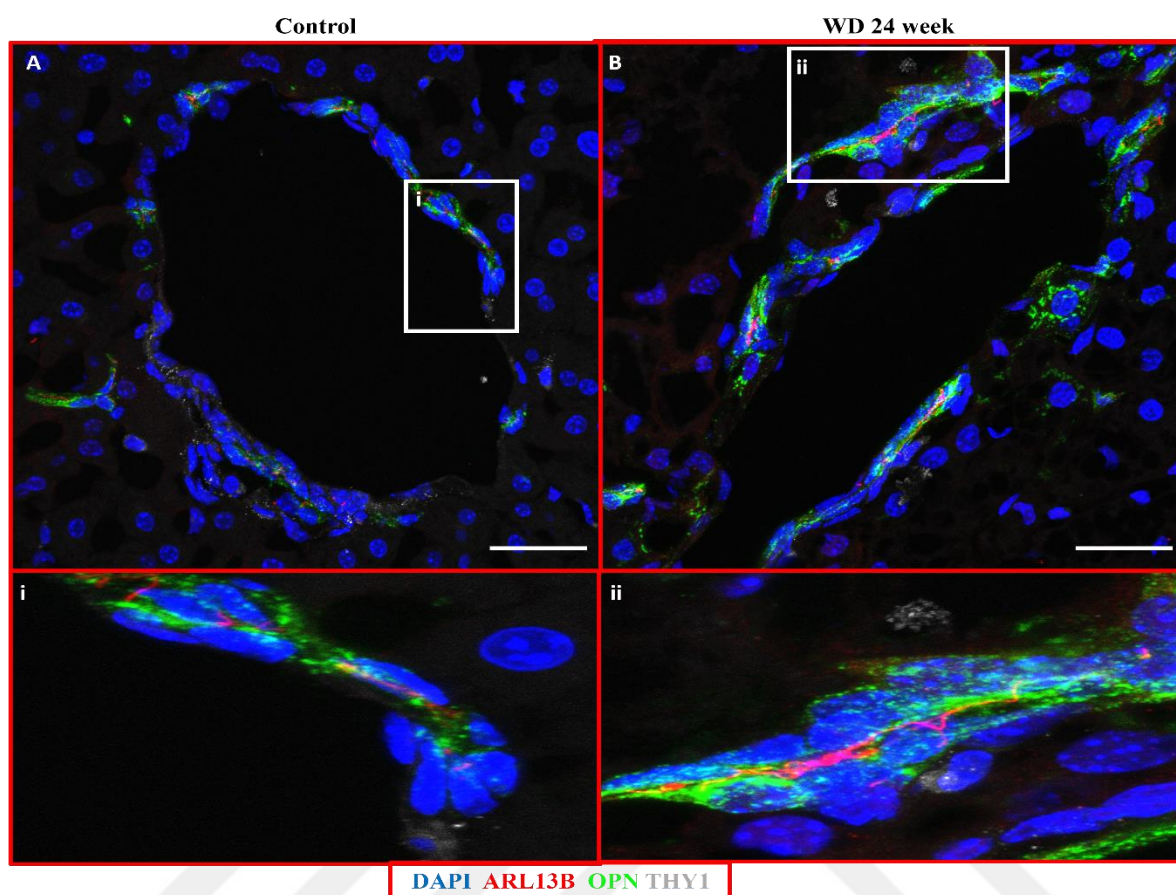
IHC analysis against CD34 (red) (n=3, n=3) and Arl13b (green) (n=5, n=7) in control (A) and High Fat Diet 24-week (B) samples. All images showed portal veins of liver. Panel i demonstrated zoomed version of a particular region in the A part whereas panel ii indicated a zoomed region in the panel B. DAPI was used for staining of nucleus. Scale bar represented 50μm.

Appendix III: Tile scan images of IHC analysis for homeostatic and HFD 24-week livers.



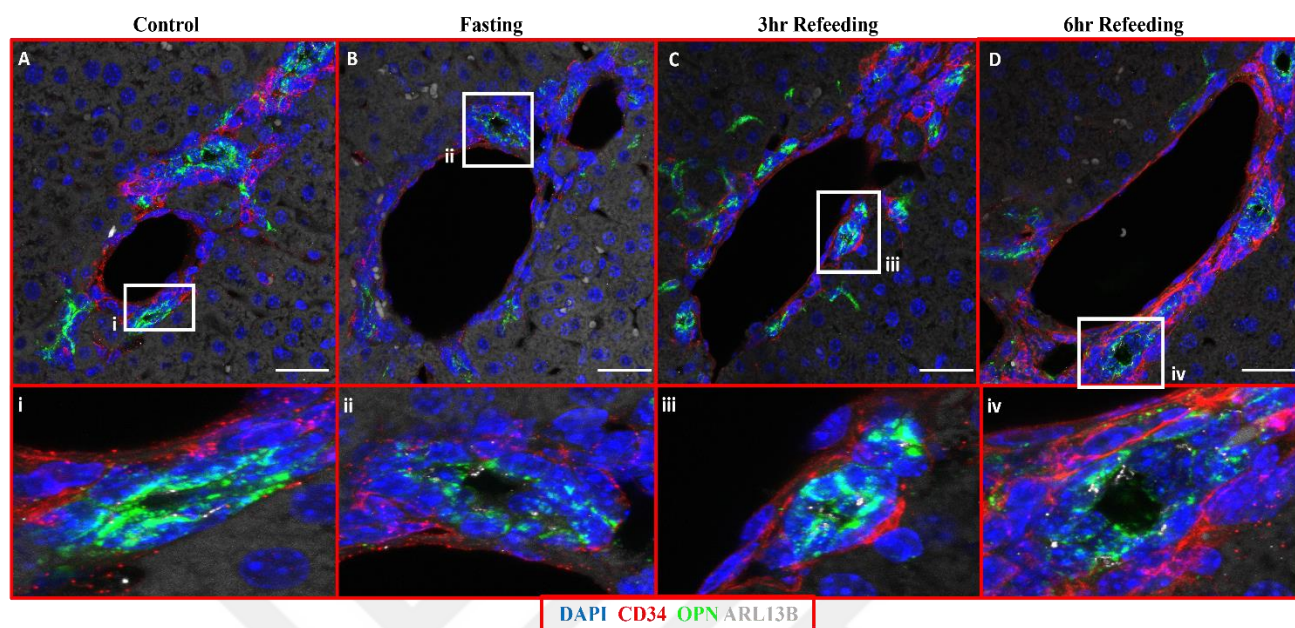
Tile scan images of Opn (red) (n=2, n=2) fluorescence staining for control (A) and High Fat Diet 24-week (B) samples. Both images included portal to central veins from left to right. DAPI was used for staining of nucleus. Scale bar represented 200 μ m. (PV: Portal Vein; CV: Central Vein)

Appendix IV: IHC analysis for control and WD 24-week samples.



Fluorescence staining performed by Arl13b (red) (n=1, n=1), Opn (green) (n=1, n=1), and Thy1(grey) (n=1, n=1) in control (A) and Western Diet 24-week (B) animals. Both images represented portal veins of the tissue. Panel i showed zoom version of specific region of panel A whereas panel ii demonstrated region in panel B. DAPI was used for staining of nucleus. Scale bar represented 50µm.

Appendix V: IHC experiment for control, fasting, 3hr refeeding and 6hr refeeding liver samples.



Fluorescence staining of CD34 (red) (n=1,n=1,n=1,n=1), Opn (green) (n=1,n=1,n=1,n=1) and Arl13b (grey) (n=6, n=5, n=5, n=5) in control (A), fasting (B), 3 hours refeeding (C) and 6 hours refeeding (D) samples. All images indicated portal veins of the liver. Panels i,ii,iii and iv showed respective areas in control, fasting, 3hr refeeding and 6 hr refeeding samples. DAPI was used for staining of nucleus. Scale bar represented 50 μ m.